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ORIGINAL ARTICLE

Three-dimensional culture of dental pulp stem cells in direct contact to tricalcium silicate cements

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Abstract
Objectives Calcium silicate cements are biocompatible dental materials applicable in contact with vital tissue. The novel tricalcium silicate cement *Biodentine*™ offers properties superior to commonly used mineral trioxide aggregate (MTA). Objective of this study was to evaluate its cytocompatibility and ability to induce differentiation and mineralization in three-dimensional cultures of dental pulp stem cells after direct contact with the material.
Materials and methods Test materials included a new tricalcium silicate (Biodentine™, Septodont, Saint-Maur-des-Fossés, France), MTA (ProRoot® MTA, DENSPLY Tulsa Dental Specialities, Johnson City, TN, USA), glass ionomer (Ketac™ Molar Aplicap™, 3M ESPE, Seefeld, Germany), human dentin disks and polystyrene. Magnetic activated cell sorting for to the surface antigen STRO-1 was performed to gain a fraction enriched with mesenchymal stem cells. Samples were allowed to set and dental pulp stem cells in collagen carriers were placed on top. Scanning electron microscopy of tricalcium silicate cement surfaces with and without cells was conducted. Cell viability was measured for 14 days by MTT assay. Alkaline phosphatase activity was evaluated (days 3, 7, and 14) and expression of mineralization-associated genes (COL1A1, ALP, DSPP, and RUNX2) was quantified by real-time quantitative PCR. Nonparametric statistical analysis for cell viability and alkaline phosphatase data was performed to compare different materials as well as time points (Mann-Whitney U test, $\alpha=0.05$).
Results Cell viability was highest on tricalcium silicate cement, followed by MTA. Viability on glass ionomer cement and dentin disks was significantly lower. Alkaline phosphatase activity was lower in cells on new tricalcium silicate cement compared to MTA, whereas expression patterns of marker genes were alike.
Conclusions Increased cell viability and similar levels of mineralization-associated gene expression in three-dimensional cell cultures on the novel tricalcium silicate cement and mineral trioxide aggregate indicate that the material is cytocompatible and bioactive.
Clinical relevance The tested new tricalcium silicate cement confirms its suitability as an alternative to MTA in vital pulp therapy.

Keywords Tricalcium silicate · Mineral trioxide aggregate · Collagen type I · Multipotent stem cells · Dental pulp · Three-dimensional cell culture

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Introduction

Biocompatible materials play an increasingly important role in the field of dental material science. In regards to tissue response, they are classified as either bioinert or bioactive [1]. Bioinert materials are tolerated by live tissues but may still elicit interposition of fibrous tissue and passive encapsulation

[2, 3]. In contrast, bioactive materials interact with the host tissue in a controlled manner, which goes beyond the characteristic of biocompatibility. They induce—by means of specific biochemical cues—desired immunological effects, cell recruitment, proliferation, and differentiation or changes in cell metabolism [1].

Past research has shed light on the complex effects of bioactive dental materials on live tissues, especially dental pulp. Calcium hydroxide is a representative material, which has been used in pulp therapies for decades [4]. Due to its alkalinity, it induces a superficial necrosis and moderate inflammatory response in the adjacent pulp tissue. Healing is initiated by resolution of inflammation, recruitment, and differentiation of stem cells and completed with the formation of tertiary dentin at the cell-material interface [5]. In recent years, mineral trioxide aggregate cements have increasingly substituted for calcium hydroxide due to less caustic effects and higher clinical success rates [5–7]. Mineral trioxide aggregate constitutes the gold standard in vital pulp therapy today. Additionally, it is applicable to treat areas of communication between endodontium and periodontium, such as perforations or root end fillings [8, 9].

MTA is a hydraulic silicate cement and mainly consists of tricalcium silicate, dicalcium silicate, and tricalcium aluminate, which form a matrix of calcium silicate hydrate during setting [10, 11]. Another important product formed during setting is calcium hydroxide as inflammatory trigger for tertiary dentinogenesis, but it does not cause the above described caustic injury as, compared to calcium hydroxide, lesser amounts are in contact with the tissue [5, 12–14]. In addition, bioactivity derives from apatitic precipitates that form in contact with phosphate-containing fluids and deposit on the material surface [10, 11].

Although clinically successful, MTA is costly, exhibits poor handling properties, short workability, a long setting time of several hours and the risk of tooth discoloration [10, 11, 15]. Therefore, new calcium silicate cements with optimized material qualities have been developed, such as the novel bioactive tricalcium silicate cement *Biodentine*™. Besides the typical components of tricalcium silicates, it contains additional calcium carbonate fillers to improve mechanical stability as well as zirconium dioxide to ensure radiopacity [16, 17]. The liquid is supplemented with a plasticizing polycarboxylate and calcium chloride to improve workability and accelerate setting [18, 19].

