Tricalcium silicate cements: in-vitro comparative studies of mechanical and biological characteristics of relevance to regenerative endodontics

Mohamed Abdul Raouf Abdul Wahab Ali
Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Thesis for the degree of Philosophiae Doctor (PhD)
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Name: Mohamed Abdul Raouf Abdul Wahab Ali
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This thesis is dedicated to my late grandfather Abdulwahab Alotaibi and my late aunt Batool Hassan Abusenina. Forever in our hearts.
“Those times when you get up early and you work hard, those times when you stay up late and you work hard, those times when you don’t feel like working, you’re too tired, you don’t want to push yourself, but you do it anyway. That is actually the dream. That’s the dream. It’s not the destination, it’s the journey.”

*Kobe Bryant*
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   Bergen, Norway.

Principal Supervisor: Athanasia Bletsa
Co-supervisors: Asgeir Bårdsen, Manal Mustafa
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Mohamed Ali
June 2020
Bergen
List of Publications

This thesis is based on the following scientific papers. They are referred to in the text by the numbers below:

   Fracture resistance of simulated immature teeth treated with a regenerative endodontic protocol.

   Tricalcium silicate cements: osteogenic and angiogenic responses of human bone marrow stem cells.

   **Differential Responses of Human Dental Pulp Stromal Cells to Bioceramic Materials: a comparative in-vitro study**
   Submitted manuscript

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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAE</td>
<td>American Association of Endodontists</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline Phosphatase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Bi₂O₃</td>
<td>Bismuth Oxide</td>
</tr>
<tr>
<td>BSP</td>
<td>Bone sialoprotein</td>
</tr>
<tr>
<td>CaO</td>
<td>Calcium oxide</td>
</tr>
<tr>
<td>Ca₂SiO₄</td>
<td>Dicalcium Silicate</td>
</tr>
<tr>
<td>Ca₂SiO₄</td>
<td>Tricalcium Silicate</td>
</tr>
<tr>
<td>CaOH₂</td>
<td>Calcium Hydroxide</td>
</tr>
<tr>
<td>CDNA</td>
<td>Complement Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CEJ</td>
<td>Cementoenamel junction</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>COL1A</td>
<td>Collagen 1 Alpha</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMP-1</td>
<td>Dentin matrix protein-1</td>
</tr>
<tr>
<td>DPP</td>
<td>Dentin phosphoprotein</td>
</tr>
<tr>
<td>DSP</td>
<td>Dentin sialoprotein</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Fe₂O₃</td>
<td>Iron Oxide</td>
</tr>
<tr>
<td>FGF-1</td>
<td>Fibroblast growth factor 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GMTA</td>
<td>Gray Mineral Trioxide Aggregate</td>
</tr>
<tr>
<td>GP</td>
<td>Gutta Percha</td>
</tr>
<tr>
<td>HBMSC</td>
<td>Human Bone Marrow Stem Cell</td>
</tr>
<tr>
<td>HDPSC</td>
<td>Human Dental Pulp Stromal Cell</td>
</tr>
<tr>
<td>HV</td>
<td>Hardness Vicker</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>Potassium sulphate</td>
</tr>
<tr>
<td>MGO</td>
<td>Magnesium oxide</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal Stem/Stromal Cell</td>
</tr>
<tr>
<td>MTA</td>
<td>Mineral Trioxide Aggregate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium</td>
</tr>
<tr>
<td>NaOCL</td>
<td>Sodium Hypochlorite</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>Sodium Sulfate</td>
</tr>
<tr>
<td>OC</td>
<td>Osteocalcin</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>OPN</td>
<td>Osteopontin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>REP</td>
<td>Regenerative Endodontic Procedure</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RRM</td>
<td>Root Repair Material</td>
</tr>
<tr>
<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
</tr>
<tr>
<td>SCAP</td>
<td>Stem cells of apical papilla</td>
</tr>
<tr>
<td>SiO₂</td>
<td>Silicon dioxide</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ti₂O₅</td>
<td>Tantalum Oxide</td>
</tr>
<tr>
<td>TDI</td>
<td>Traumatic Dental Injuries</td>
</tr>
<tr>
<td>TSC</td>
<td>Tricalcium Silicate Cement</td>
</tr>
<tr>
<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
</tr>
<tr>
<td>VPT</td>
<td>Vital Pulp Therapy</td>
</tr>
<tr>
<td>WMTA</td>
<td>White Mineral Trioxide Aggregate</td>
</tr>
<tr>
<td>ZrO₂</td>
<td>Zirconium oxide</td>
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Abstract

Tricalcium silicate cements (TSC), are bioactive ceramic materials with a wide range of clinical applications in the field of “regenerative endodontics”, i.e. tissue engineering concepts applied to regeneration of damaged or lost dental tissue. This broad definition includes both vital pulp therapy (VPT) and regenerative endodontic procedures (REP) in immature permanent teeth with necrotic pulps. Both procedures involve direct contact between stem cells and TSC: the ensuing interaction is an essential determinant of regeneration and/or repair. It is therefore important to determine the bioactivity and biocompatibility properties of TSC. In REP moreover, the application of TSC should take into account potential mechanical effects on teeth at greater risk of fracture because of the thin dentinal walls.

In this thesis three commercially available TSC were compared: White MTA-Angelus (MTA), Biodentine and TotalFill BC Root Repair Material PUTTY (TotalFill).

The surface microhardness of MTA, Biodentine and TotalFill was evaluated by the Vicker’s Hardness Test. Biodentine showed the highest microhardness whereas TotalFill, which failed to achieve a measurable level, had the lowest. Based on the Vicker’s test results, the potential effect of MTA, Biodentine and TotalFill on tooth resistance to fracture was investigated. The aim of Study 1 was to compare the fracture resistance of immature teeth treated with REP and MTA, Biodentine or TotalFill applied to the cervical area. The study utilized an acknowledged bovine tooth model. The teeth were prepared to simulate immature permanent teeth, then treated with REP and tested for fracture resistance. No differences in fracture resistance were observed in relation to the three TSC tested.

Cellular responses to MTA, Biodentine and TotalFill were evaluated and compared in cell culture experiments. Cell proliferation was assessed by MTT assay and osteogenic/angiogenic/inflammatory responses were assessed with qRT-PCR, ELISA, ALP quantification and Alizarin red staining.

In Study 2 the effect of TSC on human bone marrow stem cells (hBMSC) was investigated. Compared to MTA and TotalFill, Biodentine had the least inhibitory
effect on hBMSC proliferation. The osteogenic and angiogenic responses to the materials varied. Biodentine and TotalFill induced earlier changes at gene level. All TSC induced mineralization after 14 days, with MTA possibly demonstrating the greatest effect.

In Study 3 the effect of TSC on human dental pulp stromal cells (hDPSC) was investigated. Biodentine exhibited the least inhibitory effect on proliferation and induced upregulation of most osteogenic markers. TotalFill had an anti-inflammatory effect, expressed as downregulation of IL6. Moreover, TotalFill induced increased gene expression and production of VEGFA and had a long-lasting effect on the inhibition of ALP production.

The results indicate that although the TSC tested tend to be used interchangeably in clinical practice, these materials have not only different mechanical properties but also different biological effects. The microhardness levels of TSC differed, but there were no associated differences in fracture resistance. With respect to cellular responses, Biodentine was the most inert. It had the least effect on cell proliferation and induced pronounced expression of osteogenic markers in both hBMSC and hDPSC. TotalFill exhibited enhanced angiogenic and anti-inflammatory effects on hDPSC.

The results of these studies have potential clinical implications and further investigation is therefore warranted.
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1. Introduction

Over the past four decades, there have been great advances in medical technology in the field of hard tissue regeneration and repair. Of particular note is the innovative application of bioceramic materials (1). Tricalcium silicate cements (TSC), also known as hydraulic calcium silicate cements, are now well-established in modern dentistry, with a wide range of clinical applications in the fields of endodontics and pedodontics. TSC were originally introduced as materials for retrofilling and for perforation repair (2, 3). However nowadays they are used in vital pulp treatment such as direct and indirect pulp capping and pulpotomy, as well as regenerative endodontic procedures (REP) (4). TSC can also be used for perforation repairs and as endodontic sealers.

TSC may also be regarded as “bioceramics”: they contain glass ceramics such as bioactive glass, alumina and zirconia combined with calcium silicate and calcium phosphate. Bioceramics should meet specific requirements, e.g. they should be bioinert, meaning that the materials do not initiate a host response, bioactive and biocompatible. The most important of these properties are biocompatibility and bioactivity (5). Biocompatibility is defined as “the ability of a material to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic responses in the eventual host” (5, 6). Bioactivity on the other hand refers to the ability of a material to induce a desirable tissue response. The bioactive constituents of TSC are the dicalcium and tricalcium silicates and calcium phosphates (1, 3).

Bioactivity and biocompatibility are particularly important in dentistry, where the success of TSC application depends on the promotion of cell growth, as well as the induction of specific cellular responses important for repair and regeneration. A desirable property of TSC is the ability to stimulate differentiation of cells associated with the pulp-dentin complex, such as odontoblasts, as well as osteoblasts and cementoblasts. Osteoblast and odontoblast differentiation are crucial for repair of hard tissue, while angiogenic differentiation is desirable for regeneration of an impaired vascular supply (7, 8). Moreover, the processes of repair and regeneration also involve
the inflammatory response and resolution of inflammation; hence the regulation of proinflammatory/anti-inflammatory cytokines is a desirable characteristic of TSC (9). In recent literature, TSC are reported to upregulate the expression of gene markers directly associated with the differentiation of osteoblasts and odontoblasts, such as Osteocalcin (OC), Alkaline Phosphatase (ALP), Osteopontin (OPN), and Bone Sialoprotein (BSP), and gene markers associated with angiogenic differentiation, such as VEGFA (Vascular endothelial growth factor A) (2, 10, 11). Correspondingly, TSC also stimulate macrophages to release the inflammatory cytokines interleukin-1 beta (IL1β), interleukin 6 (IL6) and interleukin 8 (IL8) (12, 13).

1.1 Mineral Trioxide Aggregate (MTA)

In 1993, Torabinejad et al. introduced the first successful TSC used in endodontics, namely Mineral Trioxide Aggregate (MTA) (14). Because of its early introduction, much more research has been conducted on MTA than on other more recently introduced TSC. Although initially developed as a root-end filling material, MTA was gradually introduced into other dental applications, such as vital pulp therapy (VPT) and repair of root perforations (4, 15). The chemical composition of the first generation MTA was a mixture of a refined Portland cement and bismuth oxide (Bi₂O₃), with fine hydrophilic particles of silicon dioxide (SiO₂), calcium oxide (CaO), magnesium oxide (MgO), potassium sulphate (K₂SO₄), and sodium sulfate (Na₂SO₄) (16). MTA is a dimensionally stable, biocompatible and bioactive hydrophilic material which has the ability to induce osteogenic and angiogenic regeneration (14, 16-25). The hydrophilic nature of MTA is one of its major advantages, as it can set under the moist conditions of the oral environment, specifically when in contact with pulp and apical tissue (4). MTA formulations were amended to overcome some disadvantages (26). Tooth discoloration was associated with the original formulation of gray MTA (GMTA): initially this was attributed to a high iron oxide (Fe₂O₃) content (27, 28). Discoloration became an issue of concern, particularly in esthetically sensitive cases and prompted changes in formulation (29). In 2002 white MTA (WMTA) was introduced as an alternative to GMTA (4, 30). WMTA contains 90.8% Fe₂O₃ less than GMTA, as well as finer and smaller hydrophilic particles than GMTA. The smaller hydrophilic
particles are believed to enhance water absorption as well as calcium ion release (31). WMTA however did not entirely solve the issue of discoloration (32, 33). Discoloration associated with WMTA could be caused by the chemical reaction between Bi$_2$O$_3$ and the collagen in dentin (34, 35). Bi$_2$O$_3$ serves as the radiopacifying agent in conventional MTA. In more recent formulations such as Neo MTA (NuSmile) and NeoMTA plus® (Avalon Biomed) alternative radiopacifying agents such as tantalum oxide (Ta$_2$O$_5$) are used (36).

It has been shown that during vital pulp therapy (VPT), MTA stimulates odontoblasts or odontoblast-like cells to induce the formation of a dentin bridge, similar in composition to primary dentin (37, 38). This dentin bridge is reported to be significantly superior to that formed by calcium hydroxide (CaOH$_2$) treatment, which shows “tunnel defects” within the dentin bridge (39, 40). However, in a recent clinical study in which teeth were pulp capped with MTA and examined histologically, the newly formed hard tissue did not resemble “regular dentin”. The formed tissue was predominantly atubular, while primary dentin is generally tubular in nature (41).

Another disadvantage of the original MTA was its long setting time, over 2hrs (14). The presence of gypsum is thought to prolong the setting time and attempts have been made to accelerate it by adding methylcellulose and calcium chloride (CaCl$_2$) to the mixture (42). This MTA, chemically modified by the addition of 1% methylcellulose and 2% CaCl$_2$, Sets one third faster than the traditional MTA (43). In addition, MTA was challenging to handle in the clinic because of its grainy, sand-like consistency after mixing (43). In summary, clinical application of MTA requires control of several factors in order to achieve the desired effects. Alternative TSC may therefore be preferred.

1.2 Biodentine

Biodentine is one of the newer calcium silicate-based materials (44). It was made commercially available in 2009 by Septodont (45) specifically to serve as a dentin substitute or “dentin replacement” while also providing superior handling properties to that of MTA, such as ease of mixing and post-mix consistency (46). It is supplied a
powder form, to be mixed in an amalgamator with the liquid provided. The powder comprises tricalcium (Ca$_3$SiO$_5$), and dicalcium silicate (Ca$_2$SiO$_4$), calcium carbonate (CaCO$_3$) (filler material) and zirconium oxide (ZrO$_2$). The liquid is a mixture of water, CaCl$_2$ and modified polycarboxylate (47). Mixing the material in an amalgamator instead of manually provides better consistency for clinical application (3).

The setting reaction in Biodentine is comparable to that of MTA with respect to the formation of Ca$_2$SiO$_4$, Ca$_3$SiO$_5$, hydrate gel and CaOH$_2$ (3). However, the setting time of Biodentine is less than that of ProRoot MTA and most other TSC. This is due to the CaCl$_2$ in the mixing liquid, and the carbonate acting as a nucleation site for Ca$_3$SiO$_5$ hydration, thus reducing the initial chemical reaction time (46, 48, 49). Biodentine, like other TSC, has antibacterial properties, attributable to the release of CaOH$_2$ ions during the setting reaction: the pH becomes highly alkaline (12.5) and this in turn leads to inhibition of bacterial growth (3).

Biodentine is the first TSC to incorporate ZrO$_2$ as a radiopacifier, although its radiopacity is lower than that of Bi$_2$O$_3$ (50). In terms of cell proliferation, as the presence of Bi$_2$O$_3$ has been reported to be detrimental to cell viability, presence of zro2 in Biodentine serves as an advantage (51). ZrO$_2$ has also shown biocompatible properties and bioinert properties, while eliminating the risk of tooth discoloration associated with the use of Bi$_2$O$_3$ (52). Nevertheless, studies have shown that neither material has cytotoxic effects on stem cells, while ProRoot MTA showed increasing osteoinductivity compared to Biodentine, in terms of messenger RNA and protein expression of alkaline phosphatase, documented by immunocytochemistry, and alizarin red staining data (22, 53).

1.3 TotalFill

TotalFill or Endosequence Root Repair Material (ERRM) is also a relatively recently introduced bioceramic, made of Ca$_2$SiO$_4$, Ca$_3$SiO$_5$, (calcium silicates), monobasic calcium phosphate Ca$_3$(PO$_4$)$_2$, ZrO$_2$, Ta$_2$O$_5$, fillers and thickening agents (54). The difference in nomenclature stems from the origin of manufacturing: TotalFill is produced and commonly used in Europe, while Endosequence is produced and used predominantly in the USA. This bioceramic is available in a variety of forms, including
a sealer (TotalFill BC Sealer) and a root repair putty [TotalFill BC Root Repair Material (RRM)]. The sealer is available in a syringe and the RRM as either a syringable paste or a condensable putty (55). TotalFill is supplied ready-to-use and this is an advantage over other root repair materials: it is easier to handle, and the material is always consistent for each application. According to the manufacturer, TotalFill has a minimum setting time of 2hrs. The setting reaction is initiated by contact with water or moisture. In cases of extremely dry canals, the setting time may be prolonged (55).

The presence of Ca$_3$(PO$_4$)$_2$ induces positive osteoblastic responses in terms of gene expression and cellular proliferation (56) (57). Recent studies report Endosequence to have high biocompatibility and an antibacterial capacity due to its highly alkaline pH (58, 59). TotalFill has been shown to induce increased proliferation of human bone marrow stem cells (hBMSC) compared to Biodentine and MTA (60), as well as increasing proliferation of human bone marrow mesenchymal cells in serum deprived conditions compared to MTA (61). Another paper comparing TotalFill with MTA reported similar biocompatibility and bioactivity on human dental pulp cells. Both TotalFill and MTA enhanced cell proliferation, expression of odontogenic/osteogenic/inflammatory markers as well as deposition of calcium (62). These reports suggest that the effect of TotalFill on proliferation and differentiation is similar to that of MTA or MTA-like products. In this regard, TotalFill could be a suitable alternative for MTA.

In contrast to the Bi$_2$O$_3$ in MTA, TotalFill contains Ta$_2$O$_5$. Ta$_2$O$_5$ has shown excellent radiopacity, without the discoloration potential of MTA products containing Bi$_2$O$_3$ (57).

Compared with MTA or Biodentine, there is much less research evidence for TotalFill, which has only recently been introduced.

1.4 Material microhardness

The mechanical properties of a restorative material are essential as they can have implications on the longevity of restored traumatized immature teeth. This includes properties such as sealing ability, flexural strength, compressive strength, porosity,
and dimensional stability and surface microhardness. Surface microhardness is the ability of a material to resist surface deformation and can be used to provide an indication of the overall material strength and its mechanical response (63-65). In clinical settings, when TSC are applied in the coronal and cervical portion of teeth, surface microhardness plays an important role in achieving a proper coronal seal (66, 67). Occlusal loads may displace the applied TSC particularly when the material has not completed its setting and reached its peak microhardness. The faster the setting reaction of the material, the quicker it can achieve its peak microhardness and thus, better the chance of maintaining the physical seal (67).

