Clinical Oral Investigations (2022) 26:6839–6853
https://doi.org/10.1007/s00784-022-04713-5

RESEARCH

Comparative chemical properties, bioactivity, and cytotoxicity of resin-modified calcium silicate–based pulp capping materials on human dental pulp stem cells

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Received: 30 May 2022 / Accepted: 7 September 2022 / Published online: 15 September 2022

Abstract

Objectives This study investigated the cytotoxicity, the residual monomer release, degree of conversion (DC), calcium ion (Ca^{2+}) release, and crystal structure of TheraCal PT (ThPT) by comparison with TheraCal LC (ThLC) and mineral trioxide aggregate (MTA).

Materials and methods The cytotoxicity of the cured materials was evaluated on human dental pulp stem cells (hDPSCs) isolated from third molars by the water-soluble tetrazolium salt (WST-1) method. The monomer release and DC of the resin-containing materials were analyzed by high-performance liquid chromatography (HPLC) and Fourier transform infrared (FTIR), respectively. The chemical composition and Ca^{2+} release of the materials were determined by scanning electronic microscopy–energy-dispersive spectroscopy (SEM–EDS), X-ray diffractometry (XRD), and inductively coupled plasma-optical emission spectroscopy (ICP-OES), respectively. Statistical differences were evaluated with one-way ANOVA, repeated measure ANOVA, and the Tukey test ($p < 0.05$).

Results MTA showed significantly lower cytotoxicity than either ThLC or ThPT after 1, 3, and 7 days ($p < 0.05$). TEGDMA release of ThPT is significantly higher than ThLC ($p < 0.05$). All materials showed calcium Ca^{2+} release, with MTA significantly higher than the others ($p < 0.05$).

Conclusions MTA showed low cytotoxicity and high Ca^{2+} release compared to ThLC and ThPT.

Clinical relevance.

The cytotoxicity and residual monomer release of ThLC and ThPT may raise concerns about the viability of hDPSCs. Further investigations with the use of in vivo research models are required to validate in vitro bioactivity properties and the potential adverse biological effects of ThLC and ThPT on hDPSCs.

Keywords Calcium silicate · Cytotoxicity · Ion release · Monomer · Vital pulp therapy

Introduction

Vital pulp therapies, including indirect, direct capping, and pulpotomy procedures, manage the treatment of reversible pulpitis. Thus, it is aimed to preserve the vitality of the pulp, which has important roles in the nutrition and formation of dentin, as well as functions such as tooth innervation and defense [1]. In the case of pulp exposure, human dental pulp stem cells (hDPSCs) in pulp tissue can differentiate into odontoblast-like cells, stimulating hard tissue regeneration [2]. In vitro demonstration of the expression of odontogenic genes and in vivo measurement of dentin bridge formation demonstrate the ability of hDPSCs to regenerate dentin during dentinogenesis [3, 4]. In addition to the potential for hDPSCs to form a hard tissue barrier, pulp capping materials

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also promote hard tissue regeneration and prevent bacterial invasion [5].

Vital pulp therapies include the preservation of exposed pulp health and a bio-stimulus effect by the inducement of the repair mechanism, following the placement of a biocompatible material on the pulpal exposure area [6, 7]. Thus, pulpal tissue is protected from further stress which can contain operative procedures, the toxicity of restorative materials, and bacterial penetration due to microleakage. The toxicity of the pulp capping materials is an essential factor since the applied materials and pulp tissue are in contact with each other in the treatment method which aims to maintain its vitality. Therefore, the biocompatibility, antibacterial and anti-inflammatory properties, and hDPSC activity of pulp capping materials should be evaluated on the basis of chemical features and bioactivity [8, 9].

The use of calcium hydroxide-based materials has been accepted as a part of the standard treatment method for a long time in vital pulp therapies [10]. In addition, mineral trioxide aggregate (MTA; MTA Angelus, Londrina, PR, Brazil), a calcium silicate–based material, is a pulp capping material containing tricalcium silicate, tricalcium aluminate, calcium oxide, and some minerals. In comparative studies on biocompatibility, histological and clinical applications in the pulp capping applications of calcium hydroxide and MTA materials, MTA material was found to be more successful than calcium hydroxide [11, 12]. It was observed that MTA produced a less inflammatory response and pulp necrosis in the pulp exposure area and formed more homogeneous and high-thickness hard tissue [13]. MTA is currently accepted as the gold standard due to its high antibacterial performance and biocompatibility [14]. However, the major disadvantages of MTA are its long curing time, difficulty of application, long preparation time, and low resistance to discoloration [11, 15]. Also, another major disadvantage of MTA and similar hydraulic calcium silicate–based materials is that they exhibit a water-based chemical structure and therefore create an inadequate micromechanical adhesion to the overlying resin restoration [16]. It has been suggested to delay tooth restoration so as not to affect the setting reaction [17]. To overcome these limitations, new light-cured resin-modified tricalcium silicate materials are being formulated for pulp capping procedures. TheraCal LC (ThLC; Bisco Inc., Schaumburg, IL, USA) was introduced as a pulp capping material resembling the properties of MTA and containing resin monomers and calcium silicate. Among its various advantages, it can be cured immediately by exposure to light, exhibits low solubility and high mechanical strength, is easy to use, and can be fully applied to exposed pulp [18]. ThLC is transported and placed via a syringe, which, unlike conventional MTA products, eliminates the need for manual mixing procedures, thanks to its viscous nature that can flow over an exposure surface before light-curing. Therefore, a tight seal can be created, which can be easily placed on the teeth and also clinically shortened the operation time, which reduces the risk of contamination [19]. Recently, TheraCal PT (ThPT; Bisco Inc.), a new dual-cured resin-modified calcium silicate material designed for pulp capping, has been introduced for clinical use. According to the manufacturer, ThPT protects the vitality of pulp tissue by acting as a barrier to protect the dentin-pulp complex, is primarily indicated for pulpotomies, and can also be used for indirect and direct pulp capping [20, 21]. The purpose of dual-cure materials is to combine chemical polymerization and photopolymerization to achieve complete curing in the absence of a visible light source. ThLC insertion thickness is limited to 1 mm, while ThPT is dual-cure so it can be safely placed in a single layer. However, the fact that ThLC and ThPT contain resin monomers gains these materials' positive properties such as high bond strength, whereas it raises concerns about its detrimental effect on hDPSCs [22].