Recent investigations confirm the biocompatibility of the material [18–22] and its ability to induce dentin bridge formation comparable to mineral trioxide aggregate [17, 23]. Thus, the new tricalcium silicate cement appears to be a convenient and affordable alternative to MTA. However, conclusions drawn from in vitro studies are limited due to the use of monolayer cultures exposed to eluates of the test materials.

A three-dimensional culture model appears more suitable to mimic the clinical situation. It is fundamentally different from monolayer cultures as it enables 3D interaction with neighboring cells and extracellular matrix [24, 25], which stimulates cell proliferation and promotes differentiation [26–28]. A further approximation to the clinical situation is the use of cultures in direct contact to the set test materials. In eluate experiments, cells are steadily exposed to the same concentration of material compounds without influence of the material's surface morphology. A different cell response is expected after direct interaction with the material surface, where the release of soluble material compounds will be initially high and decrease over time. Little is known about the behavior of tissue-like three-dimensional cultures of human dental pulp stem cells in direct contact to bioactive dental materials.

Due to the similarities in composition between MTA and the novel tricalcium silicate cement, we hypothesize that proliferation and mineralization activity of 3D-cultured dental pulp stem cells will be alike. To justify the use of the tricalcium silicate cement as an alternative to MTA in direct contact with dental pulp, the aims of this study were (i) to evaluate the morphology of human dental pulp stem cells on the new tricalcium silicate cement and (ii) to compare the viability and mineralization activity in three-dimensional cell cultures on both materials.

Materials and methods

Materials and specimen preparation

Materials included in this study were novel tricalcium silicate cement (Biodentine™, Septodont, Saint-Maur-des-Fossés, France), mineral trioxide aggregate (ProRoot® MTA, DENSPLY Tulsa Dental Specialities, Johnson City, TN, USA), glass ionomer cement (Ketac™ Molar Aplicap™, 3M ESPE, Seefeld, Germany), and dentin disks prepared from human molars (200 μm high and 6 mm in diameter). Polystyrene for suspension cultures (Costar® 96-well plates, Corning, Lowell, MA, USA) to avoid cell attachment at the bottom of the wells was used as a control. Cell viability was tested for all materials, whereas assays for differentiation included only bioactive materials, namely MTA and the new tricalcium silicate cement.

Material specimens of 2 mm in height were prepared in 96-well plates according to the manufacturer's instructions, resulting in distinct setting protocols for each material (Table 1). After setting, pretreatment was equal for all samples. Specimens were covered with 200 μL aqua bidest. and exposed to UV-light in a laminar flow cabinet for 30 min to prevent bacterial contamination. After 24 h at 37 °C and 5 %

Table 1 Sample preparation and pretreatment of different materials in 96-well plates

Material	Sample preparation	Pretreatment for all materials	
New tricalcium silicate cement	12 min setting, laboratory conditions* 20 min setting in humid chamber**	200 µL aqua bidest for 24 h (37 °C, 5 % CO_2)	200 µL αMEM for 24 h (37 °C, 5 % CO_2)
Mineral trioxide aggregate	4 h setting, moist conditions		
Glass ionomer cement	7 min setting, laboratory conditions*		
Dentin disks	Storage in 0.5 % chloramine-T solution Rinse with phosphate buffered saline		
Polystyrene	No treatment		

* 60 % humidity, 25 °C ambient temperature

** 100 % humidity, 37 °C, 5 % CO_2

CO_2, aqua bidest. was replaced with alpha minimum essential medium (αMEM) and incubated for another 24 h.

Cell culture

Dental pulp cell cultures were established from impacted third molars as described previously [29] according to an informed consent protocol approved by an appropriate review board at the University of Regensburg. Primary cells were cultured in αMEM supplemented with 10 % fetal bovine serum, 50 µg/mL L-ascorbic acid 2-phosphate, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5 % CO_2. Cell culture media and reagents were purchased from Gibco® (Invitrogen Corporation, Carlsbad, CA, USA). At passage 3, cells were subjected to magnetic activated cell sorting (MACS) to obtain mesenchymal stem cells using a monoclonal STRO-1 antibody (primary antibody: purified anti-human STRO-1 mouse IgM, BioLegend, San Diego, CA, USA; secondary antibody: anti-mouse IgM Micro Beads, Miltenyi Biotech, Auburn, CA, USA). The sorting procedure was performed twice to gain a cell fraction reliably expressing the mesenchymal surface antigen. Successful sorting was confirmed by immunohistochemistry and differentiation was reassured after incubation with adipogenic, chondrogenic, and osteogenic culture media (StemPro® Adipogenesis, Osteogenesis and Chondrogenesis Differentiation Kit, Invitrogen Corporation, Carlsbad, CA, USA) for 4 weeks (Supplementary Fig. S1).