Furthermore, TSC may potentially reinforce the strength of immature teeth if the microhardness of the material is close to human dentin as similar microhardness allows similar “mechanical behaviour” between TSC and dentin (63). It has been shown that the microhardness of MTA is affected by factors such as pH, temperature and humidity (68, 69). Under inflammatory conditions, i.e. with increased vascularity and an acidic pH, the microstructure of the material and therefore its microhardness may be affected (69). Another factor that may affect microhardness is the thickness of material. During testing, surface microhardness was found to be directly proportional to the sample’s thickness (68). As for Biodentine, its “dentin replacing” potential can be attributed to its surface microhardness. Studies have found the microhardness of Biodentine to resemble that of human dentin implying similar mechanical properties (63). It is also considerably higher than that of MTA (63). On the other hand, TotalFill has lower surface microhardness than WMTA and GMTA, especially during the first day of setting (70). However, it showed an increase in microhardness with time; after 4 days, TotalFill reached a microhardness similar to that of both WMTA and GMTA and maintained this throughout 28 days (70). It was concluded that all the materials tested, including TotalFill, required at least 7 days to reach a complete set (70). Thus, the microhardness of TotalFill after complete setting is similar to that of MTA, but lower than that of Biodentine. Nevertheless, when TSC were used as apical plugs during apexification, there was no difference in microhardness between TotalFill and Biodentine, but MTA had a significantly higher
microhardness than TotalFill and Biodentine (71). The contradictory findings of these studies illustrate inconsistencies in the literature.

1.5 Advances in development of TSC

Work to improve handling characteristics and material properties is ongoing. This typically results in variations in the different formulations of bioceramic materials (72). In addition to the previously mentioned materials (MTA, Biodentine, TotalFill), various other types of TSC are currently in use. These include materials which enhance the flowability of MTA, such as MTA HP, which contains calcium tungstate (CaWO₄) as a radiopacifier and a mixing liquid with a plasticizer agent (72). Increased flowability may contribute to closer adaptation on the irregular surface of dentin and improve the sealing ability of the material (73). NeoMTA and NeoMTA Plus are newer formulations of MTA material in which Ta₂O₅ has replaced Bi₂O₃ as the radiopacifier. Other TSC which use Ta₂O₅ include Bioaggregate and Diaroot (50).

Another example of material reformulation is the combination of TSC and magnesium phosphate (Mg₃O₈P₂) (74, 75). Including Mg₃O₈P₂ in formulations enhances such properties as faster setting time, higher compressive strength, superior adhesion and antibacterial effects (76). The addition of hydroxyapatite has also been proposed and tested, but more research is necessary (77). Hydroxyapatite is believed to be highly osteoconductive: as it does not elicit an immune reaction it has the ability to integrate directly with bone (78, 79).

The addition and creation of nanoparticles can also lead to improvement in physical properties, as these smaller particles may penetrate into the dentinal tubules (77, 80): This may decrease porosity and in turn lead to a denser mass and an increase in the modulus of elasticity (81). When WMTA and other TSC were nanomodified with smaller radiopacifier particles, ranging in size from 40-100 nm, the physiochemical properties were significantly enhanced and improved. This includes properties such as microhardness, setting time, push-out bond strength and compressive strength (82). This effect was also observed when nanometer size bioactive glass was incorporated into the material composition of Biodentine (83). Nanoparticles have a higher surface
area and are therefore more reactive and excitable in terms of oxidizing free radicals, which may lead to an improvement of physical properties (84). The addition of nanoparticles resulted in a decrease in setting time of MTA Angelus (84). This was attributed to the higher surface area of the nanoparticles, which hastens the reaction between the powder and liquid constituents (84).

1.6 Application of TSC in regenerative endodontics

The term “regenerative endodontics” refers to the use of tissue engineering concepts to restore root canal health by promoting the continued development of the root and the surrounding tissues (5, 85). This has been an essential treatment modality for traumatic dental injuries (TDI) in vital and non-vital immature permanent teeth. A broad definition of regenerative endodontics may include two separate categories based on the site of treatment and the presence or absence of vital pulp (82): treatment of the vital pulp is termed VPT and treatment of non-vital teeth is commonly referred to as regenerative endodontic procedures (REP) (86). The ultimate aim of VPT is to preserve pulp vitality (Figure 1). The European Society of Endodontology (ESE) defines VPT as “Strategies aimed at maintaining the health of all or part of the pulp” (87). Pulp vitality is essential for continued root development and maturation. Applying TSC to pulp tissue not only restores and maintains the vascular network of the pulp (88), but also promotes the formation of hard tissue and neural tissues (82, 89-91). VPT may lead to recruitment of human dental pulp stromal cells (hDPSC) and differentiation into odontoblast-like cells which produce reparative dentin (91). VPT includes indirect pulp capping and direct pulp capping (Figure 1) as well as partial and full pulpotomy (Figure 2) (92, 93). Indirect pulp capping may be indicated when the pulp is not yet exposed but threatened with exposure by progression of dentinal caries in close proximity to the pulp. The first step in this procedure is to remove as much of the carious tissue as possible without exposing the pulp. A randomized controlled trial performed on 94 children aging 7-12 showed that indirect pulp capping with MTA was successful in 100% of cases compared to 93% of CaOH₂ cases after 1 year follow up (94). When Biodentine was compared to glass ionomer cement in a similar clinical trial in teeth with deep carious lesions and diagnosis reversible pulpitis, no clinical
differences were seen between both materials as both showed success of treatment in 83% of cases after 12 months. However, radiographic assessment with periapical radiographs and CBCT before and after treatment showed significant differences as 71% of healed lesions were treated with Biodentine and the majority of cases of new/progressed lesions (88%) were treated with GIC (95).

Direct pulp capping is indicated in cases of superficial exposure of the pulp, usually in cases of TDI, accidental perforation during excavation of carious dentin or mechanical exposure during operative procedures (92). A 2016 study performed on 70 adults aging from 18-55 found that MTA was more effective than CaOH$_2$ dressing as a direct pulp capping material as it showed 85% pulp survival rate after 3 years compared to 52% for the CaOH$_2$ group (96).

In pulpotomy, a coronally infected portion of the pulp is surgically removed, in order to maintain the vitality and normal function of the remaining pulp (97, 98). The treatment may comprise either partial or full pulpotomy. In partial pulpotomy, a 2-3mm portion of the coronal pulp is surgically removed (99, 100). This is indicated in cases of infection of a superficial portion of the coronal pulp. When a deeper portion of the coronal pulp has been infected, the entire coronal portion of the pulp is removed as far as the canal orifice/s (101). At the time of VPT, it is recommended that the involved pulp must be healthy enough to respond to treatment and the diagnosis is reversible pulpitis (93, 102). Interestingly, a recent randomized clinical trial was done on mature teeth with irreversible pulpitis treated with pulpotomy and included 37 patients aging 20-25. The criteria of clinical success were resolution of spontaneous pain and pain upon chewing, no tenderness to percussion or palpation, and normal response to cold test. Absence of internal resorption, root resorption, and periapical pathology was considered radiographic success (103). After a 2 year follow up period, MTA partial pulpotomy lead to success of 85% of cases compared to 43% of cases treated with CaOH$_2$ (103). Furthermore, the same group stipulated in a 2018 study that an irreversible pulpitis diagnosis should not be considered a contraindication for pulpotomy and that Biodentine was highly successful and a suitable alternative to conventional root canal treatment (104). This study agreed with earlier studies reporting similar findings with MTA pulpotomy (105, 106).
formulations (ProRoot MTA, OrthoMTA, and RetroMTA) were compared in a clinical trial, no significant differences in outcomes were shown between the different formulations and a high success rate of more than 92% was found at 12 months after partial pulpotomy procedures (107). While these studies confirm the reliability of VPT with TSC, they lack direct comparisons between different TSC. It may be useful to have such studies to aid clinicians to choose between the available materials.

Figure 1. *Vital Pulp Therapy: indirect and direct pulp capping* 
(A) Indirect pulp capping of a nearly exposed pulp with TSC and (B) Direct pulp capping of exposed pulp with TSC
The second category of regenerative endodontics deals with non-vital teeth (82, 108). The standard treatment for non-vital teeth with fully developed roots is conventional root canal therapy. However, the immature permanent tooth with open apices and thin dentin walls presents a challenge. Apart from the heightened risk of fracture of the thinner walls, the infected root canal space is not as easily disinfected by standard procedures and the aggressive use of endodontic files, because of the risk of removing even more dentin from the already thin walls. The open apex makes it challenging to fill the canal: in the absence of an apical barrier, root filling materials tend to be extruded into the periapical area (Figure 3).
Figure 3. Challenges in treating non-vital immature permanent teeth 1: Disinfection can be harmful to periapical tissue. 2: The open apex and absence of an apical barrier makes it challenging to fill root canal space 3: Thin dentin walls make root susceptible to fracture (109).

REP is used to treat immature permanent teeth with necrotic pulps and/or apical periodontitis: the aim is to promote root development and restore vital tissue (85). REP utilize the basic tissue engineering concept, i.e. a combination of stem cells, growth factors and scaffolds: the essential components of regenerative medicine (110).

This shift in treatment modalities attempts to replace traditional approaches involving the long-term use of CaOH₂ for apexification and the direct apexification method using TSC (111). Although relatively successful, CaOH₂ apexification is a lengthy treatment procedure, sometimes lasting for 18 months and requiring a high level of patient compliance, with multiple visits (112). Furthermore, apexification either induces the formation of a hard-tissue apical barrier or an apical TSC placement, so that conventional root canal therapy can be performed. Although treatment duration for TSC is significantly shorter than for CaOH₂, none of the methods directly addresses the issue of thin dentin walls. Thus, the high risk of fracture remains (111, 113-115).

There were reports in the literature of susceptibility to fracture of the cervical area associated with long-term CaOH₂ treatment and apexification of immature teeth with TSC: this highlighted the need for research into an appropriate treatment alternative (Figures. 4, 5) (116-119).
Figure 4. Cervical root fracture on endodontically treated tooth 11. Tooth needed endodontic treatment due to trauma at the age of 9 yo. Long-term calcium hydroxide intracanal dressing was used during treatment due to persistent infection followed by apical plugs of TotalFill and gutta percha. Two years later, tooth 11 sustained cervical fracture upon mastication (eating an apple). Courtesy of O. Iden

Figure 5. Boy 9yr old had sustained severe intrusion of tooth 11 combined with enamel/dentin crown fracture. Tooth 11 was surgically repositioned and root canal treatment was initiated when signs of root resorption were evident. Tooth was fractured while CaOH$_2$ intracanal dressing was in place. Inspection of the fractured tooth shows that fracture occurred at the root resorption area. Courtesy of A. Bletsu

REP commences with standard endodontic procedures: removal of the necrotic pulp tissue and thorough disinfection of the root canal (120, 121). The American Association of Endodontists (AAE) and the European Society of Endodontology (ESE)
recommend minimal or no instrumentation of the canal in order to preserve as much remaining dentin as possible (85, 122-124).

After disinfection of the root canal space, the canal is filled with blood from the periapical area by instrumentation of the periapical tissue (82). The intention is to transfer undifferentiated mesenchymal stem cells (MSC) from the periapical region and apical papilla into the root canal system (125). When the introduced blood coagulates, the clot acts as a scaffold for stem cells and contains platelet-derived growth factors which may influence and signal stem cell activity (85, 126). TSC is then placed in the cervical part of the root, to establish a seal and possibly stimulate stem cells to induce regeneration, ideally in the form of root completion and revascularization of the canal space (Figure 6) (120, 127-129). The concept is that the continuation of root development and the revascularization of the root canal is a superior treatment outcome to that achieved through apexification with CaOH2. A 2017 clinical randomized control study performed on patients aged 8-16 compared REP (69 cases) with apexification (34 cases) after a 12-month follow up period. Both REP and apexification lead to 100% apical healing and resolution of pain. However, REP also induced an increase in root thickness and root length in 82% of cases while apexification showed only an increase in root length in 26% of cases(130). A 2020 clinical trial quantitatively assessed with magnetic resonance imaging (MRI) the regenerated tissue of 18 necrotic teeth with periapical lesions treated with REP (131). All teeth were asymptomatic and achieved healing of the periapical lesions after a 12-month period. Interestingly, the MRI confirmed the regeneration of vital pulp-like tissue by receiving similar signals from contralateral vital teeth. In addition, 60 % of the teeth regained sensibility to cold and electric pulp tests (131). Characterization of the regenerated tissue after REP has been previously reported; In a 2018 case report, two immature teeth treated with REP were clinically and radiographically followed-up until extraction, after 54 months and 43 months respectively. The extracted teeth were assessed immunohistochemically (132). Clinically, both teeth showed resolution of symptoms and apical disease, as well as continued root development after REP (132). Furthermore, “recapitulation” of vascular and lymphatic tissue post treatment was seen, indicating a potential re-establishment of vitality (132).
The above studies demonstrate the possibility of pulp-dentin complex regeneration with REP but do not compare outcomes of REP with different TSC. REP protocols are similar, but variability still exists to a large extent as there is yet to be a preferred protocol based on scientific literature (133, 134). Comparisons between studies are hard to make as there are variations in the REP protocols regarding irrigation, intracanal medication and cervical seal with TSC.

Figure 6. Principle of Regenerative endodontics
A: Necrotic Pulp. B: After disinfection bleeding in the root canal is achieved by introducing an endodontic file beyond the apex and into the periapical area. C: A TSC material plug is placed approximately at cervical area of the root, sealing the blood clot. The clot acts as a scaffold for stem cells and source of growth factors. The TSC plug influences the microenvironment. D: Regeneration of the pulp-dentin complex, continued root development and thickening of the dentine walls. Tooth regains vitality. Adapted from (135)

1.7 Outcomes of regenerative endodontics

Preserving pulp vitality is important to ensure the development and function of a permanent tooth (136, 137). Achieving this through VPT is highly dependent on strict case selection and an elaborate treatment protocol, from pre-treatment evaluation of pulpal status (138). Initially younger patients were preferred as they were considered to have greater healing capacity of the pulp tissue and higher pulpal vascularity (138,
Nevertheless, there are reports of successful outcomes for VPT in patients ranging in ages from 6-70 years (140-142). The CaO in TSC forms CaOH$_2$ in the presence of water and induces mineralization of the adjacent pulp by the release of calcium ions (143-145). This leads to an increase in overall pH, which in turn induces an early inflammatory reaction. Finally, reparative dentin is formed at the material-pulp interface (146, 147). TSC form dentin bridges more rapidly than CaOH$_2$ (148-150). This reparative dentin bridge contains fewer tunnel defects than those formed by CaOH$_2$ and is therefore more effective in preventing bacterial leakage (144).

The formation of a dentin bridge is crucial to the outcome of VPT. The dentin bridge acts as a coronal seal and a barrier against bacterial invasion and microleakage (147, 151). Bakhtiar et al showed that partial pulpotomy of healthy human maxillary molars using RetroMTA led to disorganized pulp tissue, a discontinuous dentin bridge, and less dentin thickness than ProRoot MTA (147). These results led to the conclusion that ProRoot MTA is preferable to RetroMTA for partial pulpotomy. Whether the findings apply to carious pulp exposure is questionable, because the pulpotomy was undertaken in the absence of inflammation. This is rarely the case: under clinical conditions: pulpotomy is not undertaken on healthy teeth and inflammation is usually a factor (147). It is believed that non-resolution of inflammation can negatively influence the quality of the dentin bridge formed during pulpotomy and therefore directly affects the outcome of VPT.

Although there are reports of successful REP on mature teeth (152), according to the literature the most successful cases of REP are those on immature teeth (127). This may be due to the higher regenerative potential of younger patients as well as the presence of open apices (86). REP rely on the introduction of blood into the root canal through the root apex (86, 153). Therefore, the wide-open apex of an immature tooth is an advantage, allowing easier access into the canal space (109). An apex of more than 1.1 mm in diameter was shown to enhance the incidence of revascularization (154). Ideally TSC would stimulate the differentiation of undifferentiated MSC into odontoblasts. However, rather than dentin, the formation of bone-like tissue,
cementum-like tissue and periodontal-like tissue is reported (85), indicating that MSC differentiate into osteoblasts and cementoblasts. Histologically this outcome is considered to be repair rather than regeneration and is less than ideal, because the damaged tissue does not regain function (85, 111). Nonetheless, a 2018 case report demonstrated that REP could induce the formation of dentin-like hard tissue as well as soft tissue in the form of vascular and lymphatic structures (132).

The absence of infection is also paramount to the success of REP. Effective disinfection and coronal sealing to ensure no external leak of microbes into the root canal is important (109). The predictability of REP outcomes has however, always been a point of contention. This includes measurable clinical outcomes recognized by the ESE and AAE as criteria for determining success of REP (122, 123, 134). They are categorized into primary and secondary outcomes. Primary outcomes involve the resolution of the clinical and radiographic signs and symptoms: periapical healing, apical closure, increased root length and continuation of root development, and an increase in root dentin thickness. The secondary outcomes are also termed late-stage effects and include pulp canal obliteration and (absence of) discoloration (122, 123, 134). There are varying levels of success, depending on the primary and secondary outcomes and this has led to questions as to what may be accurately regarded as “successful” treatment.

1.8 Stem cells

Stem cells are defined as clonogenic, undifferentiated cells capable of self-renewal, which also proliferate and differentiate into other cell types (155) (156). They are an integral component of the concept of tissue engineering (110), crucial to maintenance of tissue homeostasis and play a key role in tissue repair (157). Stem cells are typically classified either according to their differentiation properties or plasticity or according to the origin and source from which they are isolated or classified (155, 158). Stem cells classified according to differentiation capacity may be totipotent, pluripotent, multipotent, unipotent or oligopotent (Figure 7) (158).
• **Totipotency** refers to the ability of a single cell to produce all differentiated cells of the organism through cell division (159).

• **Pluripotency** is the ability and potential of a cell to differentiate into any one of three germ layers: endoderm, mesoderm, or ectoderm (160).

• **Multipotency** describes a cell which can differentiate into several cells, but only those of a family closely related to the specific cell (e.g. MSC) (161). Multipotent cells self-renew through development into multiple specialized cells types within the specific tissue from which they are isolated (158).

• **Oligopotent** cells can differentiate into only a few cells (e.g. lymphoid cells) while **unipotent** cells can produce only their own cell type through self-renewal (e.g. skin cells) (161).

When stem cells are classified according to their origin, they are either embryonic, fetal, infant, adult or induced pluripotent stem cells (iPS) (Figure 7) (157).

• **Embryonic** stem cells are pluripotent stem cells isolated from the inner cell mass of a preimplanted embryo and can be maintained in an undifferentiated state in culture (157, 158, 162-164).

• **Fetal** stem cells are obtained from fetal tissues and have a greater differentiation potential than adult stem cells.

• **Infant** stem cells are derived from the placenta, the umbilical cord or amniotic fluid (157).

• **Adult** stem cells are found in developed humans postnatally, in both children and adults. Adult stem cells are also known as tissue resident cells or somatic cells and are mostly multipotent, with the exception of epidermal epithelial stem cells, which are unipotent (161, 165, 166). Adult stem cells are classified, according to their origin, into 6 different cell types: hematopoietic, mesenchymal, neural, epithelial, hepatic or pancreatic stem cells (157).