The materials used in vital pulp treatment remain in direct contact with the pulp tissue for a long time. Therefore, it should exhibit not only adequate cytocompatibility but also bioactive properties. Calcium silicate–based cements are bioactive materials with the ability to release hydroxyl and calcium ions, thereby creating an adequate environment to promote healing and repair, and providing odontoblastic cell differentiation responsible for the production of the reparative dentin bridge [23]. Calcium silicate–based materials tend to exhibit bioactivity by releasing Ca^{2+} and precipitation of apatite crystals when exposed to physiological fluids containing phosphate. Resin-modified calcium silicate cements are promising dental materials for pulp protection [24]. However, the release of unpolymerized monomers is believed to have a major impact on cytotoxicity in pulp cells, both directly by diffusion of monomers into pulp tissue and indirectly via intra-tubular and peritubular tubular dentin [25–27]. Considering that these materials are used in contact with the pulp, they should have the ability to structurally protect the pulp, as well as have a low release of monomers and a high degree of conversion to reduce the possibility of diffusion of monomers [28].

There was limited knowledge of the biochemical properties of this new material, ThPT, and its comparison with ThLC and MTA. Although there were previous studies investigating the cytotoxicity of ThPT versus ThLC using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) on hDPSCs [20, 21], a study investigating the cytotoxicity of ThPT using the water-soluble tetrazolium salt (WST-1) assay, which does not show cytotoxic effects on cells compared to the MTT assay and providing a cell viability monitoring period longer than 3 days, has not been found in the literature. Furthermore, performing the crystal phase analysis of the ThPT material with X-ray diffractometry (XRD), comparing the similarities

and differences between ThLC and MTA, and supporting this microstructural analysis with the analyzes of residual monomer, degree of conversion (DC), Ca^{2+} release, and energy-dispersive spectroscopy (EDS) will contribute to the literature. Therefore, this study aimed to compare the cytotoxicity, the residual monomer release, DC, release of Ca^{2+}, and crystal structure of ThPT with ThLC and MTA. The null hypothesis of the study was that there would be no difference among the tested materials with regard to their cytotoxicity, residual monomer release, DC, release of Ca^{2+}, and crystal structure.

Material and methods

Preparation of samples

The manufacturer, production location, composition, and lot numbers of the materials used in the study are shown in Table 1. The tested materials were prepared by placing them in sterilized cylindrical Teflon molds with a diameter of 4 mm and a height of 2 mm in accordance with the manufacturer's instructions. ThLC and ThPT samples were polymerized at a distance of 2 mm using an LED light source (Freelight Elipar II, 3 M, MN, USA) with a light output of 1200 mW/cm^2 for 20 s. MTA was prepared by mixing 1:1 MTA powder and distilled water with a metal spatula according to the manufacturer's instructions. The prepared samples were stored in an incubator (CB 160; Binder GmbH, Tuttlingen, Germany) at 37 °C and 95% relative humidity for 24 h to ensure a complete setting.

Isolation, culture, and characterization of human dental pulp stem cells

The present study and the human dental pulp isolation protocol were found medically appropriate with the ethics committee report numbered 2021/117.65 of Cukurova University Faculty of Medicine Clinical Research Ethics Committee. The healthy adult donors with permanently impacted third mandibular molars and planned orthodontic extraction were included in the study. Of the 14 contacted patients, 10 (6 women and 4 men) agreed to participate in the study, and written informed consent was obtained from each adult patient in accordance with the Declaration of Helsinki ($n = 10$). The donors aged between 18 and 30 years, with no systemic disease, no pregnancy, and no routine drug use, were determined as eligibility criteria for this study. The impacted teeth were extracted from the donors in the Clinic of Oral, Dental, and Maxillofacial Surgery of Dentistry Faculty of Cukurova University under sterile operating room conditions.

The obtained teeth were immersed in a physiological solution containing 5% penicillin/streptomycin/amphotericin (Gibco, Grand Island, NY, USA) to avoid contamination. Shortly thereafter, under sterile conditions, the dental crown was divided into several pieces to reveal dental pulp tissue and was washed in Hanks Balanced Salt Solution (HBSS; Gibco). Collagenase type I (Sigma-Aldrich, St. Louis MO, USA) was used to generate single-cell suspensions from pulp tissue. The cells were cultured in the modified Eagle's medium-alpha (MEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) and 100 IU/mL penicillin–100 µg/mL streptomycin at 37 °C in 5% CO2 conditions, and the medium was freshened every 3 days [29]. Stem cell characterization was performed within the scope of the International Society of Cellular Therapy guidelines to confirm their mesenchymal phenotype [30, 31]. The morphological characteristics of the cultured cells by sticking to the surface of the cell dish were followed by contrasting phase microscopy throughout the study. Flow cytometric analysis and immunofluorescence labeling were performed for mesenchymal stem cell markers to determine the immunophenotypic characteristics. Surface antigens of hDPSCs were analyzed in a flow cytometer (Becton Dickinson FACSCalibur; Becton Dickinson, San Jose, CA, USA) using fluorescent dye–conjugated CD45 FITC (BD 55,482), HLA-DR FITC (BD 340,688), CD 44 FITC (BD 555,478), CD34 PE (BD 345,802), CD29 PE (BD 555,443), CD73 PE (BD 550,257), CD140b PE (BD 558,821), CD146 PE (BD 550,315), CD 105 PE (BD 560,839), and Ig G1G2A FITC-PE (BD 342,409) antibodies. Mouse IgG1 FITC and IgG2a PE were used as isotype controls.