Three-dimensional cultures with sorted cells were established in scaffolds of collagen type I with a concentration of 3 mg/mL (Rat Tail Collagen Type 1, BD Biosciences, Bedford, MA, USA) and 1.5×10^5 cells were encapsulated per100 µL. After direct application onto the prepared materials and the plain bottom of 96-well plates, the collagen samples were alkalized and stored at 37 °C for 20 min to allow for gelation at physiological pH. Subsequently, each gel was covered with 200 µL media, and cells in collagen were cultured on material specimens for up to 21 days (Supplementary Fig. S2), while medium was exchanged three times a week.

Scanning electron microscopy

For the new tricalcium silicate cement, surface structure after setting without and with cells was analyzed by scanning electron microscopic (SEM) imaging. For samples with cells, 1×10^5 human dental pulp stem cells (hDPSCs) were seeded onto the material specimens in 24-well plates and allowed to adhere at 37 °C and 5 % CO_2. After 24 h, samples were fixed with 2.5 % glutaraldehyde in 0.1 M Sørensen's phosphate buffer for 30 min. Images were taken on a FEI Quanta 400 environmental scanning electron microscope with a field emitter (FEI Europe B.V., Eindhoven, The Netherlands) and operated at low-vacuum scanning electron microscopy (LVSEM) imaging mode. Images were analyzed regarding morphological aspects based on work by Camilleri et al. [30–33].

Cell viability

Viability of hDPSCs in three-dimensional cultures on materials was quantified over a period of 14 days. The MTT assay was modified for three-dimensional cell cultures by prolonged incubation and elution times (details see below) and performed with cells in collagen gels at days 1, 3, 5, 7, 10 and 14. Briefly, after incubation of the collagen gels with methylthiazolyldiphenyl-tetrazolium bromide (MTT; 0.5 mg/mL) (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, St. Louis, MO, USA) for 60 min at 37 °C and 5 % CO_2, the converted dye was eluted with DMSO for 180 min on a plate shaker at 220 rpm. Absorbance was measured on a microplate reader at $\lambda=570$ nm (Infinite® 200, Tecan, Männedorf, Switzerland).

Table 2 Primer sequences for real-time quantitative PCR

Primer	Sequence (5′–3′)	Product size	T_m
ALP		95 bp	84 °C
Sense	GACCCAAGAAACCAAAGTCTGCC		
Antisense	GAGGGAGCAAAGGCTGGAGG		
COL1A1		128 bp	85 °C
Sense	CCTCTGCTCTCCGACCTCTCT		
Antisense	CTTTGTGCTTTGGGAAGTTGTCTCT		
DSPP		97 bp	78 °C
Sense	AGCCACAAACAGAAGCAACAC		
Antisense	GACAACAGCGACATCCTCATT		
RUNX2		80 bp	79 °C
Sense	CTCCACCCACCCAAGCAGAA		
Antisense	GTTTGAGAAGGACCAGAGAACAAGG		
RPS18		80 bp	79 °C
Sense	AAATAGCCTTTGCCATCACTGCC		
Antisense	GTCAATGTCTGCTTTCCTCAACACC		

For each time point, optical density measurements of cells on polystyrene served as reference and were set to 100 %. Median values and 25–75 % percentiles were obtained from three independent experiments performed in triplicate (n=9).

Cells in collagen gels underwent live/dead staining with calcein and ethidium homodimer-1 (EthD-1) after 1, 3, 5 and 7 days of culture in 96-well plates (Live/Dead® Viability/Cytotoxicity Kit, Life Technologies, Carlsbad, CA, USA). Three-dimensional culture was illustrated by a spatial reconstruction based on single confocal laser scanning microscopy (CLSM) images without further quantification (LSM 510 META, Carl Zeiss Microscopy, Jena, Germany).

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity of 3D-cultured dental pulp stem cells in direct contact with mineral trioxide aggregate, new tricalcium silicate cement, dentin disks, and polystyrene was quantified at days 3, 7 and 14. Glass ionomer cement was excluded due to the low cell viability on this material. Collagen gels were solubilized in 60 μL phosphate-buffered saline and mixed with 60 μL alkaline buffer solution (Alkaline buffer solution, Sigma-Aldrich, St. Louis, MO, USA). After addition of 100 μL p-nitrophenylphosphate (4 μg/μL) (Phosphatase substrate, Sigma-Aldrich, St. Louis, MO, USA), samples were incubated at 37 °C and 5 % CO_2 for 60 min. Subsequently, the enzymatic reaction was stopped with 100 μL NaOH (0.3 M) and the optical density of p-nitrophenol was measured spectrophotometrically at λ=405 nm (4-Nitrophenol solution, Sigma-Aldrich, St. Louis, MO, USA). ALP activity was standardized as $\mu M \times h^{-1}$ p-nitrophenol per viable cell culture, which was defined by the MTT assay as percentage of cell viability relative to the polystyrene control.