• **Induced pluripotent** stem (iPS) cells resemble embryonic cells. They are reprogrammed from somatic adult cells such as skin fibroblasts, nerve cells, adipocytes and blood cells, into undifferentiated cells which have unlimited self-
renewal capacity (157). iPS are particularly convenient as they can be directly isolated from the patient’s own tissue.

![Classification of stem cells diagram]

Figure 7. Classification of stem cells. Adapted from (157)

1.9 Mesenchymal stem/stromal cells

The International Society for Cellular Therapy (ISCT) defines mesenchymal stem/stromal cells (MSC) as “multipotent mesenchymal stromal cells” (167). In this definition the term “stromal” describes the plastic adherence property while the “stem cell” refers to the ability for self-renewal and tri-lineage differentiation potential i.e. capable of giving rise to osteocytes, adipocytes and chondrocytes (168, 169). Found within connective tissue/stroma, MSC are a subset of cells with multipotent differentiation capacity (170). MSC are isolated and derived from mature adult human tissue and are therefore an example of adult stem cells (164, 171). The most reliable and notably enriched sources of MSC are bone marrow (172), dermis (173) and dental
tissues (174). MSC were first identified by Friedenstein et al. who reported that fibroblast-like cells in mouse and guinea pig bone marrow could generate bone and exhibited osteogenic potential (168, 175, 176).

MSC exist in a stem cell microenvironmental “niche” described by Schofield in 1978 (177). MSC are maintained in an undifferentiated state by being enclosed by the immediately adjacent surrounding tissue components (177-179). This includes non-multipotent cells as well as extracellular matrix (ECM), specific to the niche (177, 178). This niche regulates and coordinates the contribution of stem/progenitor cells towards repair and regeneration (180). This is achieved through signaling which originates from the progenitor stem cells (autocrine), the neighboring resident cells (paracrine), other tissues (systemic) or the ECM (179).

MSC are identified according to specific certain minimal inclusion and exclusion criteria established by the ISCT (167). The first of these criteria is that MSC should show plastic adherence through attachment to standard culture plates in-vitro. Another criterion is the expression or lack of expression of specific surface markers/antigens (Table 1). MSC are characterized through the expression of clusters of differentiation markers (CD) 105, CD90 (Thy-1), and CD73 (Table 1). However, no single antigenic determinant has been identified as specific for MSC. Hence, the criteria should be adopted as minimal requirements: depending on the study, further evidence can then be included for identification and characterization purposes (167).

Because of the heterogenous nature of MSC, exclusion criteria are also required for identification. These include the negative expression of hematopoietic markers such as CD34 (hematopoietic progenitors and endothelial cells marker) and CD45, and CD14 or CD11b, CD79alpha or CD19 and Human Leukocyte Antigen – DR isotype (HLA-DR) surface molecules (Table 1) (167) (181, 182).

Finally, MSC must be able to exhibit multipotency, through differentiation into odontoblasts, chondrocytes and adipocytes in standard in-vitro culture (167). The flexibility of MSC is epitomized by their differentiation plasticity. MSC have an uncanny ability to flip between differentiation pathways, depending on external stimulating conditions (183). This becomes especially significant in REP, as MSC are
ideally stimulated through TSC towards odontoblastic differentiation. However, in a clinical setting, accurately influencing these differentiation pathways through external stimuli is more challenging and complex than in in-vitro settings. MSC exhibit a broad scope of phenotypic and functional characteristics (169). Variables such as donors, tissue origin, culturing and isolation protocols, as well as passage number may influence the degree of expression of these characteristics (169).

In-vitro, MSC have also shown immunomodulatory properties: suppression of the immune response of B cells, T cells, natural killer cells and macrophages (184-186). The immunomodulation is achieved by the combined action of known immunosuppressive mediators and inflammatory molecules, such as nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), tumor necrosis factor-inducible gene 6 protein (TSG6), CCL-2, and programmed death ligand 1 (PD-L1) (186). Inactivated MSC do not show expression of these factors unless stimulated by inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (186, 187).

Thus, in the context of the limited regenerative capacity of most tissues in the body, MSC have been used to stimulate regeneration of form and function of damaged tissues.
Table 1. An outline of the minimal criteria in expressed surface markers for identification and characterization of MSC (167, 188-192).

<table>
<thead>
<tr>
<th>Surface marker</th>
<th>Name and function</th>
<th>Expression</th>
</tr>
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<tbody>
<tr>
<td>CD105</td>
<td>Endoglin (angiogenesis) (previously identified through Mab SH2)</td>
<td>+ve</td>
</tr>
<tr>
<td>CD90</td>
<td>Thy-1 cell surface antigen (osteoblastic differentiation)</td>
<td>+ve</td>
</tr>
<tr>
<td>CD73</td>
<td>5'-nucleotidase ecto (previously identified through MAb SH3 and SH4) (lymphocytes, endothelial cells and fibroblasts)</td>
<td>+ve</td>
</tr>
<tr>
<td>Stro-1</td>
<td>Heat shock protein family A (Hsp70) member 8 (endothelial antigen)</td>
<td>+ve</td>
</tr>
<tr>
<td>CD34</td>
<td>CD34 molecule (hematopoietic stem cell adhesion)</td>
<td>-ve</td>
</tr>
<tr>
<td>CD45</td>
<td>Protein tyrosine phosphatase, receptor type C (Pan-leukocyte marker)</td>
<td>-ve</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Human leukocyte antigen, antigen D Related</td>
<td>-ve*</td>
</tr>
<tr>
<td>CD14/CD11b</td>
<td>CD14 molecule/Integrin subunit alpha M</td>
<td>-ve</td>
</tr>
<tr>
<td>CD79alpha/CD19</td>
<td>CD79a molecule/CD19 molecule B cell receptor adaptor molecule</td>
<td>-ve</td>
</tr>
</tbody>
</table>

* unless stimulated with IFN-γ

In the oral region, different populations and potential sources of adult stem cells are to be found within the specific oral tissues. Bone marrow stem cells (BMSC), dental pulp stromal cells (DPSC), stem cells of the apical papilla (SCAP), periodontal ligament stem cells (PDLSC) and stem cells from human exfoliated deciduous teeth (SHED) are all examples of these various cell populations, as illustrated in Figure 8 (127). For the studies in this thesis, stem cells from bone marrow and dental pulp were used.
Figure 8. Illustration showing the different populations of stem cells present in the tissues of the oral environment. Bone marrow stem cells (BMSCs), stem cells from human exfoliated deciduous teeth (SHED), dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells of the apical papilla (SCAP), dental follicle stem cells (DFSCs), tooth germ progenitor cells (TGPCs), salivary gland stem cells (SGSCs), inflamed periapical progenitor cells (iPAPCs), oral epithelial stem cells (OESCs), gingival-derived mesenchymal stem cells (GMSCs), and periosteum-derived stem cells (PSCs). Diogenes et al., An update on clinical regenerative endodontics. Endodontic Topics 2013.

1.10 HBMSC and hDPSC

HBMSC are specialized bone marrow derived MSC which are capable of multipotent self-renewal (193). These cells are traditionally identified through their capacity to adhere to plates and form colonies in-vitro. Morphologically, hBMSC appear as a group of large, flat or cuboidal cells and spindle shaped cells (194). They express a range of non-specific cell surface markers that make them identifiable as MSC. HBMSC can differentiate into multiple cell lineages when restrictively directing conditions are imposed (195). Various treatment protocols involving the use of TSC mandate the recruitment of cells from periapical sites: this induces the influx of
hBMSC and stem cells from the apical papilla (SCAP) (82, 125). HBMSC are used extensively in research because of their clinical importance and availability (196). Dental pulp stromal cells (DPSC) are derived from dental pulp tissue (171). The dental pulp consists primarily of fibroblasts, neural fibers and vasculature as well as MSC (197). DPSC are a clonogenic population of MSC characterized by their rapid proliferative capacity compared with BMSC (174). They are harvested from both primary and permanent teeth and represent a unique cell population, with reported potential in dental pulp engineering and therapy (37, 198). DPSC are considered to be MSC because they are positive to MSC cell surface markers (199). Their gene expression profile pattern is reported to be similar to that of BMSC, with markers such as STRO-1, CD90, and CD105 identified (174, 200, 201). However, it is important to note that not all populations of DPSC will express the same surface markers (188). HDPSC have shown multiple differentiation potential and a more specific odontoblastic differentiation ability than BMSC (202). This has been observed through the expression of odontoblastic gene markers crucial for dentinogenesis, specifically dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (174, 203). In-vitro studies have shown that both BMSC and DPSC have the capacity to form calcified deposits (174, 204, 205). However, compared with BMSC, DPSC induce far less extensive calcification in the form of calcified deposits and are also unable to develop lipid-laden adipocytes (174). Numerous in-vivo and in-vitro studies have demonstrated DPSC differentiation into osteoblasts, odontoblast-like cells, adipocytes, neural cells, myocytes, and chondrocytes. However, it is important to note that the multipotent differentiation capacity of DPSC in-vivo is much less than that of BMSC (189, 206-208).

1.11 Cellular responses to TSC

During clinical applications, the material constituents and chemical composition of TSC play a significant role in the interaction of the TSC with the surrounding cellular microenvironment (209). These interactions stimulate desirable treatment outcomes such as the formation of reparative dentin during VPT and odontoblast/osteoblast differentiation during REP (210). In order to elicit such desirable outcomes, TSC must
induce cellular responses in terms of cell proliferation, cell survival, expression of cell differentiation markers, expression of inflammatory markers, cellular adhesion, and calcium deposition (210-212).

It is important that TSC do not inhibit cellular proliferation. Studies have shown that Biodentine and MTA both have a similar non-inhibitory effect on the cellular viability of hDPSC (53). Furthermore, the characteristics of TSC can be influenced by the composition ratio of silica/calcium. Increasing the ratio of silica to calcium in the composition of TSC leads to promotion of cell attachment and an increase in proliferation (213). It has been shown that the viability of fibroblasts and cementoblasts in pulp and periodontal tissue are not inhibited by Biodentine, MTA or Bioaggregate (214, 215). However, despite the considerable body of research on MTA, there is a lack of studies directly comparing the effects on cellular viability of MTA, Biodentine and TotalFill.

Cell viability and proliferation share a reciprocal relationship with differentiation and this is essential for regeneration (216, 217). TSC can affect the gene expression profile of various types of cells including hDPSC, hBMSC, SCAP, osteoblasts, odontoblasts and cementoblasts (210, 218). The silica and calcium content of TSC induces expression of osteogenic differentiation markers: this has been observed in hDPSC (210) and gingival fibroblasts (212). The calcium content of TSC leads to a release of calcium ions which induce mineralization in the form of calcium deposition. In mice, it was shown that MTA induced the osteogenic differentiation marker alkaline phosphatase (ALP) and the dentinogenic differentiation marker DSPP in cells of the dental papilla (219). BMSC treated with ProRoot MTA showed enhanced osteogenic and odontogenic responses, as evidenced by expression of markers such as ALP, runt-related transcription factor 2 (RUNX2), osteocalcin (OC), and ALP activity and deposition of calcium (220). However, another 2014 study reported that MTA did not lead to induction of osteogenic differentiation by expression of osteogenic markers in hBMSC (196). Thus, the results of studies of the osteogenic profile of TSC are inconsistent, and there is a need for further investigation of the osteogenic potential of MTA, Biodentine and TotalFill.
TSC can influence the inflammatory response by stimulating inflammatory cells such as macrophages and lymphocytes to release inflammatory cytokines. Regulation of the inflammatory response through release of inflammatory cytokines is important in the healing process (9). It might therefore be desirable for TSC to induce the expression of molecules involved in resolution of inflammation and the healing process before the cells enter the mineralization phase. Some studies however report that MTA and Biodentine do not elicit an extreme and prolonged inflammatory reaction that is detrimental to hDPSC (53). Regulation of inflammation includes the eventual resolution of inflammation, to allow differentiation of MSC into odontoblasts, osteoblasts and cementoblasts (221). Macrophages exposed to MTA formulations induced the release of inflammatory cytokines IL1β, IL6 and IL8 (12, 13). These markers are shown to inhibit osteogenic and dentinogenic differentiation of SCAP (222).

Because TSC have similar compositions, specifically silica and calcium content, it has been logically assumed that they induce similar cellular responses. However, many studies report contradictory findings. There is a lack of more detailed comparisons of the cellular responses of MTA, Biodentine and TotalFill, particularly for TotalFill which has only recently been introduced.

1.12 Rationale and aim

The use of TSC in dentistry has expanded in recent years. Manufacturers have introduced new and reformulated materials, intended to improve material properties and establish operator preference. Although numerous TSC have been studied and their use is well documented, there is still inadequate research directly comparing different TSC.

The introduction of TSC into the root canal space during REP places the material in direct contact with the surrounding and juxtaposed dental tissue. This application not only induces biological responses, but also has a direct mechanical effect on the hard tissue of the tooth, of relevance to the susceptibility of immature teeth to fracture. It is reasonable to assume that different TSC will demonstrate different levels of
microhardness and subsequently may have different effects on the structural integrity of the tooth.

It is also essential to study the effects of TSC on the mesenchymal cells recruited during REP. Specifically, MSC present within the pulp and periapical area as hDPSC and hBMSC respectively. The different composition of materials, handling characteristics, and setting times may induce different cellular responses. The question arises as to whether induction of regeneration yields tissue that replicates the original lost tissue and if so, to what extent this tissue resembles the original (223). Ultimately, \textit{in-vitro} studies which simulate the dynamic biological environment can provide initial answers to such questions and serve as pre-requisites and guides for \textit{in-vivo} studies.

In clinical practice however, TSC are used interchangeably and indiscriminately, under the assumption that they induce similar cellular responses and clinical outcomes. This is primarily due to inconsistent reports in the literature to date: due to lack of conformity of experimental designs, it is difficult to compare materials. The null hypothesis tested in this thesis is that TSC used in REP do not influence tooth fracture resistance and that TSC do not induce different biological responses by hBMSC and hDPSC. Thus, the aim of this project is to investigate and compare the effects of commonly used TSC on the surrounding biological tissues, within the context of the selected treatment method.
1.13 Specific aims

To compare the three different TSC, MTA, Biodentine, and TotalFill with special reference to:

1. Microhardness.
2. Fracture resistance of simulated immature teeth treated with REP and sealed cervically with TSC. (Study 1)
3. HBMSC proliferation, osteogenic differentiation, and the expression of markers of angiogenesis in-vitro. (Study 2)
4. HDPSC proliferation and the expression of markers of osteogenesis, angiogenesis, and the inflammatory response in-vitro. (Study 3)
2. Materials and Methods

2.1 Test Materials

The following tricalcium silicate-based cement materials were used in this project:
White MTA-Angelus (Angelus, Londrina, PR, Brazil) (MTA) (Figure 9): supplied as a powder and distilled water (Table 2).

Figure 9. Mineral Trioxide Aggregate (224)

Biodentine (Septodont, Saint-Maurdes Fosses, France) (Biodentine) (Figure 10): Supplied as a powder and a liquid (Table 2).

Figure 10. Biodentine (225)

TotalFill BC RRM PUTTY (FKG Dentaire, La-Chaux-de-Fonds, Switzerland) (TotalFill) (Figure 11): Supplied as a ready-made/ready-to-use paste (Table 2).
Table 2. Overview of tricalcium silicate materials used in the studies: composition and properties according to the manufacturers

<table>
<thead>
<tr>
<th>Material</th>
<th>Color</th>
<th>Form presented</th>
<th>Powder content</th>
<th>Liquid content</th>
<th>X-ray contrast</th>
<th>pH (After setting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTA-ANGELUS® (Angelus, Londrina, PR, Brazil)</td>
<td>White</td>
<td>Powder and liquid</td>
<td>Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, iron tetracalcium aluminate, bismuth oxide</td>
<td>Distilled water</td>
<td>Bismuth Oxide</td>
<td>10 - 13</td>
</tr>
<tr>
<td>Biodentine® (Septodont, Saint-Maurdes Fosses, France)</td>
<td>White</td>
<td>Powder and liquid</td>
<td>Tricalcium and dicalcium silicate, calcium carbonate and zirconium oxide</td>
<td>Calcium chloride</td>
<td>Zirconium oxide</td>
<td>10 - 13</td>
</tr>
<tr>
<td>TotalFill® BC RRM™ (FKG Dentaire, La-Chaux-de-Fonds, Switzerland) /Endosequence® BC RRM™ PUTTY (Brasseler, USA)</td>
<td>White</td>
<td>Ready-to-use Paste</td>
<td>Calcium silicates, zirconium oxide, tantalum oxide, calcium phosphate monobasic and filler agents</td>
<td>No liquid</td>
<td>Zirconium oxide, tantalum oxide</td>
<td>11 - 12</td>
</tr>
</tbody>
</table>
2.2 Study design flow chart

Figure 12. Flow chart: methodology

2.3 Vickers’s microhardness test

The Vickers’s microhardness test was undertaken to assess resistance of the test materials to plastic deformation of the surface after indentation (63). The test materials were prepared according to the manufacturers’ instructions. Polyvinyl chloride cylinder molds 20mm in diameter and 17mm in height were first used to prepare acrylic resin cylinders (Heraeus, MELIODENT® Rapid Repair). Five specimens of each material were then placed in scoop-like holes made on the top surface of the acrylic cylinder by an acrylic trimming bur. Composite (3M ESPE Filtek™ Supreme XTE) was prepared using a 4th generation bonding system and served as a control. The material specimens were then placed into the prepared scoop-like holes and allowed to set in a 37°C incubator at 75% humidity for 72hrs, for optimal setting (226). Prior to testing all specimens were wet polished on a Struers TegraForce-1 polishing machine (Struers Inc. Westlake, Ohio, USA) using FEPA waterproof silicon Carbide sandpaper
A Vickers MicroHardness tester (Zwick/Roell, Indentec Hardness Testing Machines Limited, Brierly Hill, UK) with a square-based pyramid-shaped diamond indenter was then used to make five different indents on the polished surface of each individual specimen, applying a 200g load force for 20 sec (Figure 13) (64, 227). The resultant indentations were immediately analyzed under the microscope and a Hardness Vickers’s (HV) reading was displayed on a digital readout (69). The Vickers’s hardness tester uses the following formula to generate the reading: \[
HV = 1.854 \times \left(\frac{F}{d^2}\right),
\]
where \(F\) is load (kg\(^{-1}\)) and \(d\) is the mean of the two diagonals (Figure 13).