Table 1 Manufacturers and compositions of the materials

Material	Manufacturer	Composition
MTA	Angelus, Londrina, PR, Brazil Lot number: 102732	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide, and calcium tungstate
TheraCal LC	Bisco Inc. Shaumburg, IL, USA Lot number: 2100004781	Calcium oxide, calcium silicate particles (type III Portland cement), strontium glass, fumed silica, and barium sulfate, barium zirconate, and resin containing bisphenol A-glycidyl methacrylate (Bis-GMA)
TheraCal PT	Bisco Inc. Shaumburg, IL, USA Lot number: 2100003403	SG-mix cement, Bis-GMA, barium zirconate, ytterbium fluoride, and initiator

In addition, adipogenic, osteogenic, and chondrogenic differentiation protocols were applied to determine the in vitro differentiation capacity of mesenchymal stem cells. In adipogenic differentiation, after inoculation of 3000 cells/cm^2 cells in the culture dish, the culture medium was replaced with a differentiation medium (DMEM culture medium with 10% FBS, 0.5 mM isobutyl-methylxanthine, 1 µM dexamethasone, 10 µg/mL insulin, and 200 µM indomethacin) when it reached 70% cell compaction. In osteogenic differentiation, after inoculating 3000 cells/cm^2 cells in the culture dish, the culture medium was replaced with a differentiation medium (DMEM culture medium containing 10% FBS, 100 nM dexamethasone, 0.05 µM ascorbic acid, and 10 mM β-glycerophosphate) when the cells reached 70% cell density. In chondrogenic differentiation, cell differentiation was performed using the micro-mass technique. Cell suspension with a density of 107 cells in 1 mL was dropped into the plastic culture dish, and the cells were adhered to in a dense environment. After waiting for 24 h at 37 °C for adhesion, the cells were cultured for 2 weeks with high glucose DMEM culture medium containing 10 ng/mL TGF-β1, 50 µg/mL ascorbic acid, 0.1 µM dexamethasone, 100 µg/mL sodium pyruvate, 40 µg/mL proline, and 50 mg/mL insulin.

Cytotoxicity assay

Cell viability with water-soluble tetrazolium salt (WST-1) was used to determine the cytotoxic effects of pulp capping materials on hDPSCs. The analysis performed is based on the principle of conversion of tetrazolium salt to formazan, which gives color by cellular mitochondrial dehydrogenase. The amount of dye produced by dehydrogenase enzyme activity is directly proportional to the number of viable cells. Twenty-four-well culture dishes were used and 25,000 cells were seeded in all wells of the experimental setup. After the cells adhered to the surface, material samples were added to the wells, and the group without any material was designed as the control group. For all experimental groups, cells were incubated in Dulbecco's modified Eagle's medium (L-DMEM; Gibco) culture medium containing 1% penicillin/streptomycin, 10% FBS at 5% CO2, and 37 °C. Cell viability was established in separate culture setups at 3 different times on the 1st, 3rd, and 7th days. At the end of the measurement times, the medium in the wells was replaced with L-DMEM containing 10% WST-1 reagent, and the cells were incubated for 2 h in the dark at 5% CO2 and 37 °C. L-DMEM containing only 10% WST-1 reagent, without both cells and any sample, was used as blank. At the end of the incubation, viable cell count measurements were completed with a spectrophotometer (Versamax microplate reader; Molecular Devices, CA, USA) at a wavelength of 450 nm. In all cell culture wells, the standards were performed as 3 replicates, and the controls and experimental groups were performed as 5 replicates.

Residual monomer release

The resin-based materials, ThLC and ThPT, were prepared as stated above ($n = 10$). The polymerized samples were placed in amber bottles containing 5 mL of 75% ethanol/water solution and kept them in a 37 °C incubator. The samples taken from these solutions at 1, 3, and 7 days were analyzed by high-performance liquid chromatography-ultraviolet (HPLC–UV; Shimadzu Nexera X2, Kyoto, Japan). A water/acetonitrile solution ratio of 30/70 was used as a mobile phase in the analysis of the monomers (BisGMA, UDMA, and TEGDMA). The flow rate of the mobile phase is 1.0 mL/min, the column temperature was 25 °C, and the absorbance readings were taken at 210 nm. Inertsil ODS-4 C18 (250 mm × 4.6, 5 µm) was used as the column. The volume of the liquid injected into the column from each sample was determined as 20 µL. The residual monomer concentrations were determined by defining the calibration points (1–5-10–50 µg/mL) for each monomer and creating the calibration curves.

Degree of Conversion (DC)

The Fourier transform infrared (FTIR) spectrometer (JASCO, FT/IR-6800, Japan) was used to measure the immediate DC of ThLC and ThPT samples ($n = 10$). The ATR crystal was placed in close contact with the bottom surface, and FTIR spectra ranging from 400 to 4000 cm^{-1} were documented by 16 scans at a resolution of 4 cm^{-1}. The peak height of around 1638 cm^{-1}, indicating the absorbance intensities of aliphatic $C=C$, was calibrated according to Rueggeberg et al. [32]. The peak of aromatic $C=C$ around 1608 cm^{-1} was taken as the internal standard and was also calibrated. The values of DC were calculated according to the formula shown below:

$$\text{Degree of conversion (\%)} = \left(1 - \frac{\left(\frac{A_{\text{aliphatic}}}{A_{\text{aromatic}}}\right)_{\text{cured}}}{\left(\frac{A_{\text{aliphatic}}}{A_{\text{aromatic}}}\right)_{\text{uncured}}}\right) \times 100$$

Calcium ion (Ca^{2+}) release

The samples were prepared from each pulp capping material and were placed in 10 mL deionized water at 37 °C in clean plastic 25-cm^3 centrifuge tubes ($n = 5$). Three hours, 24 h, 3 days, 7 days, and 14 days were collected from the tubes, and after each measurement time, the samples were transferred to new tubes containing fresh deionized water.

The storage water samples were then analyzed using an inductively coupled plasma-optical emission spectroscopy (ICP-OES; Perkin Elmer Optima 7000DV, Waltham, MA, USA) at an inductively coupled wavelength of 317.933 nm and plotting a calibration curve between 0.1 and 15 ppm. The rate of calcium Ca^{2+} released from each material was analyzed at five measurement times determined for 14 days.

Scanning electronic microscopy and energy-dispersive spectroscopy (SEM–EDS)

The disk-shaped samples with the same dimensions were prepared from each material ($n=3$). After the samples were prepared, they were subjected to a gold plating process (Quorum, Q150R ES, UK) to obtain high-quality images.

Then, the elemental quantitative analysis of the investigated material was performed from 3 randomly selected spots of each sample of each material using SEM–EDS (FEI, Quanta 650 FEG, Eindhoven, The Netherlands).

Three areas of 20 × 20 µm of each sample were scanned at × 500 magnification. A spectrum was obtained showing the elements in the basic composition of the material as a peak. In addition, the SEM images of each material were taken at × 5000 magnification.