Enzyme activity on polystyrene served as a reference for each time point and was set to 1. Median values and 25–75 % percentiles were calculated from four independent experiments performed in triplicate (n=12).

Real-time quantitative PCR

To verify the effects of new tricalcium silicate cement and mineral trioxide aggregate on the differentiation of hDPSCs, real-time quantitative PCR (RT-qPCR) was performed. Three-dimensional cultures were conducted up to 21 days as mentioned above and relative gene expression of alkaline phosphatase (ALP, NM_000478), collagen type I (α1, COL1A1, NM_000088), dentin sialophosphoprotein (DSPP, NM_014208), runt-related transcription factor 2 (RUNX2, NM_001015051) and 40S ribosomal protein S18 (RPS18, NM_022551) as housekeeping gene were analyzed. Specific primers, as shown in Table 2, were designed [34] and synthesized (Eurofins MWG Synthesis, Ebersberg, Germany).

After 7, 14 and 21 days, total RNA was isolated (RNeasy Mini Kit, Qiagen, Hilden, Germany) and quantified using a spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific, Waltham, MA, USA). Samples of 80 ng were transcribed into cDNA (Omniscript RT Kit, Qiagen, Hilden, Germany) by use of an oligo-dT Primer (Oligo-dT Primers, Qiagen, Hilden, Germany). Real-time quantitative PCR used

10 pmol of each primer, 5 μL complementary DNA diluted 1 in 2 and qPCR master mix with SYBR® Green in a total volume of 20 μL (SYBR® Select Master Mix, Applied Biosystems, Carlsbad, CA, USA). Samples were denatured at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and annealing at 60 °C for 1 min.

Amplification was performed in triplicate with the StepOnePlus™ Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) and two independent experiments were conducted. For data analysis, expression of target genes was normalized to RPS18 and expression levels were computed in relation to control groups with the comparative C_T method ($2^{-\Delta\Delta CT}$) [35]. At each time point, gene expression of cells on polystyrene served as reference and was set to 1.

Statistical analysis

Data were treated nonparametrically and results were analyzed statistically using the Mann-Whitney *U* test on the $\alpha = 0.05$ level of significance (SPSS, version 22.0, SPSS Inc., Chicago, IL, USA).

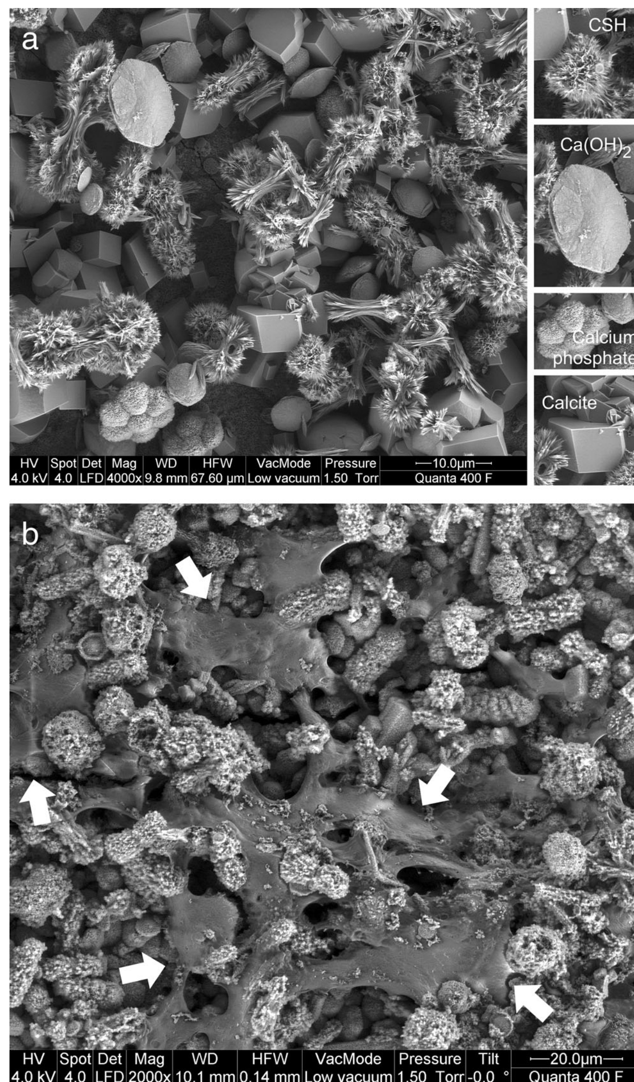

Fig. 1 SEM imaging of cement surface without and with human dental pulp stem cells. **a** Surface structure of the new tricalcium silicate cement samples after incubation in cell culture medium was analyzed according to morphological aspects based on the work of Camilleri et al. [30–33] and displays calcium silicate hydrate (CSH) deposits, calcite microcrystals, calcium hydroxide plates, and calcium phosphate particles. **b** Cell adhesion and spreading on the surface of tricalcium silicate (*arrows*)