Figure 13. **Vickers Hardness test indentation examples on three samples with different hardness levels.** The smaller the indent the harder the material. The indent on sample A is the smallest, indicating that A has the highest microhardness (228).
2.4 Bovine teeth preparation (Study 1)

Bovine mandibular incisor teeth were obtained from Fatland Slakteri, Ølen, Norway (Fatland Ølen A/S) (Figure 14) (229). The extracted teeth were cleaned and stored in 1% Benzalkonium Chloride until use (210).

The extracted teeth were examined in detail. Those with visible cracks/fractions were discarded. The sound teeth were then prepared according to a standard protocol intended to simulate the root length and thickness of immature teeth (Figure 15). The teeth were sectioned with a water-cooled low speed diamond bur, 10mm coronal to the cemento-enamel junction (CEJ) and 15mm apical to the CEJ. The root canal was thereafter instrumented and widened with a size 6 peeso reamer, until an ISO size #120 file could pass completely unhindered throughout the canal. The internal canal diameter and the remaining dentin thickness were standardized close to 2mm (Figure 15) (113, 114, 230-232).
The root canals of the controls (intact teeth) were not prepared. These teeth were sectioned to the standardized crown/root ratio only (Figure 15). The dimensions (crown/root ratio, intracanal diameter at CEJ, dentin thickness at CEJ, and cervical plug length) of all teeth were measured by digital radiography (Figure 16). A control group of “simulated immature teeth with no intervention” was not included in our study as the primary aim was the comparison of the materials at the cervical part of the root and whether they increase fracture resistance.
Figure 16. A flowchart of the tooth preparation procedure for the different groups in the study. MTA (n=11, group 1), Biodentine (n=10, group 2), TotalFill (n=10, group 3), Gutta Percha (GP) (n=10, group 4), controls (intact teeth, n=10, group 5).
2.5 Regenerative endodontic procedure (Study 1)

The regenerative endodontic protocol used at the Department of Clinical Dentistry, University of Bergen, was applied to the simulated immature teeth (Figure 17). Canals were irrigated and dried with paper points before filling with a triple antibiotic paste. The teeth were then sealed coronally with temporary filling material and stored in an incubator for 10 days (114). TSC cervical plugs were used in groups 1-3 and the teeth in group 4 were obturated with gutta percha. The teeth in groups 1-3 were then stored for 24 hours in an incubator, to allow setting of the TSC plug, and the teeth in group 4 were immediately restored with composite. The next day, groups 1-3 were restored
with composite. All teeth were then inserted into and stored in wet floral arrangement foam in an incubator until fracture resistance testing.

### 2.6 Fracture resistance testing (Study 1)

Figure 18. *Fracture resistance testing*

The fracture resistance testing procedure, including pre-testing preparation, is outlined in Figure 18. Wax-covered roots were embedded in freshly mixed acrylic resin cylinders to create a socket (231). The wax was cleaned from the root surfaces, leaving a 0.2-0.3mm margin between the root surface and the acrylic resin. The root surfaces were then coated with a thin layer of polyvinylsiloxane impression material, to simulate the periodontal ligament (PDL) (113, 230, 233-235) and re-embedded into the acrylic block socket (Figure 19). The acrylic block was firmly anchored at a 45° angle to the long axis of the tooth (Figure 19). A steadily increasing compressive force at a test rate
of 0.05mm/s was exerted on the tooth at the positioned angle until fracture occurred (113, 114, 230-232, 236). The peak load at fracture was recorded in Newtons (N).

![Fracture-testing setup](image)

**Figure 19. Fracture-testing setup**

### 2.7 TSC material extract preparation (Studies 2 & 3)

Material eluate extracts were prepared from TSC. Under sterile conditions, MTA and Biodentine were mixed and prepared according to the manufacturers’ instructions. Four plugs of MTA, Biodentine, and TotalFill were made using an amalgam carrier and placed in pre-weighed Eppendorf tubes (Figure 20). Using the same amalgam carrier for all materials ensured that the diameter and thickness of the plugs was standardized. The material plugs were allowed to set in an incubator at 37° C for 24hrs (237).
ISO Standards 10993-5 (238) were followed to prepare material eluates. Each Eppendorf tube was filled with 1 ml of serum-free Mesencult MSC Basal Medium with 1% penicillin and stored in an incubator for 24hr. The medium was filtered through sterile filters and stored for later use (239). The stock solution was serially diluted with osteogenic medium (Mesencult MSC Basal Medium, with 10% Mesencult MSC Stimulatory Supplement and 1% Penicillin (complete medium) together with 100mM dexamethasone, 10mM β-glycerophosphate, 0.05mM ascorbic acid) (240, 241) (Figure 21).
2.8 Expansion of hBMSC (Study 2)

Primary hBMSC (StemCell Technologies) were cultured and expanded in-vitro, then cultured in complete Mesencult MSC Basal Medium with 10% Mesencult MSC Stimulatory Supplement and 1% Penicillin at 37° C, 6% CO₂ and 100% humidity. The cells were usually allowed to reach a confluence of about 70-80% before being passaged. They were then detached from the plates using trypsinization and counted using a trypan blue assay in an automated cell counting machine, evaluating cell number and viability. Viability was presented as a percentage of the total number of cells and shown to be 86% - 94%. Cells from passages 2-8 were used for the experiments.

The cell morphology of the cultured cells was documented by light microscopy (Nikon Eclipse E80i, Nikon Instruments, Tokyo, Japan).

After the cells have been seeded into the appropriate wells for each experiment, they were left for 24hrs to allow complete cellular attachment to the bottom of the well plate before exposure to the material extracts for various periods of time (6hrs, 1 day, 3 days, 7 days and 14 days). Untreated hBMSC served as controls.

2.9 Isolation and expansion of human Dental Pulp Stromal Cells (Study 3)

Human Dental Pulp Stromal Cells (hDPSC) were isolated from the third molars of healthy adults at the Department of Clinical Dentistry, University of Bergen, Norway. The following protocol was approved by the ethics committee, Norway (225.05, 3.2008.1750, 2009/610 and 2013/1248). The teeth were extracted during routine clinical procedures (impacted tooth removal) and collected after informed consent of the patients. The teeth used for pulp cell isolation were removed in toto and were caries free. The cells were then isolated using enzymatic dissociation according to previously reported protocols (242) (174, 199, 243). Briefly, a groove (0.5 – 1mm) was created along the CEJ using a high-speed bur. A chisel was then used to split the tooth to expose the canal chamber and harvest the pulp tissue. The pulp tissue was then minced into very small pieces before being exposed to enzymatic dissociation. The minced tissue
was placed in an enzymatic digestive solution of collagenase type 1 (4mg/ml) and dispase (2mg/ml) for 1hr at 37°C. The digested tissue was centrifuged at 1400rpm for 10min and then filtered through a 70µm strainer. Single cell suspensions were cultured and expanded with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Sigma-Aldrich), 4mM L-glutamine (Thermofisher), 100U/ml penicillin (HyClone) and 100µg/ml streptomycin (HyClone) in a 5% CO₂ incubator at 37° C. The culture medium was changed twice weekly. At 75% confluence, cells were either subcultured or stored in liquid nitrogen for future use.

The hDPSC were phenotypically characterized and evaluated for mesenchymal stem cell markers. Flow-cytometric analysis on the 1st passage (primary cells) was carried out using fluorescein isothiocyanate (FITC) mouse anti-human CD90 (BD Biosciences), allophycocyanin mouse anti-human CD105 (Southern Biotech), PerCPCy5.5 mouse anti-human STRO-1 (Santa Cruz Biotechnology, Inc.), and phycoerythrin mouse anti-human CD24 (R&D System) as positive markers (199, 242). A 20µl blocking reagent (0.5% bovine serum albumin (BSA) in phosphate buffered saline (PBS) at a pH of 7.4) was used on cells at a cell density of 5×10⁵ per tube at 37°C for 10min. They were then stained by incubation in the dark for 1hr by conjugated antibodies STRO-1 (1:125), CD90, CD24 and CD105 (1:50). The cells were then washed with 200µl PBS and centrifuged for 5min at 4°C and a rate of 300 g. After centrifugation, the cell pellet was resuspended in 300µl of 10% paraformaldehyde and stored at 4°C. During flow cytometric analysis (BD LSRFortessa™ Flow Cytometer System (BD Biosciences), 100,000 events were used for each individual sample (199). The flow cytometric and immunophenotypical analysis showed that at the 1st passage, the hDPSC were positive for CD90 (45.4%), STRO-1 (6.1%), CD105 (5.8%), and CD24 (95.9%) (199). The hDPSC used in this study were from passages 3-7.

The cell morphology of the cultured cells was documented by light microscopy (Nikon Eclipse E80i).

Three different donors with a mean age of 22 were used in this study, referred to as DM1 (male, 20yrs), DM10 (female, 22yrs) and DM17 (male, 24yrs).
After the cells had been seeded in the appropriate wells for each experiment, they were left for 24hrs to allow for complete cellular attachment before being exposed to the material extracts for various periods of time (1 day, 3 days, 7 days). Untreated hDPSC served as controls.

2.10 MTT proliferation assay (Studies 2 & 3)

HBMSC and hDPSC were seeded at a density of $0.1 \times 10^5$ cells per well on 96-well plates. They were then stimulated by exposure to serially diluted TSC extract concentrations (1:2, 1:4, 1:8, 1:16) for different experimental periods (1 day, 3 days and 7 days). The 3-(4,5-dimethyl-thiazoyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay was used to test cell metabolism. A microplate reader was used to record absorbance at a wavelength of 570 nm.

2.11 RNA extraction and quantitative reverse transcription PCR (Studies 2 & 3)

HBMSC and hDPSC were seeded in 6-well culture plates at a density of $0.3 \times 10^6$ cells per well. The cell monolayer was exposed to TSC extract medium (eluate of 1:4 dilution) for various periods (6hrs 1 day, 3 days and 7 days). At the end of each experimental period, the cell culture supernatant was collected in 300µl aliquots and stored at -80°C for future protein assessment. The cell monolayer was washed with PBS and the plate was then stored at -80°C for later RNA analysis. Total RNA was then isolated using a commercially available kit (Maxwell 16 Cell LEV Total RNA purification Kit, Promega, Wisconsin, USA). NanoDrop Spectrophotometer (ThermoFisher Scientific NanoDrop Technologies, Wilmington, Delaware, USA) was used to measure the quantity and quality of RNA.

After RNA extraction, a cDNA reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit by Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 50 µg of total RNA to complementary DNA (cDNA). Quantitative reverse transcriptase PCR (qRT-PCR) was completed to quantify the relative changes in gene expression. Amplification was done in triplicate replicates with a reaction volume of
10 µl for every marker. The PCR reaction in this thesis was recorded using the following TaqMan™ probes (Applied Biosystems, Foster City, CA, USA): Alkaline phosphatase (ALP), Collagen 1alpha (Col1A), Osteoprotegerin (OPG), osteocalcin (OC), Runt-related transcription factor 2 (RUNX2), Vascular endothelial growth factor A (VEGF-A), Fibroblast growth factor (FGF-1), Interleukin 1 beta (IL1β), Interleukin 6 (IL6), and Tumor necrosis factor alpha (TNFα) as summarized in Table 3. The osteogenic markers were chosen as they can provide a general overview of the hard tissue regenerating potential of the material whereas the angiogenic and inflammatory markers chosen could provide preliminary information and guide for further investigations. Furthermore, comparison between the responses of the two cell types could be made. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as the endogenous control gene (Table 3). The comparative $2^{-\Delta\Delta Ct}$ method was used to compute relative gene expression. The target gene value was calculated in relation to the control samples, setting the control value to 1.0. GAPDH was stable throughout the experiments.
Table 3. *Overview of TaqMan probes used in this thesis*

<table>
<thead>
<tr>
<th>GENE</th>
<th>ASSAY ID</th>
<th>MARKER</th>
<th>AMPLICON LENGTH</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Hs99999905_m1</td>
<td>Endogenous control</td>
<td>122</td>
<td>Study 2 &amp; 3</td>
</tr>
<tr>
<td>ALP</td>
<td>Hs01029144_m1</td>
<td>Osteogenic</td>
<td>79</td>
<td>Study 2 &amp; 3</td>
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<tr>
<td>COL1A</td>
<td>Hs00164099_m1</td>
<td>Osteogenic</td>
<td>68</td>
<td>Study 2 &amp; 3</td>
</tr>
<tr>
<td>OPG</td>
<td>Hs00900358_m1</td>
<td>Osteogenic</td>
<td>74</td>
<td>Study 2 &amp; 3</td>
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<tr>
<td>OC</td>
<td>Hs01587814_g1</td>
<td>Osteogenic</td>
<td>138</td>
<td>Study 2 &amp; 3</td>
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<td>Hs01047973_m1</td>
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<td>VEGF-A</td>
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<td>59</td>
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<tr>
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<tr>
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<td>80</td>
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</tr>
</tbody>
</table>
2.12 Enzyme-Linked Immunosorbent Assay (ELISA) (Studies 2 & 3)

After exposure of cells to 1:4 dilution of TSC eluates, the protein levels of VEGF-A in the cell culture supernatants from hBMSC and hDPSC were measured with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s recommendations.

2.13 Alkaline phosphatase quantification (Studies 2 & 3)

Extracellular ALP was measured with a colorimetric assay kit (Sigma-Aldrich) in previously collected and stored culture supernatant of cells exposed to material eluates (1:4 dilution) for various time periods. ALP activity was expressed as optical density measured at 405nm using a microplate reader (FLUOstar OPTIMA).

2.14 Alizarin Red staining (Study 2)

Alizarin Red staining was used for qualitative evaluation of in-vitro mineralization (deposition of calcium) of hBMSC exposed to TSC eluates. HBMSC were seeded at a density of $0.3 \times 10^4$ cells per well onto 24-well culture plates. The cells were then stimulated by exposure to TSC eluate (1:4 dilution) for 1, 3, 7 and 14 days. The cells were then washed with PBS and fixed using 10% neutral buffered formalin before incubation with Alizarin Red S staining solution (Carl Roth, Karlsruhe, Germany). The treated cellular monolayer was observed with light microscopy at a magnification of 10× (Nikon Eclipse E80i).

2.15 Statistical analysis (Studies 1-3)

The statistical package programs used in this thesis were GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA) (Study 1-3). The analyses used in this thesis are outlined in Table 4. In addition, boxplots were prepared for the presentation of the results.
Table 4. **Overview of statistical analyses**

<table>
<thead>
<tr>
<th>Study</th>
<th>One-way ANOVA with Bonferroni’s post hoc test</th>
<th>Two-way ANOVA with Bonferroni’s post hoc test</th>
<th>Two-way ANOVA with Dunnett’s post hoc test</th>
<th>Kruskal-Wallis with Dunn’s post hoc test</th>
<th>Mann-Whitney test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Study 2</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Study 3</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3. Results

3.1 Vickers’s Microhardness Test

Analysis of the images showed that materials with a higher surface microhardness were better wet polished and consistently resulted in a smoother surface finish. This was particularly evident on the surface of composite. The smaller the size of the indent created, the higher the microhardness, as more resistance was present (Figure 22). TotalFill did not have the surface microhardness necessary to allow a measurable HV reading. This was evident as the 10× magnification did not allow for complete accommodation of the diamond shape indent made on the material surface, leading to inability to read the final indent (Figure 22). Composite (106.3 ± 11.47 HV) was the hardest material in the Vickers test compared with the tested TSC (Figure 23). Biodentine (87.12 ± 10.4 HV) displayed a significantly higher surface microhardness than MTA (14.2 ± 3.8 HV) (Figure 23).
Figure 22. *Diamond indent created on wet polished surface of Composite, Biodentine, MTA, and TotalFill.* The size and clarity of the indent are a direct representation of the material microhardness. Small clear indents on a smoother finish surface indicate higher microhardness, as seen for the composite and the Biodentine. The indent created on the surface of TotalFill was unsuitable for measurement and analysis by the Vickers MicroHardness Test, indicating very low surface microhardness. Bars: 200µm.
Hardness Vickers (HV) test. The tested materials exhibited significant differences in surface microhardness. Composite had the highest microhardness ($106.3 \pm 11.47\text{ HV}$), followed by Biodentine ($87.12 \pm 10.4\text{ HV}$) and MTA ($14.2 \pm 3.8\text{ HV}$). The results are presented as mean±SEM, Kruskal-Wallis test with Dunn’s multiple comparison, ***p < 0.001.

In this context, it was of interest to investigate whether the differences in material microhardness would result in differences in fracture resistance of teeth treated with these materials, particularly for TSC in the cervical area of immature teeth.

3.2 Simulated immature teeth (Study 1)

To guarantee experimental standardization, the crown-root ratios, the intracanal diameter at the CEJ and dentine thickness were measured. These measurements indicated no intergroup differences among the simulated immature teeth. Nor were there any differences among the TSC groups (1-3) in terms of the length of the cervical plug. All prepared teeth (groups 1-4) had a significantly higher canal diameter and lower dentin thickness at the CEJ than the intact teeth (group 5, p < 0.05) (Figure 24).
Figure 24. *Intracanal and dentin thickness differences between intact teeth and simulated immature teeth.* The intracanal diameter was significantly higher in simulated immature teeth. The dentin thickness at the CEJ was significantly higher in intact teeth than in simulated immature teeth. The data are presented as mean ± SEM, Mann-Whitney test, *p < 0.05; **p < 0.001.

3.3 Fracture testing (Study 1)

Fracture testing showed that intact teeth required a significantly higher peak load to fracture (1669 ± 60.77 N) than all the other test groups (groups 1-4). Among the “immature” teeth there were no differences in peak load required to fracture (Figure 25).

Figure 26 shows the fracture pattern observed after testing. Simulated immature teeth fractured at the interface of the material plug/or gutta percha and the coronal composite filling. The fracture line pattern of intact teeth was within the crown. (Figure 26).
Figure 25. Fracture testing results. A significantly higher peak load was needed to achieve fracture in intact teeth than in all groups of simulated immature teeth (group 1-4). However, there were no differences among groups 1-4 in the peak load required to fracture. Data presented as mean ± SEM, Kruskal-Wallis test with Dunn’s multiple comparison, *p < 0.05; **p < 0.01.
Figure 26. Examples of typical fracture patterns of tested immature teeth showing lingual and lateral aspects. a-b: MTA. c-d: Biodentine. e-f: TotalFill. A diagonal fracture line is seen through the canal buccolingually. This line crosses the interface between material plug and composite filling. Intact teeth (g-h) showed a similar fracture line pattern located within the crown. Scale bars = 2mm.
3.4 Cellular morphology (Studies 2 & 3)

Cellular morphology of hBMSC and hDPSC was assessed microscopically during culture procedures. Both cell types shared similar fibroblast-like morphological characteristics, with a flat elongated or narrow spindle shape (Figure 27). Morphology was unchanged throughout culture and passaging and no morphological differences between hDPSC donors (Study 3) were seen.