X-ray diffractometry (XRD)

The phase analysis of the materials was performed using XRD. The samples were dried in a vacuum desiccator and crushed to a very fine powder using an agate mortar and pestle. The diffraction meter (Empyrean XRD, PANalytical, UK) used Cu Kα radiation ($\lambda=0.15406$ nm) at 40 mA and 45 kV, and the detector was set to rotate between 10 and 90° with a sampling width of 0.05 and a scan rate of 1°/min at 15 cycles. Phase identification was performed using a search matching software and using the ICDD database (International Center for Diffraction Data, Newtown Square, PA, USA).

Statistical analysis

Statistical analysis was performed by using the SPSS program for Mac version 26 (IBM SPSS Inc., Chicago, IL, USA). The homogeneity of variance and normal distribution of the data was confirmed by the Shapiro–Wilk test. The statistical differences were analyzed by using one-way ANOVA followed by Tukey's post hoc test for pair-wise comparison. Time-dependent changes were evaluated by repeated measure ANOVA. All data were evaluated with a 95% of confidence interval ($p=0.05$).

Results

Characterization of human dental pulp stem cells

The particles such as dead cells and debris were eliminated using the R1 gate in flow cytometry. All markers of all cells scattering around the R1 gate were shown in the histograms (Fig. 1a).

The flow cytometry analysis of the hDPSCs isolated was shown to be positive for the markers of CD44 FITC (96%),

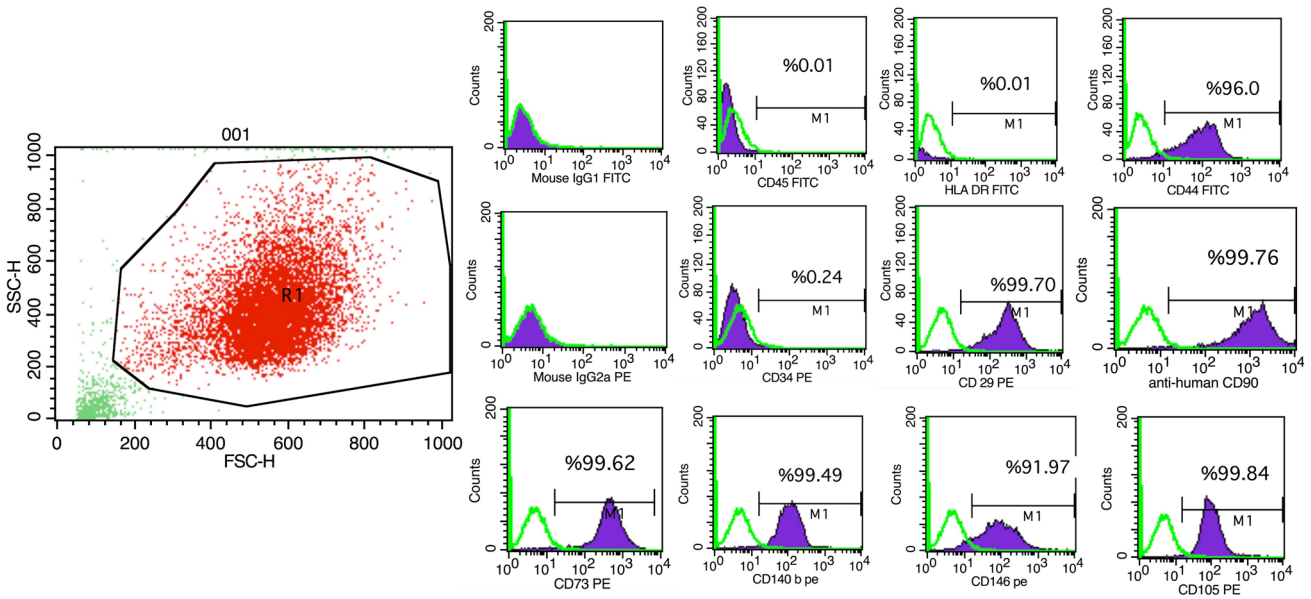


Fig. 1 Characterization of hDPSCs by flow cytometric analysis

CD29 PE (99.7%), anti-human CD90 (99.76%), CD73 PE (99.62%), CD140 PE (99.49%), CD146 PE (91.97%), and CD105 PE (99.84%) and negative for the markers CD45 FITC (0.01%), HLA DR FITC (0.01%), and CD34 PE (0.24%) (Fig. 1b). Mouse IgG1 FITC and IgG2a PE were used as isotype controls. The percentages are represented relative to the proper expression of mesenchymal stem cell surface markers.

The multilineage differentiation ability of stem cells in terms of adipogenic, osteogenic, and chondrogenic was observed in the culture medium. As a result of adipogenic differentiation, lipid vesicles were observed in the cytoplasm of the cells after 20 days; as a result of osteogenic differentiation, mineral deposits in the cytoplasm of cells after 4 weeks; and as a result of chondrogenic differentiation, phosphate proteoglycans in the cartilage matrix after 2 weeks.

Cytotoxicity

The absorbance in all cell culture wells prepared for standards, controls, and experimental groups was measured at 450 nm after incubation. Then, from the data obtained with the standards, an equation was found which gives the number of viable cells and was shown as followed: $y = 21171x + 794.27$, $R^2 = 0.9953$. The number of viable cells in the experimental groups and in the control was determined according to this equation, and the normalization of the experimental groups was compared with the number of cells in the control group and is shown in Fig. 2.

The number of viable cells in MTA (4177.4), ThLC (1031.4), and ThPT (2183.1) groups decreased significantly compared to the control group (8902.8) for day 1 ($p < 0.0001$), and MTA was significantly higher than in ThLC and ThPT ($p < 0.002$). There was no difference between ThLC and ThPT ($p = 0.078$).

The number of viable cells was further reduced in the MTA (3376), ThLC (726.4), and ThPT (670.4) groups compared to the control group (8304.2) for day 3 ($p < 0.0001$). There was no difference between the ThLC and ThPT materials in terms of the number of viable cells ($p = 0.999$), while these two groups contained significantly fewer viable pulp cells than the MTA ($p < 0.0001$).

When the differences in the measurement times were compared, there was no difference in the number of viable cells among the day 1 (4177.4), the day 3 (3376), and the day 7 (3357.3) measurements of the MTA group ($p = 0.221$). The number of viable cells of ThLC and ThPT from day 1 to day 3 was significantly reduced, and by day 7, there were no viable cells in either material group ($p < 0.0001$). The control group had similar viable pulpal cells in terms of measurement times ($p = 0.811$).