Results

Scanning electron microscopy

Representative scanning electron microscopic images of the new tricalcium silicate cement without and with cells are depicted in Fig. 1. Specimens without cells exhibited the characteristic appearance of tricalcium silicate cement after immersion in a buffered solution.

As a product of gelation, calcium silicate hydrate crystals were present with their typical honeycomb morphology. The surface showed predominantly rhomboid calcite microcrystals of variable sizes (Fig. 1a), which are associated with the material's process of hydration and improve the mechanical properties. Calcium hydroxide crystals shaped as hexagonal plates, which are formed during setting reaction, were distributed over the entire surface. Furthermore, spheroidal calcium phosphate particles were observed between the calcium silicate hydrates.

The SEM images of cells cultured on tricalcium silicate cement showed adhesion to and spreading onto the material surface (Fig. 1b). Cells extended elongated processes on the cement surface.

Cell viability

Data collected from cell viability assays are shown in Fig. 2. The statistical analysis regarding differences between materials is summarized in the supplementary information (Supplementary Table S1).

Cell viability of hDPSCs on novel tricalcium silicate cement was significantly higher compared to the polystyrene control at all time points. Viability of cells cultured on MTA was significantly lower compared to that of cells on the new tricalcium silicate cement until day 7 and not significantly different for the last two time points. Viability on dentin disks was not significantly different to controls, whereas it was significantly lower on glass ionomer cement. As the left insert shows, viability on polystyrene decreased during the measurement period. Live/dead staining at days 1, 3, 5 and 7 in

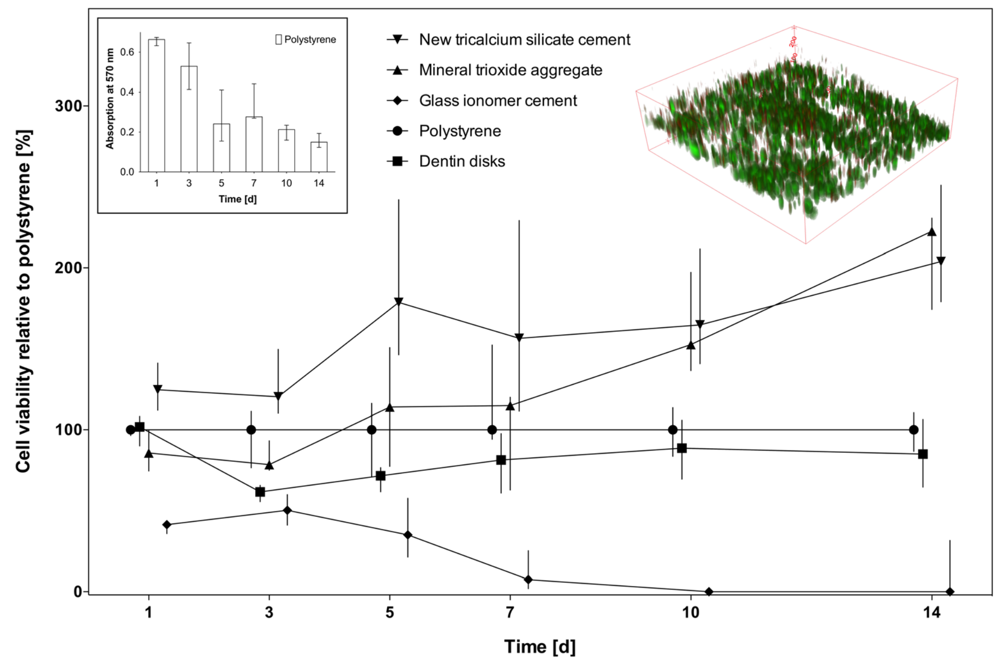

Fig. 2 Cell viability of hDPSCs in three-dimensional cultures on different materials during a 2-week culture period. For each time point, optical density measurements of cells on polystyrene served as reference (*left insert*) and were set to 100 %. Depicted are median values and 25–75 % percentiles computed from three independent experiments performed in triplicate (n=9). Representative three-dimensional reconstruction of a collagen gel with hDPSCs after 3 days in culture on polystyrene (*right insert*). Live/dead staining shows the majority of cells as viable (*green*)

collagen on polystyrene, however, showed that viable cells (green) dominated the image, whereas nonviable cells (red) were sparse; both were distributed equally inside the gel. The right insert in Fig. 2 shows a representative 3D image of cells in collagen after 3 days on polystyrene. Additional images from different time points can be viewed in the supplementary information (Supplementary Fig. S3).