Figure 27. Human bone marrow stem cells (A) and human dental pulp stromal cells (B) in culture under 10× magnification showing fibroblast-like spindle shaped morphology. HBMSC are in culture with complete Mesencult MSC Basal Medium with 10% Mesencult MSC Stimulatory Supplement and 1% Penicillin. HDPSC are in culture with DMEM supplemented with 10% FBS, 4mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Scale bars = 100μm

3.5 MTT assay (Studies 2 & 3)

In both cell types, the MTT results showed similar responses by the cells to the TSC. One day of exposure to the TSC eluates did not affect cell proliferation. Differences between the materials were apparent after 3 days, with TotalFill showing a greater inhibitory effect on cell proliferation of both hBMSC and hDPSC. After 7 days’ exposure to TSC, MTA and TotalFill exhibited the highest inhibitory effect on cell proliferation of both hBMSC and hDPSC, whereas Biodentine had the least inhibitory effect (Figure 28).
Figure 28. Proliferation of hBMSC and hDPSC after exposure to TSC. Generally, both cell types responded similarly to the TSC eluate exposure. The longer the exposure time, the greater the inhibition of cell proliferation, in a dose-dependent manner. It was apparent at 7 days that compared to MTA and TotalFill, Biodentine had the least inhibitory effect on hBMSC and hDPSC proliferation. [For the hDPSC, OD values were normalized in relation to the control samples and presented as relative cell proliferation (controls values set at 1)]. The data are presented as mean ± SEM. Detailed statistical analysis is presented in in papers 2 & 3.

3.6 Osteogenic, angiogenic and inflammatory gene expression (Studies 2 & 3)

A 1:4 ratio concentration/dilution of material eluate was used for cellular stimulation at all experimental timepoints for both hBMSC and hDPSC.
RNA quantity and quality for hBMSC and hDPSC

<table>
<thead>
<tr>
<th>RNA</th>
<th>VALUES</th>
</tr>
</thead>
<tbody>
<tr>
<td>QUANTITY</td>
<td>100 ng/µl - 350 ng/µl</td>
</tr>
<tr>
<td>QUALITY</td>
<td></td>
</tr>
<tr>
<td>260/280</td>
<td>1.87-2.2</td>
</tr>
<tr>
<td>260/230</td>
<td>2.00-2.20</td>
</tr>
</tbody>
</table>

RNA quantity ranged from 100 to 350ng/µl. NanoDrop spectrophotometry indicated good RNA quality and quantity (Table 5).

Gene expression analysis showed variations among the tested TSC. Specifically, Biodentine induced consistent upregulation of osteogenic markers such as ALP, OPG and OC in both hBMSC and hDPSC (Table 6). On the other hand, TotalFill enhanced the angiogenic VEGF-A expression in both hBMSC and hDPSC (Table 6). Compared to the controls, MTA did not induce many changes in the tested genes in either cell type. Inflammatory markers were tested in hDPSC only. IL1β and TNFα were not detected under the experimental conditions. The only difference in inflammatory markers among the TSC tested was that for the longest experimental period, TotalFill downregulated IL6 expression (Table 6).
Figure 29. Differential expression of osteogenic and angiogenic markers by hBMSC after exposure to MTA, Biodentine and TotalFill. Data are presented as Tukey’s boxplot. Statistical significance was determined using two-way ANOVA followed by Bonferroni’s post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.
Figure 30. Differential expression of osteogenic, angiogenic and inflammatory markers by hDPSC after exposure to MTA, Biodentine and TotalFill. Data are presented as Tukey’s boxplot. Statistical significance was determined using two-way ANOVA, followed by Dunnett’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001 compared to control.

3.7 Enzyme-linked immunosorbent assay (ELISA) (Studies 2 & 3)

Both hBMSC and hDPSC showed an increase in VEGF-A production over time. In hBMSC, Biodentine induced a statistically significant increase in VEGF-A production compared to the control and TotalFill at 7 days. In hDPSC however, TotalFill yielded the highest VEGF-A production, compared to the control, at both 3 and 7 days (Figure 29). This finding was also supported by upregulation of VEGF-A gene expression in hDPSC.
3.8 ALP Quantification (Studies 2 & 3)

ALP activity in hBMSC and hDPSC was reduced over time by all TSC. In hBMSC, there were no differences among the tested TSC materials with respect to their inhibitory effect on ALP production (Figure 30). In hDPSC, at 7 days TotalFill maintained the inhibitory effect on ALP activity compared to the control (Figure 30).
ALP activity decreased over time in both cell types. There were no differences between materials in the responses of hBMSC whereas in the hDPSC, TotalFill significantly decreased ALP at 7 days compared to the control. The data are presented as mean ± SEM. The statistical analysis is presented in detail in papers 2 & 3.

Figure 32. *ALP activity in hBMSC and hDPSC.*

3.9 Alizarin Red staining (Study 2)

The stained hBMSC monolayer disclosed osteogenic capacity of the cells *in-vitro.* Mineralization was present after exposure to all the tested TSC but also to the control and this effect increased over time for all groups. This was seen as calcium deposits in the cells.

Although no quantification was made, after 14 days of exposure, cells stimulated with MTA exhibited more pronounced mineralization than the other TSC tested and the control. (Figure 31).
Figure 3. *Alizarin red staining of hBMSC after 14 days of exposure to TSC.* Cells exposed to MTA showed more pronounced mineralization than the control, Biodentine and TotalFill after 14 days. Scale bars = 500µm
### 3.10 Summary of results (Studies 2 & 3)

#### Table 6. Comparison of TSC effects

<table>
<thead>
<tr>
<th></th>
<th>Cell morphology</th>
<th>MTT assay</th>
<th>Gene expression analysis</th>
<th>ELISA</th>
<th>ALP Quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MTA</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hBMSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Inhibition of proliferation at 7 days</td>
<td>COL1A ↑ 7 days, VEGFA ↑ 1 day</td>
<td>No effect on VEGFA production</td>
<td>Extracellular ALP ↓ at 1, 3 and 7 days.</td>
</tr>
<tr>
<td>hDPSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Inhibition of proliferation at 3 and 7 days</td>
<td>OPG ↓ 7 days, IL6 ↑ 3 days</td>
<td>No effect on VEGFA production</td>
<td>Extracellular ALP ↓ at 3 days</td>
</tr>
<tr>
<td><strong>Biodentine</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>hBMSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Inhibition of proliferation at highest concentration at 7 days</td>
<td>ALP ↑ 6 hrs, OPG ↑ 1 day and 3 days</td>
<td>VEGFA ↑ at 7 days</td>
<td>Extracellular ALP ↓ at 1, 3 and 7 days</td>
</tr>
<tr>
<td>hDPSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Inhibition of proliferation at highest concentration at 7 days</td>
<td>ALP ↑ 3 days, OPG ↑ 3 days, OC ↑ 7 days, FGF-1 ↑ 3 and 7 days, IL6 ↑ 3 days</td>
<td>No effect on VEGFA production</td>
<td>Extracellular ALP ↓ at 3 days</td>
</tr>
<tr>
<td><strong>TotalFill</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>hBMSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Inhibition of proliferation at 3 and 7 days</td>
<td>ALP ↓ 3 days, OPG ↑ 1 day, VEGFA ↑ 1 day and 3 days</td>
<td>No effect on VEGFA production</td>
<td>Extracellular ALP ↓ at 1, 3 and 7 days</td>
</tr>
<tr>
<td>hDPSC</td>
<td>Fibroblast-like spindle shaped</td>
<td>Enhance proliferation at 1 day, Inhibition of proliferation at 3 and 7 days</td>
<td>ALP ↓ 7 days, VEGFA ↑ 3 and 7 days, IL6 ↑ 3 days</td>
<td>VEGFA ↑ at 3 and 7 days</td>
<td>Extracellular ALP ↓ at 3 and 7 days (long-lasting effect)</td>
</tr>
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↑ = Upregulation, ↓ = Downregulation
4. Discussion

In regenerative endodontics, the clinical implications of choice of TSC are considerable. Mechanical properties of the TSC need to be taken into account: the teeth being treated are more vulnerable to fracture because of the thin dentinal walls. Biological aspects must also be considered: there is direct contact between TSC materials and the cells and a positive interaction is a major determinant of regeneration and/or repair.

The present project was designed to investigate mechanical and biological aspects of the application of TSC in regenerative endodontics, using an experimental design which simulated clinical conditions. Two types of mesenchymal cells from different sources were utilized. The effect of TSC was investigated not only on cell proliferation but also the potential effects on cellular expression of osteogenic, angiogenic and inflammatory markers.

4.1 TSC effect on tooth fracture resistance

Composite microhardness was tested to illustrate the visual differences in surface polish and indent quality between materials of different microhardness levels as is seen in Figure 22. In addition, the composite’s close proximity to the cervical plug and its use as a coronal restoration justified the comparison to TSC. It is possible that the fracture resistance of the treated teeth could be primarily due to the reinforcement by the significantly harder composite material, a speculation worth investigating further in future projects.

The results of the studies show that the microhardness of Biodentine is higher than that of MTA or TotalFill. Other recent reports also show a higher surface microhardness for Biodentine than for MTA (244, 245). This may be attributable to its low water to cement ratio (246), achieved by adding water soluble polycarboxylate to the powder and a hydrosoluble polymer to the liquid (246, 247). It was of interest to note that after 72 hrs, TotalFill had not achieved the baseline hardness required to register a HV reading, indicating a much lower microhardness for TotalFill than for Biodentine. The microhardness of TotalFill putty is reported to be slightly lower than that of MTA after
7 days, supporting the present results that TotalFill has the lowest microhardness (248, 249). Prior to testing, the materials were allowed to set for a period of 72 hrs, as opposed to 7 days. This shorter setting time may have influenced the significantly lower microhardness of TotalFill: it may not have reached its final set form at 72 hrs. The microhardness levels of Biodentine observed in this study are similar to those of human dentin as reported in the literature (63, 250, 251). As Biodentine was initially developed as a dentin substitute, the current findings are in accordance with that concept.

Based on the findings on microhardness, it was logical to investigate further whether these differences in TSC microhardness are reflected in the fracture resistance of teeth treated with REP. We did not include a control group of non-treated immature teeth as the main scope was to compare the TSC as far as their potential effect on fracture resistance. Furthermore, previous studies have consistently shown a significantly lower fracture resistance of simulated immature teeth when compared to intact teeth (252-254). This is primarily due to the thickness of the dentinal walls. Accordingly, the intact teeth with the thickest CEJ showed significantly higher fracture resistance in the current study. Although no intergroup differences in fracture resistance were observed among the treated immature teeth, a larger sample size of teeth may ultimately have disclosed some differences. Nevertheless, the results support the overall conclusion that TSC applied to the cervical area do not negatively influence the fracture resistance of the teeth.

The results indicate no differences in fracture resistance between teeth in which the canals were obturated with gutta percha and those treated with TSC, nor were there any differences with respect to the individual TSC tested. However, a previous in-vitro study has shown that compared to gutta percha obturation, total obturation of the root canal with MTA or Biodentine significantly increases the fracture resistance (255, 256). This is attributed to “chemical bonding” of TSC and dentin by the formation of an intermediate layer of hydroxyapatite (255, 257, 258). As the TSC in the present study were limited to the cervical area, it may be that the contact surface area between the TSC and dentin was too small to influence fracture resistance. A 2016 study
suggested that filling the whole root canal of immature teeth with MTA reinforced the roots to levels comparable to that of intact teeth (252).

However, our findings are in agreement with another 2016 study comparing intact teeth with simulated immature teeth root-filled throughout the whole canal length with MTA or Biodentine. The intact teeth were significantly more resistant to fracture than the simulated immature teeth (259). This is not unexpected, as resistance to fracture is directly proportional to the remaining tooth structure and the amount of dentin in the cervical area (260, 261). Furthermore, consistent with our findings, no differences in fracture resistance were reported between teeth filled with MTA or Biodentine (259). Although the present findings do not imply that TSC strengthens the cervical area, successful REP results in root completion and development; thus, it is reasonable to assume that the fracture resistance of the tooth would increase. There are however, in-vitro studies reporting that the fracture strength of teeth filled with MTA decreases over time (236). These studies however, may not be able to simulate the process of further root development which can occur in a clinical setting. Considering the susceptibility to fracture of endodontically treated immature teeth (117), continued root development is crucial for long-term tooth survival. Because of the increase in length and thickness of the root, REP is therefore preferable to apexification (262).

In this context, the differences observed in microhardness of the TSC were not manifest as differences in the peak load required to fracture. This may imply that the microhardness of the material in this case had no bearing on the fracture resistance of the teeth: the fracture resistance of the simulated immature teeth may have been related to the composite filling material used as the coronal seal.

4.2 TSC effect on cell proliferation

Cell proliferation and differentiation are crucial indicators of progression of the regenerative process and/or healing of the dental pulp and the surrounding tissues (85, 263, 264). In this project, cell proliferation was assessed by MTT assay. The assay measures cell proliferation through metabolic activity: in the presence of metabolically active cells, the yellow MTT reagent (tetrazolium salt) changes into a purple formazan
product. This can then be measured colorimetrically: the more pronounced purple color indicates higher numbers of viable cells (265). The similarities in methodology allow comparison of the tested materials across studies 2 and 3.

HBMSC and hDPSC showed similar proliferation responses to TSC. In the MTT assay, both cell types demonstrated a dose-related response to material eluates. This is presented as the inhibition of cell proliferation being directly proportional to the eluate concentrations. However, after 7 days of exposure, only Biodentine did not induce a decrease in cell proliferation in both hBMSC and hDPSC. The MTA used in this thesis contains Bi₂O₃ which is reported to have cytotoxic effects on cells (51). When the Bi₂O₃ in MTA was replaced with alternative radiopacifying agents, cell viability improved (266). The presence of Bi₂O₃ in the MTA used in this study may explain the lower proliferation rates of hBMSC and hDPSC with MTA than with Biodentine. This is also reported in the literature: Biodentine supported greater hDPSC viability than MTA during the first 7 days (267, 268). There are however, contradictory reports in the literature, with some studies reporting no difference between Biodentine and MTA with respect to their effect on cellular proliferation (22, 269).

Material eluates in this thesis were prepared through a standard volume of material plug placed in medium after initially setting for 24hrs. If a material has a higher rate of dissolution of its components into the medium it may lead to a higher concentration of stock eluate solution. This may be the case with TotalFill, where the decrease in cell proliferation may be attributed to a higher dissolution rate of material into the medium, possibly due to the long setting time. Guo et al. and Charland et al. report that TotalFill has a longer setting time than MTA (270, 271). Guo et al. measured the setting time of MTA and TotalFill in an incubator (37 °C, > 95 % relative humidity) while Charland et al. compared the ability of the materials to set in wet environments (human blood, culture media, with saline as the control). The setting time proposed by the manufacturer for TotalFill was questioned. Guo et al. suggested that both MTA and TotalFill took 7 days to set completely while Charland concluded that regardless of medium exposure, TotalFill took longer to set than MTA (270, 271). The setting time for TotalFill was reported to be longer than for Biodentine (272). Considered in this context, it is probable that the TotalFill material plugs in the present study may not
have reached final set form in 24hrs and possibly effused more composition into the stock medium than MTA or Biodentine. When dental pulp fibroblasts were exposed to material eluates, TotalFill had a greater inhibitory effect than MTA on proliferation during the first 48hrs. After 5 days the inhibitory effect of TotalFill was similar to that of MTA, whereas after 8 days TotalFill showed more enhanced dental pulp fibroblast proliferation than MTA (273). These results suggest an inhibitory component in TotalFill which decreases, over time, as the material sets.

Overall, the study demonstrated that Biodentine was less harmful to the proliferation of hBMSC and hDPSC than MTA or TotalFill. There are studies showing that compared to MTA, Biodentine increases proliferation of hDPSC (267, 268). However, TotalFill has also been reported to decrease the proliferation of Human Saos-2 osteoblast-like cells compared to MTA (274). While in studies to date there is a lack of direct comparison among MTA, Biodentine and TotalFill, it can be concluded from the available literature that Biodentine is more favourable than MTA to the viability of stem cells. In contrast to our findings however, Sultana et al. showed greater enhancement of hBMSC proliferation by TotalFill than by Biodentine (60). These contradictory findings may be attributable to differences in experimental designs. Sultana et al. seeded cells onto the specimen cement and thus tested the direct effect of contact between the material surface and cells. In contrast, the experimental model in the current studies is more appropriate for comparison of material concentrations. The model is based on the assumption that the soluble products leached from the TSC are the active components. As the results showed that Biodentine was less inhibitory to cell proliferation than MTA or TotalFill, it can thereby be considered the superior material with respect to cell viability.

4.3 TSC effects on cell differentiation

The studies showed that both hBMSC and hDPSC, when stimulated by TSC, expressed markers of osteogenic differentiation. Biodentine exhibited the most pronounced and consistent effect on both cell types. In hBMSC, Biodentine induced upregulation of ALP early and Col1A and OPG late; in hDPSC it induced upregulation of ALP and
OPG earlier and OC later. ALP and OC are typically expressed in a reciprocal manner, where ALP is an early marker and OC is a late marker of osteogenic differentiation (201, 268, 275). ALP expression indicates an initial phase of mineralization and as the cells exit this phase, ALP is substituted with the upregulation of OC (201, 268). These results indicate that Biodentine has a superior osteogenic profile to MTA or TotalFill. This is supported by previous reports that Biodentine has a higher osteogenic differentiation potential than MTA, in hDPSC (276) and in SCAP (8). In the latest study, ProRootMTA and RetroMTA were used (8).

There are, however, reports of pulp canal obliteration of more than 50% in pulpotomies with Biodentine (Figure 32) (277). This has led clinicians to view the pronounced osteogenic response induced by Biodentine as less than ideal. Obliteration of the canal is a sign of increased odontoblastic activity, which demonstrates reparative and regenerative potential (277-279). However, this reparative response can be less than ideal if it overstimulates odontoblastic activity, leading to pulp canal obliteration. Overstimulated odontoblastic activity can also be a result of an unresolved inflammatory reaction, from caries or trauma, regardless of the TSC used in treatment. In other words, it is unclear whether obliteration of the canal is a direct effect of the applied TSC, or due primarily to pulpal inflammation independent of the applied TSC, or a combination of the two (Figures 32 and 33).