Residual monomer release

The time-dependent monomer releases of the materials are presented in Fig. 3. BisGMA release was greater in ThLC than ThPT at all measurement times ($p < 0.0001$). TEGDMA release was significantly lower for ThLC on day 3 and day 7 compared to ThPT ($p < 0.0001$). BisGMA release from ThLC was higher than TEGDMA for all measurement times ($p < 0.0001$). While ThPT showed similar releases of the monomers on day 1, TEGDMA released from ThPT on days 3 and 7 was considerably higher than BisGMA ($p < 0.0001$).

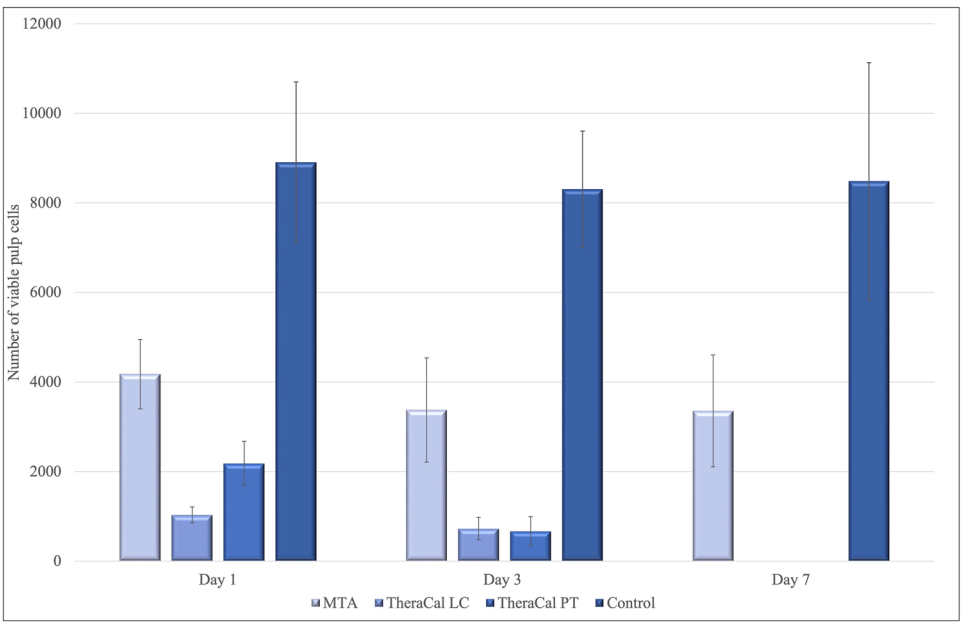
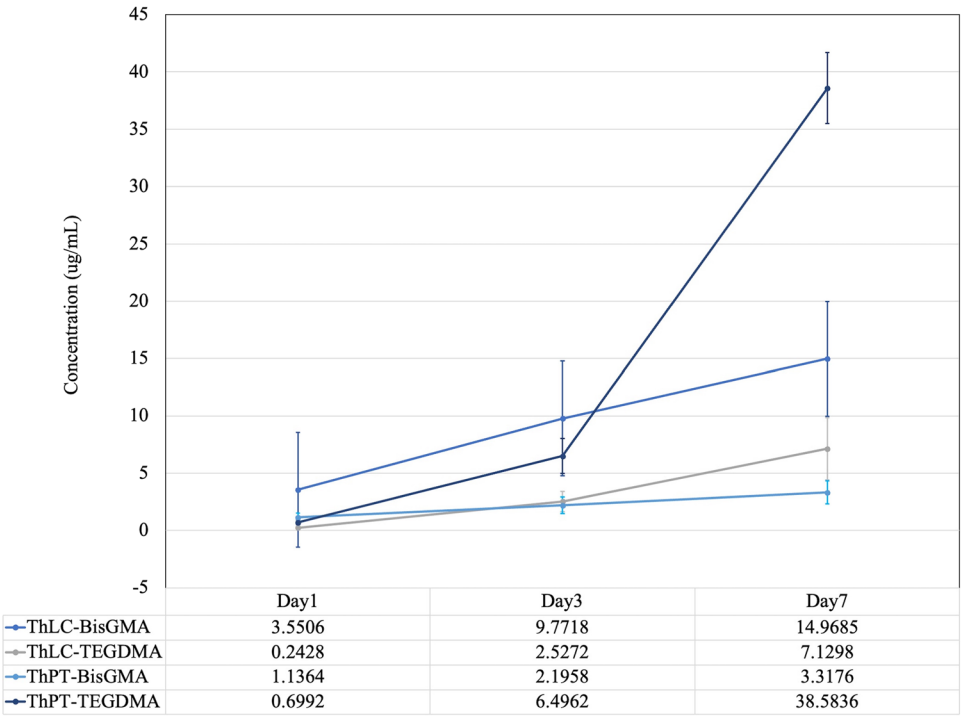


Fig. 2 In vitro cytotoxicity of hDPSCs after exposure to extracted medium prepared from MTA Angelus, TheraCal LC, and TheraCal PT on days 1, 3, and 7



	Day1	Day3	Day7
ThLC-BisGMA	3.5506	9.7718	14.9685
ThLC-TEGDMA	0.2428	2.5272	7.1298
ThPT-BisGMA	1.1364	2.1958	3.3176
ThPT-TEGDMA	0.6992	6.4962	38.5836

Fig. 3 The release of average monomer concentration (ug/mL) from the TheraCal LC and TheraCal PT, respectively, was observed on days 1, 3, and 7

UDMA monomer was not found in the monomer release concentration curve of the tested materials.

Degree of conversion

The representative graphic of the DC of ThLC and ThPT is presented in Fig. 4. As a result of statistical analysis of homogeneously distributed the data of DC by independent *t*-test, the DC of ThLC and ThPT was found to be significantly higher than ThLC ($p < 0.0001$).

Ca^{+2} release

The time-dependent change of Ca^{2+} released from the materials and the differences between them is presented in Fig. 5 with a line chart and table. At all measurement times except day 14, the Ca^{2+} release of MTA was significantly higher than that of ThLC and ThPT ($p \leq 0.02$). There is no difference in Ca^{2+} release between ThLC and ThPT at hour 3, day 1, and day 7 ($p \geq 0.340$), but the Ca^{2+} released from ThLC on days 3 and 14 was higher than ThPT ($p \leq 0.03$).