Alkaline phosphatase activity

ALP activities in three-dimensional cultures on different specimens are depicted in Fig. 3. Cells on polystyrene (no material) served as control (Fig. 3, insert). They showed the highest level of ALP activity at day 3 (400.67 $\mu M \times h^{-1}$) and lower levels at days 7 and 14 (115.63 $\mu M \times h^{-1}$and 140.43 $\mu M \times h^{-1}$).

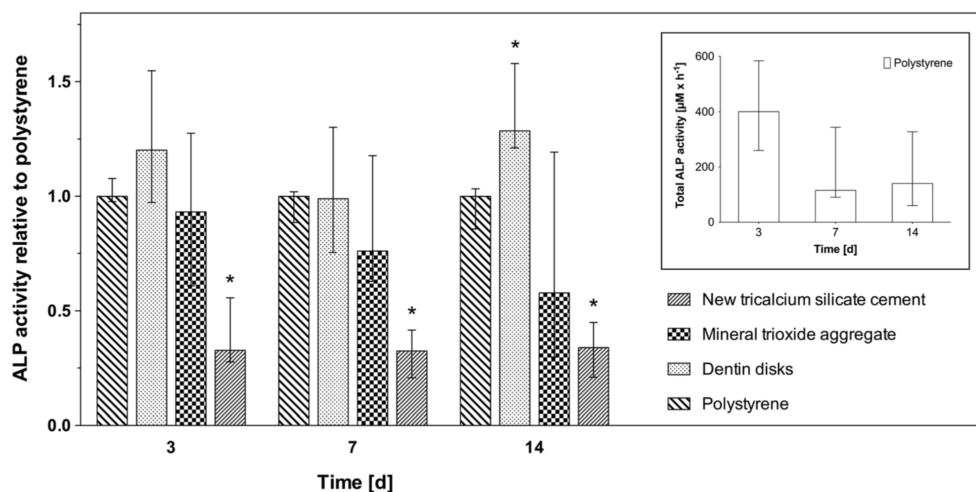

Fig. 3 Quantification of ALP levels in three-dimensionally cultured hDPSCs on mineral trioxide aggregate, new tricalcium silicate cement, dentin disks, and polystyrene for 3, 7 and 14 days. For each time point, ALP activities per viable cell culture were depicted as relative enzyme activity compared to polystyrene (*insert*), which was set to 1. Median values and 25–75 % percentiles were calculated from four independent experiments performed in triplicate (n=12). *Asterisks* denote statistical differences in experimental groups compared to the reference (polystyrene) at each time point

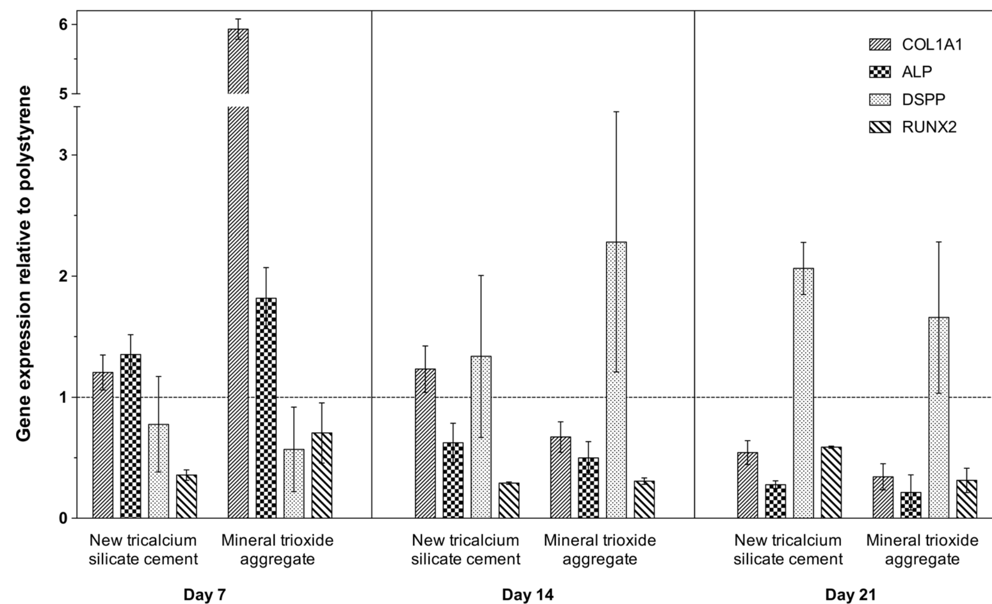

Fig. 4 Effect of mineral trioxide aggregate and the new tricalcium silicate cement on expression of mineralization-associated marker genes in three-dimensionally cultured hDPSCs at three time points. Target gene expressions are depicted relative to the untreated control. Median values were calculated from two independent experiments, *error bars* illustrate the measured values