Figure 34. *Asymptomatic 11-year-old girl treated with pulpotomy with Biodentine due to pulp exposure upon caries excavation*: pre-op (A) and post-op (B) radiographs. One year later, obvious obliteration of the root canal system (mesial root) is seen (C). Courtesy of N. Bletsa
Before osteogenic differentiation reaches the level of mineralization and late maturation, it passes through stages of early commitment and matrix synthesis (241). This is corroborated by Alizarin red staining images of hBMSC, showing minimal mineralization by all groups at 7 days and eventually marked mineralization and calcium deposition at 14 days. The deposition of calcium ions in the extracellular matrix indicates a commitment of cells towards osteoblastic and osteogenic differentiation. In Study 3 this can be observed by the upregulation of OC by hDPSC stimulated by Biodentine at later timepoints, possibly indicating the initiation of osteogenic differentiation. These findings support those of an earlier study of mesenchymal stem cells stimulated by Biodentine, in which OC expression was upregulated increasingly over time (280). In this context, it may be concluded that under the experimental conditions, Biodentine caused greater expression of osteogenic markers than MTA or TotalFill.

In terms of VEGF-A, TotalFill induced significantly higher gene and protein expression in hDPSC than MTA or Biodentine. In a recent report, MTA and Biodentine induced VEGF-A expression in hDPSC (281). In mice, TotalFill was shown to induce angiogenic responses in the form of VEGFA expression at levels comparable to MTA.
The expression of angiogenic markers of differentiation is a prelude to the formation of vasculature. Although VEGF-A is primarily recognized for its role in angiogenesis, it also contributes to bone development and repair. As bone is a highly vascular organ, blood vessel formation is closely associated with bone formation and therefore these two processes are closely coordinated (283-285). This may explain why when there is an increase in the late expression of osteogenic markers of mineralization, there is also enhanced production of VEGF-A at protein level. This was observed in hBMSC stimulated with Biodentine, in which VEGFA production was higher than in the control.

In hDPSC, the expression of osteogenic markers such as OC and the development of mineralization are also associated with the early expression of inflammatory markers in-vitro, such as IL1β, IL6 and TNFα (286-288).

All TSC induced early expression of the proinflammatory cytokine IL6 in hDPSC but only TotalFill stimulated a late decrease. IL1β and TNFα were not expressed by hDPSC. Previous reports also show early expression of IL6 in SCAP, but in contrast to the present findings, there was also expression of IL1β and TNF from cells stimulated by MTA and Biodentine (289). Unfortunately, the expression of inflammatory markers in hBMSC was not investigated in this thesis. This information might have contributed to a more extensive comparative profile of hDPSC and hBMSC. Non-resolution of inflammation is detrimental to the healing process, but the initial inflammatory reaction is desirable and can be essential to healing (290, 291). The current findings in hDPSC suggest greater anti-inflammatory potential for TotalFill than for MTA or Biodentine. The TSC may affect the immune response by stimulating cells to inhibit the expression of proinflammatory cytokines after the acute phase of inflammation. A decrease in IL6 expression indicates resolution of inflammation, which in turn is conducive to healing and repair (292-294). In a 2018 study, the authors carried out pulpotomies using ProRootMTA and RetroMTA and later extracted the teeth to study the effect of the materials on the quality of dentin bridge formation (147). Histological evaluation revealed that the quality of the dentin bridge was superior in teeth treated with ProRootMTA than with RetroMTA. Furthermore, histological evaluation of the pulp revealed that none of the teeth treated
with ProRootMTA showed signs of pulpal inflammation, whereas 28% of the teeth treated with RetroMTA developed mild pulp inflammation (147). The implication is that inflammation and its resolution influence the outcome of VPT. Based on the expression of inflammatory markers, it can be concluded that although none of the tested TSC induced an exaggerated inflammatory response in hBMSC, TotalFill had an anti-inflammatory effect over time.

4.4 Methodological considerations

In Study 1 it was decided to simulate immature roots, with a root length approximately 2/3 the length of the completely developed root. This is consistent with stage 3 root development according to Cvek’s classification (117, 295). This length was chosen because shorter roots, typical of the earlier stages of root development, were found to be easily dislodged from the acrylic mold during loading. The crown was also standardized for all test teeth and the canals of the prepared teeth were enlarged to a diameter of approx. 2.2 mm, significantly larger than in the intact teeth (ca 1.8 mm).

Obtaining human single-rooted teeth in large numbers proved impractical and an alternative was therefore needed. In order to ensure sufficient numbers of teeth which could be standardized according to the experimental protocol, bovine teeth were used. This bovine tooth model for simulating immature human teeth is well established and has been applied extensively (113, 114, 119, 230, 231, 233, 235, 296). Several studies have demonstrated that bovine incisors are a suitable alternative for mechanical testing as the dentin modulus of elasticity and tensile strength are similar to those of human teeth (113, 297, 298). An advantage of the adoption of bovine teeth is the feasibility of standardization because bovine teeth exhibit fewer morphological variations than human teeth (113, 299). Although it would be an advantage to validate the present findings by tests on human teeth, this would be fraught not only with practical issues but also with concerns about ethics.

The cells types used in this project are human primary cells. They are directly isolated from human tissue and have a limited proliferative capacity and lifespan (81, 300). Primary cells differ from immortal cell lines which are used in numerous areas of research (301). The most significant difference is that primary cells retain most of the
key morphological and functional features of their tissue of origin (302). This characteristic makes the *in-vitro* use of primary cells clinically relevant. On the other hand, due to the heterogeneous nature of primary cells, one may expect differences in the results due to donor variations.

The hDPSC used in Study 3 were harvested by enzymatic dissociation, which has been shown to generate cells with enhanced, high biomineralization potential (303). Usually hDPSC are obtained from extracted third molar teeth and from premolars removed on orthodontic indications. The hDPSC used in Study 3 were obtained from young adult patients (mean age 22 yrs). The relatively young age of the donors may have contributed to heterogeneity of the isolated cells, as the roots of the extracted third molars might not have been fully developed. The dental pulp of immature teeth contains a larger pool of undifferentiated cells; hence isolation may garner a less homogenous population (304). The heterogeneous hDPSC still maintain and display multipotent differentiation capabilities (207) and have been shown, in immunocompromised mice, to produce tissue resembling pulp/dentin tissue (174). *In-vivo* studies have shown more mineralization and matrix formation regenerated from implantation of unsorted/heterogeneous populations of hDPSC than from sorted/homogenous populations of hDPSC (305).

### 4.5 Concluding remarks

Based on the results of the three studies comprising the research for this thesis, the following conclusions can be drawn:

- Biodentine has a higher surface microhardness than MTA or TotalFill.
- The use of MTA, Biodentine and TotalFill during REP does not negatively affect the fracture resistance of the tooth.
- The proliferation of hBMSC and hDPSC *in-vitro* is less inhibited by Biodentine than by MTA or TotalFill.
- Osteogenic and angiogenic responses of hBMSC were differentially affected by the materials. Biodentine and TotalFill induced earlier changes at gene level.
TotalFill enhanced the expression and production of VEGFA and reduced the expression of IL6 in hDPSC.

4.6 Summary

This thesis was aimed to be a comparative analysis of TSC in the context of their application in regenerative endodontics. The ultimate goal of regenerative endodontics is to regenerate functional pulp tissue and particularly in the case of non-vital immature permanent teeth to achieve resolution of apical disease as well as, continuation of the root development (306). Considering the decreased dentin thickness of the roots of immature teeth and their susceptibility to fracture, the aim is to reinforce the strength and structural integrity of teeth treated with regenerative endodontics. Therefore, it is evident that the goal of regenerative endodontics is 2-fold, on both a biological/physiological level as well as a mechanical/strength-structural level. To have a comprehensive comparison of TSC it is paramount to explore possible differences across all levels.

Although being performed in-vitro, the findings of this thesis are clinically relevant to both REP and VPT considering the use of hBMSC in study 2 and hDPSC in study 3. This is primarily based on the assumption that hBMSC are recruited during REP due to the over-instrumentation into the periapical area to induce bleeding into the canal. Study 3 is designed to relate to VPT where the existence of pulp tissue naturally allows the recruitment of hDPSC during treatment with TSC. This experimental design further allows an auxiliary comparison between the responses of hBMSC and hDPSC to TSC stimulation. However, it must be emphasized that this comparison pertains only to the stimulation by TSC and does not offer an analysis of the characteristics and properties of the cells. Under the standardized experimental conditions, the TSC in this thesis elicited a consistent cellular response from both cell types (Table 6).

It is important to consider that before a physiological change such as hard tissue formation or completion of root development can occur, the risk of root fracture
remains for immature teeth. For this reason, the immediate effect of TSC introduction into the root canal during regenerative endodontics was explored. We hypothesized that differences existed between the TSC on fracture resistance of the treated teeth. This hypothesis was rationalized by the preliminary findings of significant differences between the TSC in the microhardness test. However, the results did not support the hypothesis. The cervical area of immature teeth treated with REP remained a vulnerable area regardless the choice of TSC.

It is important to notice the clinical significance of the current findings bearing in mind the experimental limitations. Although Biodentine exhibited a more favorable viability response from both hBMSC and hDPSC, additional consideration of bioactivity is needed when making clinical recommendations for choice of TSC in regenerative endodontics. When comparing the immediate mechanical effects of TSC it is evident that Biodentine may be more favorable in contact with dentin as its microhardness is closer to that of human dentin compared to MTA. The longer setting time required for TotalFill is a disadvantage as it may impair the immediate sealing ability compared with TSC with shorter setting time. It can be speculated that the continuity of microhardness between Biodentine and dentin could lead to a favorable transfer of occlusal and masticatory forces between the two structures. This may be particularly relatable to REP where fracture resistance is of primary concern. With that being said, this speculation was not supported by our fracture resistance findings. However, as previously mentioned, a larger sample size may have indicated such differences.

In terms of making recommendations for specific TSC, it is important to consider the treatment outcome desired. During REP it is preferable to achieve formation of pulp-dentin tissue and continuation of root development. Biodentine however, may induce root completion with bone-like hard tissue formation compared to MTA and TotalFill. The osteogenic potential of Biodentine is an advantage in perforation repair. On the other hand, in VPT overstimulation of hard tissue formation may be detrimental to the success of treatment as it may lead to excessive pulp canal obliteration. In such cases one must therefore consider using a TSC with a less potent
osteogenic effect compared to Biodentine such as MTA or TotalFill. Ultimately, a specific odontoblastic response to induce dentin formation is preferred over an osteogenic response. Unfortunately, the dentin forming potential of the TSC was not explored in this thesis. Although the angiogenic and anti-inflammatory potential of TotalFill must be interpreted with caution, the findings suggest that TotalFill may be a better alternative for VPT than Biodentine. Particularly when considering that VPT can be successful even with the diagnosis of irreversible pulpitis as previously mentioned (103, 104). TSC that displays promising findings about resolving inflammation and promoting angiogenesis should be considered a better option for VPT.

It is evident that the tested TSC exhibited different microhardness and induced different biological responses from stem cells. The findings of this project put these differences in the proper context in an attempt to aid operators in making reasonable choices for TSC use in regenerative endodontics.

4.7 Future perspectives

Because of the relatively small sample size in Study 1, differences in fracture resistance among the test TSC may not have been disclosed. For future reference, a larger sample size is necessary. Different methods of applying force to achieve fracture should also be considered. In the present study teeth were subjected to a steadily increasing compressive force to the point of fracture. This is intended to simulate a single traumatic blow that results in fracture. A relevant force would be repetitive hitting of the tooth (at a 45° angle to the long axis). This would simulate the cumulative effect of frequent trauma to the tooth and provide insight into the effect of a repetitive stress cycle on the modulus of elasticity of the tooth.

It would also be of interest to analyse the composition of the material eluate prior to application, particularly with reference to differences in the material density and effusion rates. For instance, it may be useful to measure calcium ion release by inductively coupled plasma-mass spectrometry (307). It would also be of interest to explore the effect of altering the cell culture conditions of the experimental model.
the present studies, a 1:4 dilution of eluate was chosen to stimulate cells for gene expression analysis. However, it may be of interest to compare the effects of different material eluate concentrations on gene expression. For comparison of materials it is important to stipulate longer timepoints in the experimental design. The application of TSC in VPT and REP is long-term, and therefore effects over time are clinically relevant. Longer observation times may be useful for functional assays such as the Alizarin red staining assay, as the deposition of calcium nodules takes longer than the expression of markers of differentiation. It then becomes important to investigate other osteogenic, angiogenic, and dentinogenic markers expressed during the later stages of differentiation of osteoblasts/odontoblasts. It is also relevant to investigate the expression by hBMSC of the inflammatory markers as well as other molecules involved in the resolution of the inflammatory process, such as transforming growth factor beta family proteins and interleukin 10 (308).

The in-vitro model applied in Studies 2 and 3 is commonly utilized for evaluating the biological effects of dental materials. However, it may not readily relate to the true biological environment in which these materials must function. The monolayer 2D culture does not accurately reflect the complex cellular structure within human tissue (309). In the past decade there has been an increase in the application of 3D cell culture techniques, as the 3D model more closely simulates in-vivo like conditions within an in-vitro setting (309). 3D cell culture models mimic the biological microenvironment by attempting to restore the morphological characteristics of functional human tissue (309). These include models such as multicellular spheroids, hydrogels, and scaffolds created by 3D bioprinting.

The results and conclusions presented in this thesis warrant further evaluation, in prospective long-term clinical trials comparing the effects of the three TSC. The differentiation profile observed in the present studies should be confirmed by applying TSC to VPT and REP, in order to determine to what extent, the in-vitro findings apply to in-vivo regeneration of dental tissues.
5. References


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Fracture resistance of simulated immature teeth treated with a regenerative endodontic protocol

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ABSTRACT

This study aims to evaluate fracture resistance of simulated immature teeth after treatment with regenerative endodontic procedure (REP) using tricalcium silicate cements (TSCs) as cervical plugs. Bovine incisors were sectioned to standard crown/root ratio. Pulp tissue was removed and canals were enlarged to a standardized diameter. Teeth were then treated with a REP protocol consisting of NaOCl and EDTA irrigation, intracanal medication with triple-antibiotic paste for 14 days followed by a TSC cervical seal and composite restoration. Teeth were divided into groups according to the material used; Mineral-Trioxide-Aggregate (MTA), Biodentine, TotalFill. Teeth filled with gutta-percha (GP) and intact teeth served as controls. All teeth subjected to an increasing compressive force (rate of 0.05 mm/s at a 45° angle to the long axis of the tooth) until fracture. All treated teeth exhibited significantly lower resistance to fracture compared to the intact teeth but no difference was found between the TSC groups (Kruskal-Wallis, Dunn’s multiple comparison, p < .05). TSCs applied at the cervical area of simulated immature teeth treated with REP did not reinforce fracture resistance.

INTRODUCTION

Endodontic treatment of non-vital immature permanent teeth presents quite a challenge in dental clinics due to wide open apices and thin dentinal walls. A relatively high incidence of cervical root fracture (>60%) has been reported in such teeth after a long-term intra-canal treatment with calcium hydroxide (CH) in order to achieve a hard-tissue barrier at the apical area (apexification) [1,2]. These fractures may occur with minor impacts or spontaneously over time [1,3]. In the latest years, tricalcium silicate cements (TSC) have been widely used as endodontic repair materials and dentin substitutes [4]. The use of TSC materials to achieve a root-end closure at the apical area of necrotic immature teeth (direct apexification) has replaced the traditional treatment with CH. However, with this method the dentinal walls remain thin, and the risk of fracture is still present [5,6].

Regenerative endodontic procedures (REP) have been advocated as an alternative treatment modality to apexification for immature permanent teeth with necrotic pulp [7]. Regenerative endodontics have been defined as “biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex” [8] with the optimal goal to regenerate functional pulpal tissue and subsequently further root development. Although there is no consensus regarding the clinical regenerative protocols [9,10] the common step in all suggested ones is cervical sealing with a TSC barrier. This biocompatible cervical plug provides a bacterial-tight seal and acts as pulp space barrier [11,12].

The fact that non-vital immature teeth, due to fragile root, are more prone to fracture represents a substantial clinical problem. The risk of fracture of endodontically treated immature teeth relates to the degree of root development, with lower degree of development associated with higher fracture risk [1]. REPs aim at inducing further root development and eventually strengthening the tooth. However, even...
with REPs, the cervical area does not develop further. Furthermore, placement of TSCs at this exact area may mechanically affect the susceptibility of treated immature teeth to fracture. Little is known about the immediate effect of TSCs on the fracture resistance of immature teeth treated with REPs. The aim of this in vitro study was to investigate the fracture resistance of simulated immature teeth treated with REP and sealed at the cervical area with three different TSC materials; White MTA ANGELUS® (MTA), Biodentine† Septodont (Biodentine), and TotalFill‡ BC RRM Putty (TotalFill).

The null hypotheses tested:

\( H_0 \), there is no difference in fracture resistance between intact immature bovine teeth and immature bovine teeth treated with different TSCs as coronal seal during REP.

**Material and methods**

**Bovine teeth preparation**

Bovine mandibular incisor teeth were extracted, cleaned and stored in 1% Benzalkonium Chloride [13]. Teeth were examined thoroughly and teeth with visible cracks/fractures were discarded. Intact teeth were then prepared according to a standard protocol in order to simulate immature teeth. Briefly, they were sectioned with a water cooled low speed diamond bur, coronally 10 mm above the cemento-enamel junction (CEJ) and apically 15 mm below the CEJ. The root canal was thereafter instrumented and widened with a size 6 peeso reamer so that an ISO size #120 file could pass completely unhindered throughout the canal. In that way, the internal canal diameter and the remaining dentin thickness were standardized close to 2 mm [5,6,14–16] (Figure 1).

Controls (intact teeth) were sectioned according to the standardized crown/root ratio but the canal was not prepared (Figure 1).

Dentine thickness and canal diameter at the cervical area of all teeth was measured with buccolingual and mesiodistal radiographs using the DIGORA Optime UV system (Unident, Falkenberg, Sweden) and the measurements were averaged.

**Tricalcium silicate cement materials**

The TSC shown in Table 1 were mixed according to the manufacturer’s instructions and used in the regenerative endodontic procedure and throughout this study.