There is a statistically significant difference among the five measurement times of the Ca^{2+} release for all materials ($p < 0.0001$). MTA showed the highest Ca^{2+} release on day 1. MTA exhibited similar ion release at hour 3 and day 3, and the release significantly decreased for days 7 and 14 compared to other measurement times ($p \leq 0.02$). ThLC showed the highest Ca^{2+} release on days 3, 7, and 14, and it was observed similar values while ThPT showed the highest Ca^{2+} release on days 7 and 14.

SEM/EDS

The representative SEM images of the investigated materials at × 5000 magnification are presented in Fig. 6. MTA exhibited a surface containing interspersed particles in similar

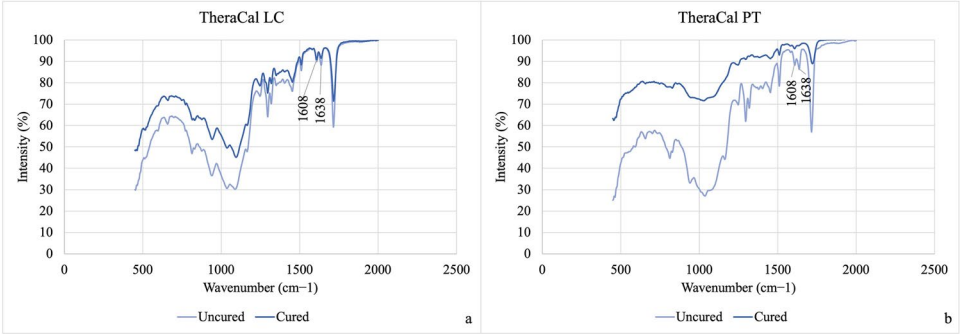
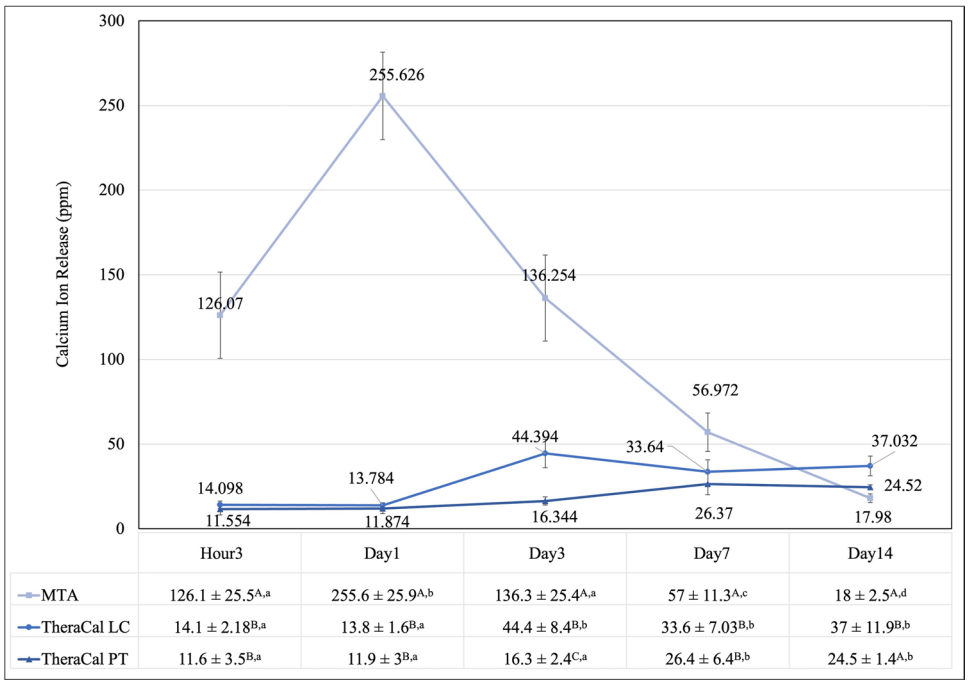


Fig. 4 The representative graphs of "**a**" and "**b**" demonstrate the degree of conversion (%) of the TheraCal LC and TheraCal PT, respectively. Contrary to graph "**a**," a significant decrease in the ratio of the absorbance intensities of the aliphatic $C=C$ peak at 1638 cm^{-1} is observed in graph **b**



	Hour3	Day1	Day3	Day7	Day14
MTA	$126.1 \pm 25.5^{A,a}$	$255.6 \pm 25.9^{A,b}$	$136.3 \pm 25.4^{A,a}$	$57 \pm 11.3^{A,c}$	$18 \pm 2.5^{A,d}$
TheraCal LC	$14.1 \pm 2.18^{B,a}$	$13.8 \pm 1.6^{B,a}$	$44.4 \pm 8.4^{B,b}$	$33.6 \pm 7.03^{B,b}$	$37 \pm 11.9^{B,b}$
TheraCal PT	$11.6 \pm 3.5^{B,a}$	$11.9 \pm 3^{B,a}$	$16.3 \pm 2.4^{C,a}$	$26.4 \pm 6.4^{B,b}$	$24.5 \pm 1.4^{A,b}$

Fig. 5 Time-dependent $Ca.^{2+}$ release values (ppm) from the tested materials. The table below the line chart represents the significant differences among the materials (column, uppercase letters) and among the time measurements (row, lowercase letters) ($\alpha = 0.05$)