Whereas alkaline phosphatase activity in cells on dentin disks was higher compared to the polystyrene control (significant at day 14), it was reduced in cells cultured on mineral trioxide aggregate and the new tricalcium silicate cement. The latter evoked a distinct reduction of enzyme activity with statistical significance to the control.

Real-time quantitative PCR

Figure 4 shows the expression of mineralization-associated genes (Table 2) in three-dimensionally cultured hDPSCs on different materials at different time points of a 3-week culture period.

On both bioactive materials, expression of mRNA for COL1A1 and ALP was initially upregulated, especially in cells cultured on mineral trioxide aggregate, and decreased steadily until day 21. For DSPP expression, cells cultured three-dimensionally on tricalcium silicate cement and mineral trioxide aggregate showed a similar pattern of upregulation at days 14 and 21. Throughout the duration of culture, expression of RUNX2 was downregulated.

Discussion

In the present study, we investigated the potential of a new tricalcium silicate cement to enable adhesion and proliferation and to promote differentiation of dental pulp stem cells in comparison to MTA. Human DPSCs were cultured three-dimensionally in collagen scaffolds in this study; thus, the observed effects arise from direct contact and interaction between cells and material surface as well as from soluble compounds released from the material into the cell culture media.

Scaffold material

Collagen type I was chosen as scaffold material as it represents the major component of extracellular matrix in dental pulp. The material is highly cytocompatible, biodegradable, promotes cell adhesion and allows for cell migration [36]. Confocal images after live/dead staining confirmed cell vitality and showed equal distribution within the carrier material. Three-dimensional cultures of dental pulp stem cells in collagen mimic the characteristics of a connective tissue and appear to be a more adequate model than monolayer cultures [24]. Comparisons between monolayer and spheroid cultures of dental pulp cells revealed increased odontoblastic marker gene expression and mineralized nodule formation in three-dimensionally cultured cells, indicating a promotion of cell differentiation [26]. Moreover, $\alpha_2\beta_1$-dependent cell adhesion to a collagen type I-specific sequence (GFOGER) has been shown to advance odontoblast differentiation [37, 38].

Material pretreatment

Pretreatment of the specimens before cell seeding mimics the effect of tissue fluids on a dental material at the pulp-material interface. Buffer capacity and ion concentration of cell culture medium correlates with tissue homeostasis in vivo and likewise is able to reduce initially high concentrations of calcium hydroxide and thus alkalinity [10, 11]. This is essential to overcome the dose-dependent toxic effect of calcium

silicate-based materials on dental pulp stem cells [21, 39] and to allow for the formation of calcium phosphate precipitates, which contribute to a cytocompatible environment [10, 11].

Scanning electron microscopy

SEM imaging showed the typical morphology of tricalcium silicate cement [30, 33]. As a product of gelation, calcium hydroxide particles were present on the surface, which reportedly play a key role in dentinogenesis in vivo [5]. Dental pulp stem cells adhered directly to the surface and extended processes, affirming the cytocompatibility of tricalcium silicate cement.

Cell viability

MTT assay was modified for three-dimensional cell culture. Whereas an incubation time of 60 min was adequate to reach all cells in the collagen gels, the elution time was prolonged (180 min) to ensure the complete liberation of the converted dye from the scaffold. Cell viability declined over time, which may not be misinterpreted as cell death (Fig. 2, left insert). Three-dimensional cultures of hDPSCs in type I collagen were characterized predominantly by viable cells, which was confirmed by live/dead staining at different time points of the culture period (Supplementary Fig. S3). A decline of absorbance values might be due to a high seeding density, which was chosen after pilot experiments on cell numbers to establish acceptable optical densities for initial measurements and to enable an intense cellular communication in three-dimensional culture. Material stiffness might also be a contributing factor; the low elastic modulus of collagen scaffolds can cause a change in cell-matrix adhesion and affect cell proliferation negatively [25, 40, 41]. Furthermore, a progressive contraction of the collagen gels over the culture period was observed, which may induce contact inhibition and a decrease in cell metabolism. Thus, normalization of data from three-dimensional cultures on test materials on three-dimensional cultures without material (polystyrene) appears legitimate and necessary for a comparative and clear interpretation of the outcomes.