**Regenerative endodontic procedure**

Simulated immature teeth were by a random procedure allocated into the 4 groups; MTA (n = 11) (group 1), Biodentine (n = 10) (group 2), TotalFill (n = 10) (group 3), Gutta Percha (GP) (n = 10) (group 4). In addition, untreated teeth served as controls (intact teeth, n = 10) (group 5) (Figure 1). Intact teeth were stored in a wet flower arrangement foam in a 37°C and 100% humidity incubator until testing. All teeth in groups 1–4 were treated with the protocol followed at the dental clinics of the University of Bergen: irrigation with 10 ml Dakin’s solution (0.5% buffered sodium hypochlorite) followed by 5 ml of 17% ethylendiamine tetraacetic acid (EDTA) and 5 ml sterile water. The canals were then dried with paper points and filled with a triple antibiotic paste consisted of equal volumes of 500 mg Metronidazole, 500 mg Ciprofloxacin and 500 mg Amoxicillin mixed with sterile water in a slurry paste placed with a lentulo spiral. The access cavities were then sealed with Cavit® temporary filling material and the roots inserted into a wet flower-arrangement foam. The teeth were stored in incubator (37°C and 100% humidity) for 10 days [6]. The Cavit® was then removed and the triple antibiotic paste was washed out with the same irrigation protocol as above. Teeth in groups 1–3 were sealed with a cervical plug of TSC. Teeth in group 4 were obturated with gutta-percha using lateral condensation technique and sealer (AH Plus® DENTSPLY, Germany) and avoiding over-filling by applying finger pressure at the apex. Those teeth served as negative controls (Figure 1).
Buccolingual and mesiodistal radiographs were taken to measure the material plug length (measurements were averaged as stated previously) and to confirm the uniformity of the gutta-percha obturation using the DIGORA Optime UV system. A wet cotton pellet and Cavit™ temporary filling material was placed at the access cavity and the teeth were stored overnight in the incubator to allow the TSC to set. After complete setting of the TSC material, a composite filling (3 M ESPE Filtek™ Supreme XTE) was placed as the coronal seal using a 4th generation bonding system involving the use of 38% phosphoric acid (TOP DENT etch gel 2.5 ml, DAB DENTAL, Sweden) followed by a primer application (Optibond™ FL and Adhesive (Optibond™ FL). Group 4 teeth were filled with composite immediately after filling with GP and stored in a wet flower arrangement foam in the incubator (37°C and 100% humidity) until testing (Figure 1). Buccolingual radiographs were again taken to confirm the integrity of the composite fillings using the DIGORA Optime UV (Unident, Falkenberg, Sweden).

### Fracture resistance testing

All teeth were dipped into molten wax leaving a 0.2–0.3 mm thick layer of wax covering the root (2 mm below the CEJ to the root apex) [14]. Thereafter, the roots were embedded in acrylic resin cylinders (Heraeus, MELIODENT® Rapid Repair, Denture acrylic self-curing, Kulzer, Germany) that were prepared using polyvinyl chloride cylinder molds measuring 20 mm in diameter and 17 mm high [15]. As soon as polymerization of the acrylic resin started, the teeth were removed from the resin, and the wax was cleaned from the root surfaces using a curette. The cleaned root surfaces were then coated with a thin layer of polyvinylsiloxane impression material (Affinis®, Coltene/Whaledent AG, Altstetten, Switzerland) to simulate the periodontal ligament (PDL) [5,14,17–19] and then re-embedded into the acrylic resin block. The acrylic block with the prepared teeth was mounted onto an MTS® Hydraulic test System and subjected to an increasing compressive force at a test rate of 0.05 mm/s while being positioned at 45° angle to the long axis of the tooth until fracture occurred [5,16–18,20]. Peak load at fracture was recorded in Newton (N).

### Statistical analysis

For statistical analysis GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA) was used. D’Agostino-Pearson omnibus normality test validated the distribution of the data. For normally distributed data one-way analysis of variance (ANOVA) with Bonferroni’s post hoc test was used. When normality test was not passed, Kruskal-Wallis with Dunn’s post hoc test was used for the comparison between the groups or Mann-Whitney test for comparison between two groups (e.g. prepared vs intact teeth). All tests were performed at a level of significance α=0.05. Results are presented as mean ± SEM, (*p < .05; **p < .01; ***p < .001).

### Results

#### Simulated immature teeth

In the interest of experimental standardization, there were no differences between the simulated immature teeth (groups 1–4) in terms of crown/root ratio, intra-canal diameter at CEJ and dentin thickness (Table 2). Moreover, there were no differences between the TSC groups (1–3) in terms of the TSC cervical plug length (MTA: 3.9 ± 0.193 mm; Biodentine: 4.015 ± 0.122 mm; TotalFill: 3.481 ± 0.125 mm) (Table 2). All prepared teeth (groups 1–4) had a significantly higher canal diameter and lower dentin thickness measured at the CEJ compared to the intact teeth (group 5) (Figure 2 and Table 2, p < .05).

#### Fracture testing

All teeth were looked under ×1 magnification for fracture patterns. The diagonal fracture line extends from the buccal aspect of the crown to the lingual aspect of the teeth and exposes the root canal of all

### Table 1. Summary of the Tricalcium Silicate Cements (TSC) cements used in the study.

<table>
<thead>
<tr>
<th></th>
<th>Biodentine™ (Septodont, Saint-Maurdes Fosses, France)</th>
<th>TotalFill™ BC RRMTM PUTTY (FKG Dentaire, La-Chaux-de-Fonds, Switzerland)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder:</td>
<td>Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, iron tetraalcum aluminate, bismuth oxide;</td>
<td>Ready-made paste: Calcium silicates, zirconium oxide, tantalum pentoxide, calcium phosphate monobasic and filler agents</td>
</tr>
<tr>
<td>Liquid:</td>
<td>distilled water</td>
<td></td>
</tr>
<tr>
<td>Mixing ratio:</td>
<td>1 scoop of powder to 1 drop of liquid</td>
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#### Table 2, p < .05: Comparing the cervical plug length between groups (e.g. prepared vs intact teeth). Results are presented as mean ± SEM, (*p < .05; **p < .01; ***p < .001).
tested teeth. The fracture line of the simulated immature teeth (groups 1–4) crosses the interface between the material plug or gutta-percha and composite filling (cervical area) whereas the fracture line of the intact teeth (group 5) is mainly located within the crown (Figure 3).

The result of fracture testing showed that intact teeth (Group 5) had a significantly higher peak load to fracture (1,669 ± 60.77 N) in comparison to all other test groups (Figure 4). TotalFill had a higher peak load to fracture (804.5 ± 147.8 N) in comparison to MTA (724.2 ± 128.2 N) and Biodentine (779.4 ± 104.7 N) whereas the GP control group 4 exhibited the lowest peak load to fracture among all simulated immature teeth (675.8 ± 86.84 N). However, there were no statistical significant differences among the simulated immature teeth (groups 1–4) (Figure 4).

**Discussion**

The experiment model in this study emphasizes the immediate effect of the TSCs on treated immature teeth with REPs. We implemented a continuously increasing load of force model to measure fracture resistance. Traumatic dental injuries involve mostly anterior teeth [2]. The absence of high occlusal forces at the incisors may imply that the type of force that leads to dental trauma in such cases is a single impact that overwhelms the structural integrity of the tooth at that moment. Untreated immature bovine teeth had a higher fracture resistance than immature bovine teeth treated with TSCs therefore, the null hypothesis was rejected. Under the experimental set-up, the treated immature teeth fractured at the cervical area and thus, REP and cervical seal with bioceramic materials does not seem to reinforce fracture resistance of bovine teeth.

Bovine teeth were used and prepared to simulate immature teeth. Use of human teeth for the same purpose would have allowed for testing the hypothesis in a more clinically relevant substrate. However, difficulty to obtain sufficient quantity and with adequate quality, as well as ethical issues led to use of bovine teeth. All teeth used were extracted from animals of approximately same age shortly after slaughtering and stored under the same conditions until preparation. Thus, minimizing variations in morphology and composition. Previous studies comparing human and bovine teeth showed similar dentin tensile strength and modulus of elasticity [21], fracture strength of composites [22], as well as dentin Knoop hardness [23], properties relevant to the current experimental model. Although human teeth are

<table>
<thead>
<tr>
<th>Table 2. Dimensions (mean ± SEM) of the bovine teeth used in the study.</th>
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<tbody>
<tr>
<td><strong>Group (n = number of teeth)</strong></td>
</tr>
<tr>
<td>1: MTA (n = 11)</td>
</tr>
<tr>
<td>2: Biodentine (n = 10)</td>
</tr>
<tr>
<td>3: TotalFill (n = 10)</td>
</tr>
<tr>
<td>4: Gutta-percha (n = 10)</td>
</tr>
<tr>
<td>5: Intact teeth (n = 10)</td>
</tr>
</tbody>
</table>

*p < .01 compared to group 1, 2 and 3.**
**p < .001 compared to group 4; Kruskal-Wallis test with Dunn’s multiple comparison.

Figure 2. The simulated immature teeth (groups 1–4, n = 41) had a statistically significant larger canal diameter (2,153 ± 0,07 mm) and lower dentin thickness measured at the CEJ (1,857 ± 0,027 mm) compared to the intact teeth (1,780 ± 0,13 mm and 2,704 ± 0,096 mm, respectively) (group 5, n = 10); Results are presented as mean ± SEM, Mann-Whitney test, **p < 0.05; ***p < 0.001.
generally preferred for in vitro dental research, bovine teeth were a valid substitute in this study. We opted to simulate immature roots with a certain root length (15 mm) consistent with stage 3 development [1,24–26]. This length was chosen as shorter roots, typical of earlier root development stages, were easily dislodged from the acrylic mold during loading. Furthermore, the crown was also standardized (9 mm) for all tested teeth and the canals of the prepared ones were enlarged to a canal diameter of approximately 2.2 mm, significantly larger than the canal of the intact teeth (ca 1.8 mm). An earlier report with similar experimental set-up concluded that teeth with a canal diameter of 1.5 mm or less does not need canal wall reinforcement after endodontic treatment [27] and the intact teeth in the current study exhibited a similar lumen diameter. The majority of teeth treated with REP s are teeth in stages 2 through 5 [28] and thus, the current preparation was suitable for the scope of this study.

In addition, the experimental set-up included simulation of the PDL. An elastomeric impression material was used as in previously evaluating ex vivo tooth fracture resistance models [5,29]. The modulus of elasticity of human PDL ranges from 0.12–0.96 MPa [30], which is comparable to various elastomeric impression materials [31] as the thin layer of polyvinylsiloxane used in the current study. Soares et al showed that PDL simulation had a significant effect on fracture resistance in a similar ex vivo laboratory model [29]. The presence of PDL is important when teeth are subjected to trauma. It plays a major role in the stress distribution of forces.

Figure 3. Typical fracture pattern of the immature teeth under the fracture test. (a) and (b): Biodentine group; (c) and (d): Gutta-percha group; (e) and (f): intact teeth group. The diagonal fracture line extends from the buccal aspect through the canal to the lingual aspect of the tooth. The treated immature teeth fractured at the interface between the material plug/or gutta-percha and composite filling (a-d). The fracture line of the intact teeth is mainly located within the crown (e-f). Lingual aspects: (a), (c) and (e); Lateral aspects: (b), (d) and (f). (× 1 Magnification).
applied to teeth [32]. For all of the above, the present model is suitable for testing the hypothesis.

Previous studies investigating the intraradicular reinforcement of structurally compromised roots showed that the resistance to fracture was directly related to the remaining tooth structure and to the amount of dentin at the cervical area [33,34]. This was confirmed by the current study as the intact teeth with wide canal but higher dentin thickness at the cervical area (control teeth), required double the force in order to sustain fracture under the experimental set up.

Simulated immature teeth treated with REP showed no difference in fracture resistance compared to teeth filled with gutta-percha in the current study. Group 4 (GP) acted as negative controls and was expected to exhibit lower resistance to fracture compared to the intact teeth. In clinical situation, immature teeth filled with gutta-percha represent cases treated with apexification technique. Apexification with long-term calcium hydroxide treatment has been banned as responsible for cervical fractures due to the effect of calcium hydroxide on dentin structure [1,35]. Therefore, direct apexification techniques with the use of an apical plug of bioceramic materials have been advocated as the preferable method of treatment for necrotic immature teeth. In the current study, the GP group was not filled with an apical plug of TSCs in order to facilitate the obturation of the wide canal. The reason was purely financial, and it would not have any effect on fracture resistance at the cervical area. Moreover, we did not treat the GP group with long-term calcium hydroxide dressing prior to root canal obturation with gutta-percha. All teeth were treated with the same REP protocol and all prepared canals were subjected to the same chemical treatment with irrigation and antibiotic dressing in order to avoid possible structural changes of the dentin. There is evidence that long-term and periodic changes of the intracanal dressing may negatively affect fracture resistance of teeth [19]. It is unlikely that the low resistance to fracture exhibited by the negative controls was due to structural changes of the dentin after the chosen REP protocol since it was a short-term treatment. Nevertheless, all treated and filled immature teeth in this study showed low resistance to fracture regardless of filling material.

The sample size used in this study was sufficient to demonstrate differences between intact and treated teeth. Lack of reinforcement in fracture resistance of simulated immature teeth when bioceramic materials were applied at the cervical area was the main finding of this study. There were small, non-significant differences in fracture resistance between the tested TSCs. However, increased number of teeth would be needed in each group in order to detect possible differences between the tested TSCs as indicated by the current results. It would have been interesting to further investigate if the choice and the thickness of bioceramic material at the cervical area plays a role in fracture resistance at this vulnerable area.

There are several studies trying to address a similar question with conflicting results but the difference from the current study was that the entire immature canal was filled with TSCs [20,36–39]. Within the limitations of in vitro studies, canal filling with MTA, or other bioceramic materials e.g. calcium phosphate bone cement, or BioAggregate have been reported to reinforce fracture resistance in some studies [20,36,39,40] whereas in others, the materials used did not [17,37,38]. Most of the studies have used MTA as the golden standard. However, the discoloration caused by MTA even when placed below the CEJ [41] have led to the use of other TSC during REP such as
Biodentine or TotalFill. To the best of our knowledge, this is the first study applying these three commonly used TSC at the cervical area only according to advocated REPs and evaluating their effect in fracture resistance ex vivo.

The use of composite resin systems has been recommended for the reinforcement of the cervical area of treated immature teeth [16,42,43] and placement of composite restoration is often the final step in the treatment of traumatized immature teeth. The lack of difference in fracture resistance shown under the current experimental set-up between the treated immature teeth (groups 1–4) may also attributed to the composite restoration.

Conclusions
Within the limitations of this study, we can conclude that TSC such as MTA, TotalFill and Biodentine do not influence either negatively or positively, the fracture resistance of immature teeth during regenerative endodontic therapy. Further material tests and clinical trials are necessary to validate these results.

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We would like to thank Fatland Slakteri, Ølen, Norway for providing us with bovine teeth, Odd Johan Lundberg for technical assistance and Marit Øilo and Nils Roar Gjerdet for fruitful comments on study execution. We would also like to thank biostatistician Stein Atle Lie for helpful comments to the statistical issues, and sample size considerations in the article.

Disclosure statement
All authors declare and attest to no conflicts of interest in relation to this study.

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References


Tricalcium silicate cements: osteogenic and angiogenic responses of human bone marrow stem cells

Ali MRW, Mustafa M, Bårdsen A, Bletsa A. Tricalcium silicate cements: osteogenic and angiogenic responses of human bone marrow stem cells.


Tricalcium silicate cements (TSCs) are used in endodontic procedures to promote wound healing and hard tissue formation. The aim of this study was to evaluate and compare the effect of commonly used TSCs [mineral trioxide aggregate (MTA), Biodentine, and TotalFill] on cellular metabolism and osteogenic/angiogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) in vitro. We tested the null hypothesis of no difference between MTA, Biodentine, and TotalFill in stem cell responses. Cells were subjected to eluates of the tested materials for up to 14 d. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Real-time PCR was used to determine the levels of expression of the osteogenic factors alkaline phosphatase (ALP), osteoprotegerin (OPG), osteocalcin (OC), and collagen 1A (COL1A1), and the angiogenic factors vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 1 (FGF1). ELISAs were used to measure the levels of VEGFA and ALP in culture supernatants. Mineralization in vitro of hBMSCs was assessed using Alizarin Red staining. The hBMSCs tolerated exposure to TSCs well, with Biodentine showing the most favorable effect on cell viability. Expression of ALP, COL1A1, OPG, and VEGFA were differentially affected by the materials, with Biodentine and TotalFill inducing earlier changes at gene level. Increased mineralization was observed with time, after exposure to all TSCs tested, with MTA showing the greatest effect. The results revealed different responses of hBMSCs to TSCs in vitro.

Tricalcium silicate cements (TSCs) are used in modern endodontics for different procedures, such as pulp capping and pulpotomy (vital pulp therapy), perforation repairs, root-end fillings during apicoectomies, apexification, and regenerative endodontic procedures (1–4). During these procedures, the materials come into direct contact with vital pulp, periapical tissues, and stem cells. It is therefore important that these dental materials are biocompatible, preferably promote wound healing and hard tissue formation, and are hydrophilic (as they are applied and function in a wet environment) (5). They should also demonstrate dimensional stability, and are known to have antibacterial effects because of their high pH (6, 7).

Mineral trioxide aggregate (MTA), the first bioactive TSC on the market, has a stimulatory effect on the cells and induces osteogenic and angiogenic regeneration (8–12). Biodentine, a relatively new tricalcium silicate-based material, is used clinically and applied similarly to MTA (13). Like MTA, Biodentine may modify the proliferation of pulp cell lines (1). When MTA and Biodentine were compared in a recent in vitro study, neither material showed a cytotoxic effect on human bone marrow mesenchymal stem cells (hBMSCs) after 14 d of exposure but ProRoot MTA exhibited greater osteoinductivity than Biodentine regarding expression of alkaline phosphatase (ALP) mRNA and ALP protein (11).

TotalFill or EndoSequence Root Repair Material (ERRM) is a recently introduced bioceramic material made of calcium silicates, monobasic calcium phosphate, zirconium oxide, tantalum oxide, proprietary fillers, and thickening agents (14). As it is presented as a ready-to-use paste in a tube, the material is easy to handle and the formulation of the product is consistent between batches. Some studies have shown EndoSequence to have a higher mitogenic effect on periodontal ligament stem cells than MTA, but no differences between these materials on hBMSCs have been found (15) and neither showed any cytotoxic effects on human gingival fibroblasts (16).

Currently, the different TSC brands are used interchangeably in dental clinics for the same applications, without any distinctions or specific recommendations. The choice of material depends mainly on personal preference in clinical situations. However, a direct comparison of the effects of the TSCs currently available,
in terms of their ability to elucidate different biological responses, might help to develop specific guidelines for the use of these different materials. Cell-culture techniques are useful for evaluating the biocompatibility of different materials (17). Indeed, in vitro assays with cell cultures are commonly used to investigate cell behavior and biological responses in specific situations. The results of these in vitro assays are relevant to clinical conditions and provide an appropriate model for screening the properties of different dental materials (18) prior to in vivo studies.

Thus, the aim of the present study was to assess and compare the effects of three TCA materials, namely white MTA-Angelus, Biodentine, and TotalFill BC RRM PUTTY, on stem cell metabolism and osteogenic/angiogenic differentiation in vitro. We tested the null hypothesis that there was no difference between these three commonly used TCA materials in stem cell responses.

Material and methods

Preparation of extracts

The following tricalcium silicate-based cement materials were used to prepare the extracts:

(i) White MTA-Angelus (Angelus, Londrina, PR, Brazil) (MTA): Powder comprised of Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, iron tetracalcium aluminate, bismuth oxide; liquid: distilled water.