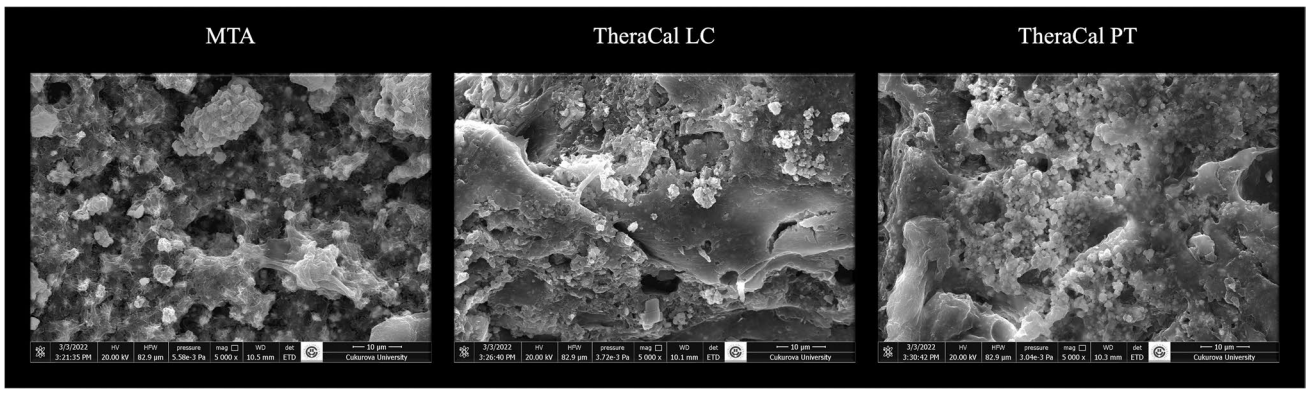


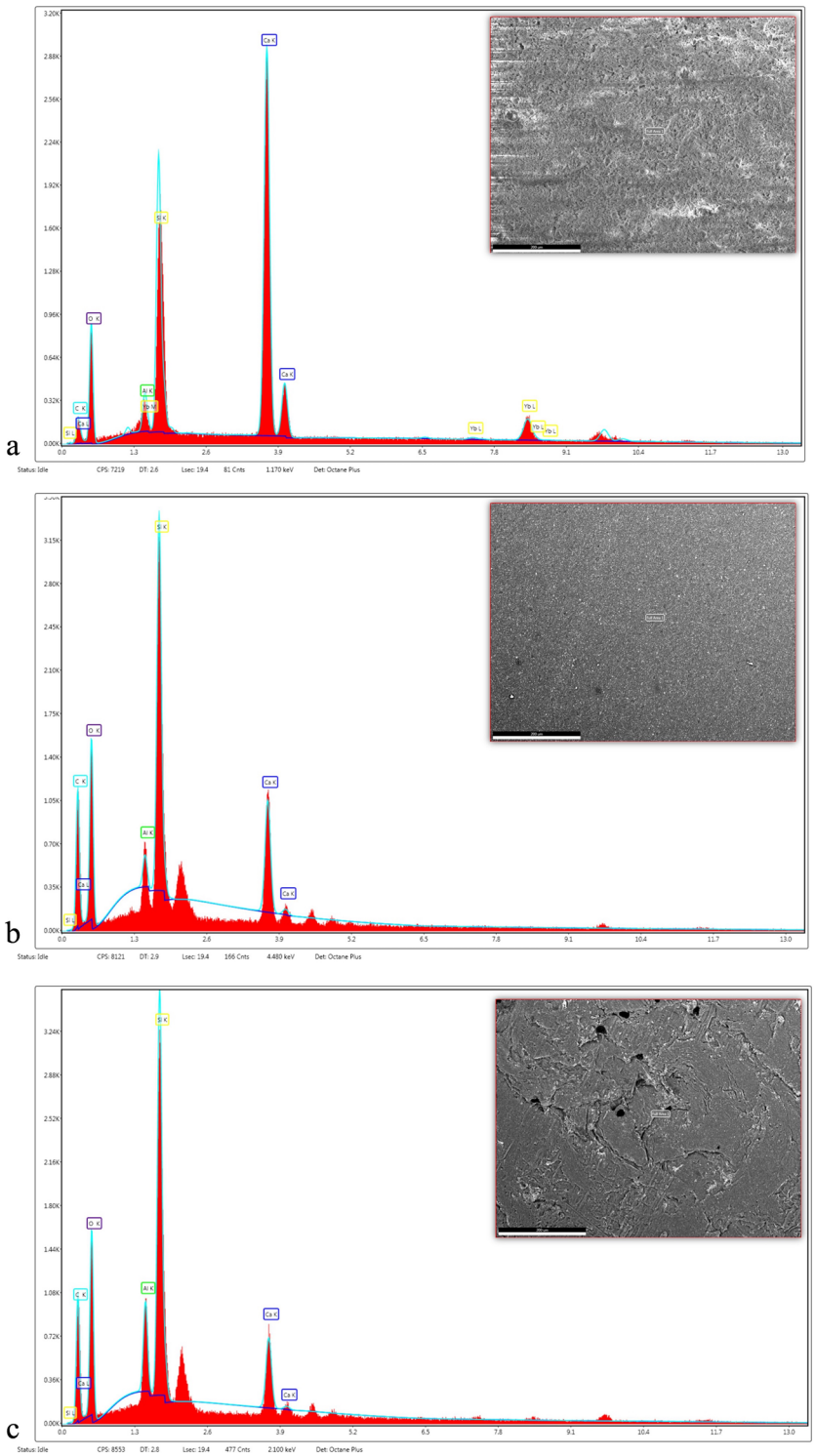
Fig. 6 Representative SEM images at × 5000 magnification of the materials

proportions and a dense microstructure with a porosity under 10 µm. MTA surface also showed mixtures of relatively large and small particles composed of aggregate deposition. ThLC and ThPT that showed a relatively more homogeneous and less rough surface than MTA exhibited mineral particles dispersed in the organic resin matrix, similar to the resin composite. Compared to ThLC, globular-shaped structures including elements with relatively higher atomic numbers were intensely observed on the resin matrix surface of ThPT.

A representative EDS spectrum was obtained from a randomly scanned region for each material examined, and the average weight and atomic percentage of the main elements in the composition of the material obtained from 3 different regions of 3 samples for each investigated material are presented in Fig. 7. Carbon (C), oxygen (O), aluminum (Al), silicon (Si), and Ca were found in all materials. Also, MTA contained ytterbium (Yb). There are significant differences among the materials in terms of percentage weights of C, Al, and Ca ($p < 0.0001$). The ratio of C (5.07%) in MTA is lower than that in ThLC and ThPT materials ($p = 0.001$). The ratio of Ca in MTA (37.59%) is significantly higher than in other materials ($p < 0.0001$). The ratio of Ca in ThLC (9.55%) was significantly higher than that in ThPT (5.94%) ($p = 0.034$). ThPT has a higher ratio of Al than the other materials ($p < 0.0001$).