Increased cell viability on bioactive materials, such as the new tricalcium silicate and MTA compared to controls was observed in this study. Similar results were reported previously for monolayer cell cultures with material eluates [20–22]. Whereas the influence of dentin disks on cells was negligible, glass ionomer cement had a negative impact on cell viability as reported before [39, 42]. As pH value was physiological during culture period, the adverse effect on cells may be attributed to leachable components, such as aluminum, fluoride, strontium and zinc ions [1, 43].

Enzyme activity and gene expression of ALP

Alkaline phosphatase plays an important role during dentin matrix mineralization. The enzyme hydrolyzes pyrophosphates (PP_i) and generates inorganic phosphates (P_i) that are necessary for hydroxyapatite crystallization [36, 44]. ALP serves as an early marker for calcification; its expression and activity are upregulated at the initial phase of mineralization and declines as soon as other noncollagenous proteins are formed [36]. Our results showed only small changes in ALP activity throughout the measurement period for each material and revealed a reduction of ALP activity on new tricalcium silicate cement and MTA compared to control cells. A similar profile of reduced ALP activity was described in a recent report on the effect of tricalcium silicate cement on monolayer cells, which was interpreted as cellular maturation [20]. In this study, ALP activity is related to cell viability and furthermore to the negative control (cells on polystyrene without material), which allows for direct comparison of data columns. Reproducibility was confirmed by an increased sample size for this experiment.

RT-qPCR data confirmed the trend observed for ALP activity, where mRNA expression for alkaline phosphatase decreased during the observation period. Results for day 7 showed an inconsistency, where ALP gene expression was slightly increased, but this increase was not found on the protein level. This may be attributed to a potential posttranscriptional regulation; however, a direct comparison is disputable, because of normalization of ALP activity on viable cell culture (percentage viability defined by MTT assay).

Expression of mineralization-associated marker genes

The analysis of additional mineralization-associated genes included collagen type I (α1, COL1A1), dentin sialophosphoprotein (DSPP), and runt-related transcription factor 2 (RUNX2).

At the beginning of dentinogenesis, upregulation of genes for extracellular matrix formation, like COL1A1, takes place [36]. Collagen type I is the major component of the connective tissue in dental pulp and acts as matrix for calcification and crystal formation [5, 36]. In this study, the transcription of mRNA for COL1A1 was initially upregulated, especially on mineral trioxide aggregate, and declined for the last two time points simultaneously to ALP; similar observations were presented in a recent investigation using eluates on monolayer cultures [20].

Another initiator and modulator for hydroxyapatite growth is dentin phosphoprotein (DPP), which is cleaved off the inactive precursor dentin sialophosphoprotein together with dentin sialoprotein (DSP). DPP binds to collagen fibrils and enables initial formation of apatite crystals. Our results

showed a continuously increasing transcription of mRNA for DSPP with both bioactive cements.

The multifunctional transcription factor RUNX2 plays a pivotal role in odontoblast differentiation and dentin mineralization by regulating the expression of noncollagenous matrix proteins, such as DSPP [36, 45]. The controlled downregulation of its transcription is essential for odontoblast differentiation and cell maturation, whereas increased expression obviates odontoblast terminal differentiation and promotes trans-differentiation into osteoblasts [46–48]. Lower expression of RUNX2 in three-dimensional cultures on the new tricalcium silicate cement and mineral trioxide aggregate compared to control at all measuring time points may be regarded as an indicator for the initiation of odontoblast differentiation by bioactive cements. A reduced expression of RUNX2 along with an increased expression of DSPP has been described for pre-odontoblast-like cells by several groups [46, 49].

Thus, gene expression patterns of mineralization-associated proteins and transcription factors were alike with both bioactive materials, confirming similar effects on hDPSCs in three-dimensional culture. This is in accordance with clinical studies comparing MTA and the new tricalcium silicate cement in vital pulp therapy [17]. In addition to gene expression analysis, further quantification of protein levels of Runx2 and dentin sialophosphoprotein might be interesting. However, the respective experiments require high cell numbers, which are difficult to establish in the chosen setup with three-dimensional cultures on test materials.

Conclusions

The tested novel tricalcium silicate cement is cytocompatible, stimulates cell proliferation, and exerts a bioactive effect on three-dimensionally cultured dental pulp stem cells by induction of mineralization-associated gene expression similar to MTA. The results from this study support its clinical use as a suitable alternative to mineral trioxide aggregate in vital pulp therapy.

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Conflict of interest The authors declare that they have no conflict of interest.

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