(iii) TotalFill BC RRM PUTTY (FKG Dentaire, La-Chaux-de-Fonds, Switzerland) (TotalFill): ready-made paste composed of calcium silicates, zirconium oxide, tantalum pentoxide, calcium phosphate monobasic, and filler agents.

The MTA and Biodentine materials were mixed and prepared according to the manufacturer's instructions under sterile conditions. Four plugs of MTA, Biodentine, and TotalFill were made using an amalgam carrier and placed in preweighed Eppendorf tubes. The diameter and thickness of the plugs were standardized by using the same amalgam carrier for all materials. The weight of the plugs was determined using a Bergman AG204 DeltaRange weight scale (Mettler-Toledo, Greifensee, Switzerland) and the Eppendorf tubes were then placed in an incubator (37°C, 6% CO₂, and 100% humidity) Heracell (ThermoScientific, Waltham, MA, USA) for 24 h to allow for complete setting of the materials.

Thereafter, the eluates were prepared according to asceptic conditions according to ISO Standards 10993-5 (19). One milliliter of serum-free Mesencult MSC Basal Medium (Human) (StemCell Technologies, Vancouver, BC, Canada) with 1% penicillin (HyClone, GE Healthcare, Logan, UT, USA) was added to each Eppendorf tube as extraction vehicle and then stored in the incubator (37°C, 6% CO₂, and 100% humidity) Heracell (ThermoScientific) for 24 h. The collected medium (eluate) was filtered through sterile Acrodisc syringe filters (pore size 0.2 μm diameter; Pall Life Sciences, New York, NY, USA) and stored at −80°C until required (20). The stock extract was serially diluted (1:2, 1:4, 1:8, and 1:16) with osteogenic medium (Mesencult MSC Basal Medium; StemCell Technologies), with 10% Mesencult MSC Stimulatory Supplement (StemCell Technologies) and 1% penicillin (HyClone) (complete medium) with 5 μl of dexamethasone (Sigma-Aldrich, St Louis, MO, USA), 250 μl of L-ascorbic acid 2-phosphate (Sigma-Aldrich) and 175 μl of beta-glycerophosphate (Sigma-Aldrich) added per 50 ml of complete medium before use at the experiments.

Cell culture and reagents

Commercially available primary hBMSCs (StemCell Technologies) were cultured in complete medium consisting of Mesencult MSC Basal Medium (StemCell Technologies) plus 10% Mesencult MSC Stimulatory Supplement (StemCell Technologies) and 1% penicillin (HyClone), and expanded in an incubator at 37°C, 6% CO₂, and 100% humidity. After reaching 70%–80% confluence, the cells were trypsinized and counted using a trypan blue assay in an automated cell counting machine (Countess; Invitrogen, Carlsbad, CA, USA) to check cell numbers and cell viability. Cells at passages 2–8 were used in the experiments.

After seeding in the appropriate wells for each experiment, cells were left for 1 d to allow complete cellular attachment. They were then exposed to the different TSC extracts for various periods of time (6 h, and 1, 3, 7, and 14 d). Untreated hBMSCs served as controls.

Cell morphology was assessed, for all groups at the time points tested and under all experimental conditions, using light microscopy (Nikon Eclipse E80i; Nikon Instruments, Tokyo, Japan).

MTT assay

The hBMSCs were seeded onto 96-well plates, at a density of 0.1 × 10⁵ cells per well, and exposed to different concentrations of TSC extracts (1:2, 1:4, 1:8, 1:16) for different experimental time periods (1, 3, and 7 d), as described above. Cell viability was tested using the 3-(4,5-dimethylthiazoyl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich), according to the manufacturer's instructions. Absorbance was recorded and quantified at a wavelength of 570 nm using the FLUOstar OPTIMA (BMG LABTECH, Leicester, UK) microplate reader.

The MTT assay was performed in three independent experiments, with quintuplicate samples used in each.

RNA extraction and quantitative real-time PCR

The hBMSCs were seeded in six-well culture plates at a density of 0.3 × 10⁵ cells per well. They were then exposed to the different TSC extracts (1:4 dilution of eluate) for 6 h, and 1, 3, and 7 d, as described above. At the end of the experimental period, the cell culture supernatant was removed and stored at −80°C for later assessment of protein expression. The cells were washed with PBS and the plate was then stored at −80°C until required for RNA analysis. Total RNA was isolated using a Maxwell 16 Cell LEV Total RNA purification Kit (Promega, Madison, WI, USA) according to the protocol provided, in conjunction with the Maxwell 16 Instrument (Promega). Total RNA was quantified using a Nanodrop Spectrophotometer (Thermo Scientific NanoDrop Technologies, Wilmington, DE, USA).
Fifty micrograms of total RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR (qRT-PCR) was performed to quantify the relative changes in gene expression using the ABI StepOnePlus Real-Time PCR System (Applied Biosystems). Amplification was performed in triplicate for every marker, in a 10-μl reaction volume [5 μl of TaqMan PCR Master Mix (Applied Biosystems), 3.5 μl of nuclelease-free water, and 0.5 μl of the designated probe]. The TaqMan probes used in the PCR reaction were as follows: alkaline phosphatase (ALP: Hs01029144_m1), collagen 1 alpha (COL1A1: Hs00164099_m1), osteoprotegerin (OPG: Hs00900358_m1), osteocalcin (OC: Hs01587814_g1), vascular endothelial growth factor A (VEGFA: Hs00900555_m1), and fibroblast growth factor 1 (FGF1: Hs01092738_m1). Glycer-aldehyde-3-phosphate dehydrogenase (GAPDH: Hs99999905_m1) served as the endogenous control gene. Relative gene expression was quantified using the comparative CT method. The value of the target gene was calculated according to the following formula:

\[ \frac{C_{T_{\text{target}}}}{C_{T_{\text{endogenous}}}} = 2^{-\Delta C_{T}} \]

where \( \Delta C_{T} \) is the difference between the target gene and endogenous control expression. The results were obtained from two experiments performed in triplicate.

ELISA

The levels of VEGFA in the culture supernatants (after exposure for 1, 3, and 7 d to a 1:4 dilution of the different TCA eluates) were determined using commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. They were then incubated with Alizarin Red S staining solution. Briefly, hBMSCs were seeded in 24-well culture plates at an initial seeding density of 0.3 × 10^4 cells per well and stimulated with a 1:4 dilution of the different TSC eluates, using a colorimetric ALP assay kit (Sigma-Aldrich), according to the manufacturer’s instructions. Absorbance was recorded and quantified at a wavelength of 405 nm using the FLUOstar OPTIMA microplate reader, expressing ALP activity as absorbance. Results presented are from two independent experiments performed in triplicate.

ALP quantification

Extracellular ALP was measured in culture supernatant, collected after exposure for 1, 3, and 7 d to a 1:4 dilution of the different TSC eluates, using a colorimetric ALP assay kit (Sigma-Aldrich), according to the manufacturer’s instructions. Absorbance was recorded and quantified at a wavelength of 405 nm using the FLUOstar OPTIMA microplate reader, expressing ALP activity as absorbance. Results presented are from two independent experiments performed in triplicate.

Alizarin Red staining

Detection of calcium deposits (mineralization) in vitro of hBMSCs exposed to the different TSC eluates was performed with Alizarin Red staining. Briefly, hBMSCs were seeded in 24-well culture plates at an initial seeding density of 0.3 × 10^4 cells per well and stimulated with a 1:4 dilution of the different TSC eluates for 1, 3, 7, and 14 d. At the end of the experimental period, the cells were washed with PBS and fixed for 45 min using 10% neutral buffered formalin. They were then incubated with Alizarin Red S staining solution (Carl Roth, Karlsruhe, Germany), pH 4.1, at room temperature in the dark for 45 min. Visualization of the cellular monolayer was performed under 10× magnification using bright-field microscopy (Nikon Eclipse E800). The results were obtained from two experiments performed in triplicate.

Statistical analysis

For statistical analysis, GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) was used. Data were analyzed using a two-way ANOVA followed by Bonferroni post-hoc testing to explore differences in cell responses to the TSC materials in individual experiments. All tests were performed at a level of significance α = 0.05. Results are presented as mean ± standard error of the mean.

Results

Cell viability

Cell morphology was not affected under the experimental conditions. Exposure for 1 day to the TSC eluates did not reduce viability of hBMSCs, regardless of the TSC material and/or concentration tested. Differences between the TSC materials, regarding their effect on hBMSC viability, were seen after 3 d of incubation; simple main-effect analysis showed that only TotalFill reduced cell viability at the highest concentration at 3 d (P < 0.001). Moreover, after 7 d of incubation with TSC materials, a statistically significant (P = 0.0036) concentration–effect interaction was observed for each TSC: MTA showed the strongest inhibitory effect, followed by TotalFill and then by Biodentine, and inhibition occurred in a dose-dependent manner (Fig. 1).

Based on these results, a 1:4 dilution of TSC material was selected for the rest of the experiments.

Osteogenic and angiogenic differentiation

Cells were stimulated with TSCs diluted 1:4. This concentration was chosen based on the MTT results, in which lower concentrations showed no effect on cell viability and higher concentrations reduced cell viability. A two-way ANOVA was conducted to examine the effect of TSC and duration of exposure on gene expression levels. For all genes investigated, there was a statistically significant interaction between the effects of TSC and duration of exposure on gene expression. For all genes investigated, there was a statistically significant interaction between the effects of TSC and duration of exposure on gene expression levels. For all genes investigated, there was a statistically significant interaction between the effects of TSC and duration of exposure on gene expression levels. For all genes investigated, there was a statistically significant interaction between the effects of TSC and duration of exposure on gene expression levels. For all genes investigated, there was a statistically significant interaction between the effects of TSC and duration of exposure on gene expression levels.
downregulated \((P < 0.05)\). TotalFill and Biodentine stimulated upregulation of \(\text{OPG}\) at 1 and 3 d, respectively \((P < 0.05\) for TotalFill and \(P < 0.01\) for Biodentine) but MTA had no effect on this gene. TotalFill upregulated \(\text{VEGFA}\) at 1 and 3 d \((P < 0.001\) for both time points), whereas MTA had the same effect at 1 d only \((P < 0.001)\). Upregulation of \(\text{COL1A1}\) was seen only after exposure to MTA for 7 d \((P < 0.05; \text{Fig. 2})\). These results show that hBMSCs exhibit differential responses at gene level when exposed to the bioceramic materials tested in this study.

**ELISA**

ELISA results showed that production of \(\text{VEGFA}\) was significantly enhanced over time (effect of exposure time: \(F_{(2,56)} = 10.22, P = 0.0002\)). However, this effect was not attributed to exposure to TSC. Nevertheless,
Biodentine led to enhanced production of VEGFA compared with the control ($P < 0.01$) and TotalFill ($P < 0.01$) at the 7-d experimental time point (Fig. 3A).

**ALP quantification**

There was no statistically significant interaction between the effects of TSC and duration of exposure on ALP activity, as shown with two-way ANOVA. Simple main-effect analysis showed that ALP activity of hBMSCs was reduced over time ($P < 0.0001$). Moreover, exposure to TSC materials induced a decrease in ALP activity compared with the control at all experimental time points ($P < 0.0001$). However, there were no differences between the TSC materials tested regarding their inhibitory effect on ALP production (Fig. 3B).

**Alizarin Red staining**

Cells stained with Alizarin Red and visualized under 10× magnification showed mineralization and formation of calcium nodules in all groups, including the control, after longer periods of incubation. Only very low levels of calcification were seen in all groups at 1 and 3 d. At 7 d, minimal calcium deposition and staining were seen in all groups, including the control groups. Although no quantification was made, slightly more mineralization and staining were evident for the MTA group (Fig. 4). Pronounced calcium deposition...
and mineralization in all groups stimulated with TSC materials, as well as in control groups, were apparent at 14 d of stimulation. Again, the nodules in the MTA-stimulated cells stained more strongly compared with nodules in the other groups (Fig. 4).

Discussion

The present study compared the effects of three TSC materials, namely MTA, Biodentine, and TotalFill, on stem cell viability and osteogenic/angiogenic differentiation in vitro. We found that the hBMSCs exhibited diverse responses to the tested TSC materials under most of the experimental set-ups and, based on the results of the study, we reject the null hypothesis. However, a conclusion as to which of the three tested TSCs was overall superior could not be drawn as the results varied for the different assays.

Most differences between the three TSC materials on hBMSC responses were seen at gene level. As shown, Biodentine enhanced expression of ALP very early after exposure of the cells, whereas the other materials did not. On the contrary, TotalFill downregulated ALP at 3 d. The osteogenic gene, OPG, was upregulated after exposure to Biodentine and TotalFill at 1 and 3 d, whereas MTA did not have any effect on OPG but upregulated expression of COL1A1 at 7 d. The angiogenic marker, VEGFA, was upregulated by MTA and TotalFill at 1 d of exposure, with this effect lasting longer for TotalFill. Some results obtained in this study were in agreement with those presented in previous reports (11, 21) and some were novel as not many reports compare these three TSCs. The osteogenic markers investigated are all implicated in osteogenic differentiation, dentine and mineral formation, regulation of osteoblastic differentiation, and osteogenesis (22–25). The angiogenic markers chosen are vital for enhancement of endothelial cell proliferation, migration, and differentiation (21, 26). Expression of these molecules is crucial in the healing process of the periodontium after endodontic procedures requiring use of TSC, such as perforation repairs and apicoectomies. It seems that Biodentine and TotalFill induce a larger number of changes at transcriptional level early after exposure, implying a stronger effect of these TSCs on hBMSCs compared with MTA. Of the genes investigated, ALP, OPG, and VEGFA reached comparable levels of expression after 7 d of incubation with TSCs. Whether the early differences in gene expression, induced by Biodentine and TotalFill, have clinical significance in the healing process is worth investigating further.

Cell morphology of the stem cells was not affected by exposure to TSCs and none of the three TSC materials enhanced cellular viability. Biodentine was the most favorable TSC material in terms of cell viability: only the highest concentration of Biodentine, after the longest incubation period, induced a significant decrease in cell metabolism and this decrease was not as large as induced by MTA and TotalFill. Moreover, MTA and TotalFill exhibited their inhibitory effects earlier, indicating either that these materials are more potent than Biodentine or that they have a higher dissolution rate after setting. It should be noted that in the current study the amount of TSC material used to obtain the material eluates was standardized by volume and not by mass. Nevertheless, a dose-dependent response was seen because more concentrated eluates had a higher inhibitory effect on cellular viability for both MTA and TotalFill. Similarly to the present findings, previous reports showed that high concentrations of TSCs (i.e. concentrations of >2 mg ml⁻¹) or undiluted material eluates, may decrease cell viability (27, 28). Furthermore, differences between the materials with respect to the time frame of their effect on cell proliferation have been reported previously (28). Endosequence (e.g. the same material as TotalFill) has been shown to exhibit a greater inhibitory effect on the proliferation of human dermal fibroblasts than MTA at 48 h but this effect was reversed after 8 d of exposure, suggesting that, over time, Endosequence may have a favorable effect (29). Moreover, TotalFill/Endosequence has a longer setting time than MTA and Biodentine, which may explain the differences in its early and late effects. A recent study showed that the highest hBMSC viability was observed after 7 d of exposure to ERRM, followed by MTA and, lastly, Biodentine (30). In the same study, enhanced proliferation of hBMSCs was seen after exposure to Biodentine for 3 d (30). In contrast to our findings, ZHOU et al. (28) reported no differences between MTA and Biodentine in their effect on cell viability of human gingival fibroblasts.

Although Biodentine was the most inert material tested, even at early time points there are reports of its initial high cytotoxicity because of the presence of calcium chloride in the liquid and/or the cement’s higher initial pH (pH) compared with other cements during the setting period (31–33). In the current study, the eluate was prepared after complete setting of the materials, thus explaining the favorable results of Biodentine. Many studies have investigated the cytotoxicity of MTA. Bismuth oxide, the radiopacifying agent in MTA, has been implicated in the cytotoxicity exhibited by the material, being shown to decrease or inhibit cellular proliferation of the Saos-2 osteosarcoma cell line (34). On the other hand, replacing bismuth oxide with niobium oxide in MTA promoted higher cell viability in the Saos-2 osteoblastic cell line (ATCC HTB-85) (35). The MTA used in this study contained bismuth oxide, which might have contributed to its inhibitory effect on the viability of hBMSC. This response was only observed at the longest study time point (7 d), and is in agreement with previous results (34). As healing of the periodontium is a long process, it would have been of interest to study cell viability for even longer periods of time.

Analyses of ALP protein consistently showed a significant decrease in production of ALP after exposure to TSCs, but the response did not differ between the three materials. Surprisingly, increased production of VEGFA was seen over time but this was not attributed to the
materials. Both mRNA expression and protein production followed a similar pattern.

All tested TSC materials induced mineralization at later time points, as seen with Alizarin Red staining. These findings are consistent with previous results from a similar study in hBMSCs in which, after 14 d of culture in differentiation medium, both MTA and Biodentine, as well as control groups, exhibited significant mineralization (11). The current report is the first to show that TotalFill induced the same effect. However, it seems that calcium deposits appeared earlier, and were more pronounced, with MTA than with either Biodentine or TotalFill, again underlying a difference in the response of hBMSCs to the different TSC materials. The earlier mineralization observed with MTA may be attributed to a higher pH and further activation of ALP, which in turn leads to release of a higher concentration of Ca^{2+} ions (36). This high pH, however, can have a cytotoxic effect on cells, which may also explain the less favourable effect of MTA on hBMSC viability in our MTT assay.

The composition of TSCs has a clear effect on their physical, chemical, and biological properties (37, 38). The calcium silicate composition of the bioresorbable materials tested in the present study may, in fact, be responsible for constant release of calcium ions. When MTA and Biodentine come into contact with water, calcium hydroxide is created, which in turn leads to the release of calcium ions. This has been shown for MTA and Biodentine (38) but also applies for TotalFill and other materials of similar composition. The silica component of TSCs may also have an effect on the osteogenic potential of the materials (39). Silica inhibits bone resorption and osteoclast formation, suggesting that it is an essential component in the mechanism of action of TSCs.

This study utilized a relatively short experimental period, and it is possible that a longer time period of incubation with the tested materials might identify further effects on cell viability/metabolism rate, and gene and protein expression, and consequently alter the outcome of the study. It is also important to recognize that substances leaching out from the materials may diffuse into the medium at differing rates, yielding different concentrations of components with potentially different effects. The findings in this study, while demonstrating differences between the TSCs in their overall effects on hBMSCs, are inconclusive in terms of establishing a true superior material for clinical recommendation. Therefore, in vivo studies would be the next step in providing a better and more accurate understanding of these materials and their ultimate effect on cellular responses. We believe that the clinical relevance of these findings should be further investigated.

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