XRD

The chemical composition and the crystal structure of the tested materials are represented in Fig. 8. The components of MTA, tricalcium silicate (ICDD:98–009-4742), calcium silicate (ICDD:98–024-0448), calcium oxide



a

Element	Weight %	Atomic %
C K	5.07	9.46
O K	40.58	56.9
Al K	1.75	1.45
Si K	13.74	10.97
Ca K	37.59	21.04
Yb L	1.28	0.17

b

Element	Weight %	Atomic %
C K	32.99	44.81
O K	40.78	41.58
Al K	1.38	0.83
Si K	15.3	8.89
Ca K	9.55	3.89

c

Element	Weight %	Atomic %
C K	33.98	45.74
O K	39.51	39.93
Al K	3.79	2.27
Si K	16.77	9.65
Ca K	5.94	2.4

Fig. 7 Representative EDS spectrum and representative SEM images of the focused region of MTA Angelus (**a**), TheraCal LC (**b**), and TheraCal PT (**c**) (scale bar: 200 µm). The mean weight and atomic weight for each material were presented in the column next to the EDS spectrum, respectively ($n = 3$)

(ICDD:98–002-6959), and silicon oxide (ICDD:98–019-3155), showed the highest peaks at 29.478, 36.648, 37.621, and 29.029 2Θ, respectively. The chemical compositions of ThLC and ThPT were similar. Barium peaks with zirconium and the particles of Si, strontium aluminum, and Ca were observed in the matrix of these materials. ThLC showed the

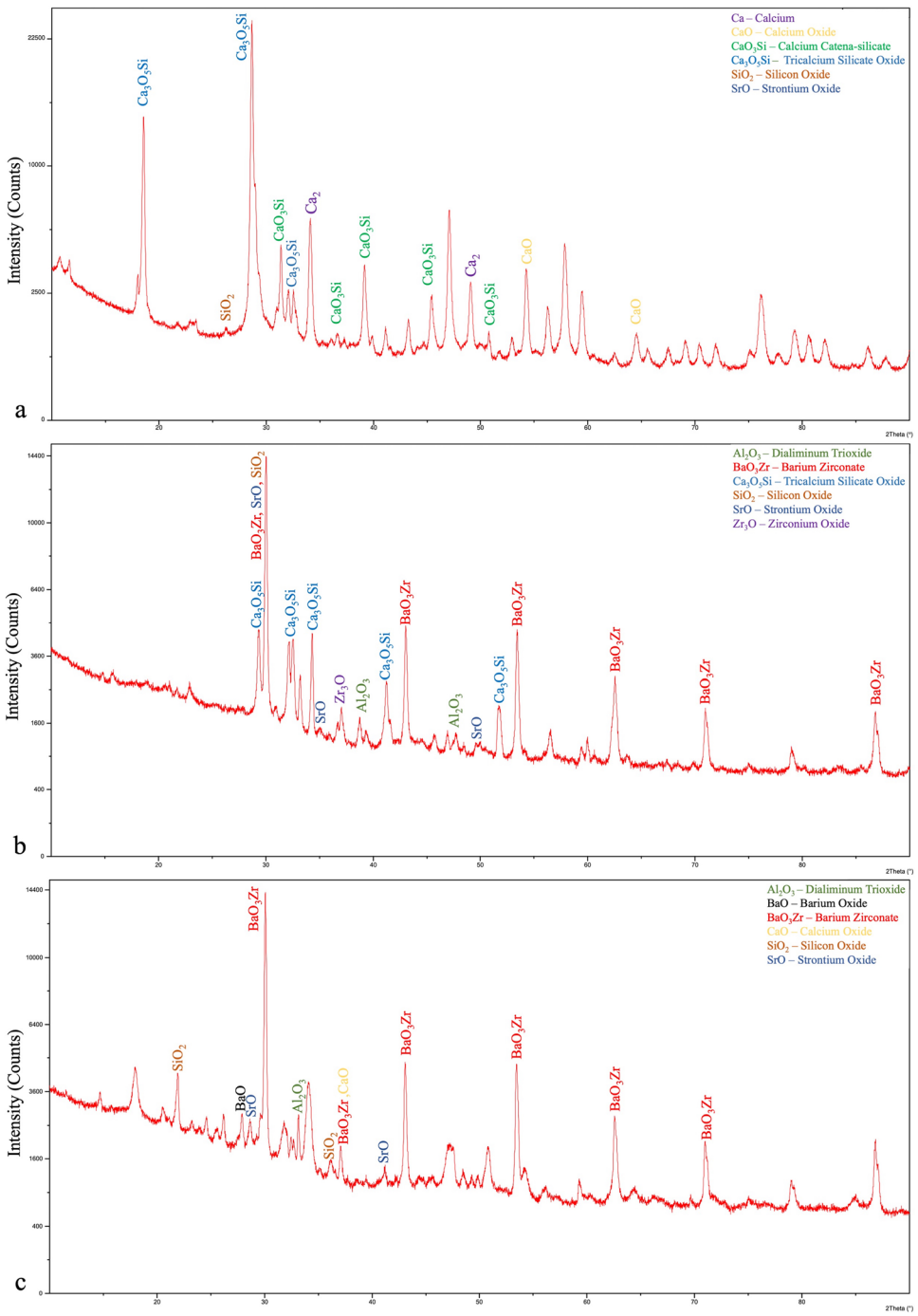


Fig. 8 X-ray diffraction patterns of MTA (**a**), TheraCal LC (**b**), and TheraCal PT (**c**) show peaks representing the crystal phases

highest peak of tricalcium silicate (ICDD: 98–008-1100) at 32.193 2Θ, while ThPT indicated only calcium oxide (98–018-0198) at 37.153 2Θ. ThLC displayed the highest peaks at 30.073, 38.593, and 30.117 2Θ for barium zirconate (98–009-7462), aluminum oxide (98–015-1589), and silicon oxide (ICDD:98–015-8531), respectively. ThPT showed the peaks of barium zirconate (ICDD: 98–009-7461), aluminum oxide (ICDD: 98–017-3713), and silicon oxide (ICDD: 98–007-7457) at 30.095, 33.215, and 21.909 2Θ, respectively.

Discussion

The present study compared the current vital pulp capping materials in terms of the cytotoxicity on hDPSCs, the release of residual monomer, the DC, the release of Ca^{+2}, and the crystal structure. Biocompatibility and bioactivity are considered essential features due to the direct contact of the material with vital tissues in choosing a material for the vital pulp procedure [33]. This study compared ThPT, a recent-developed material for vital pulp therapy,

with currently used ThLC and MTA materials in terms of cytotoxicity on days 1, 3, and 7. At the end of 7 days, it was found that there were no viable cells in ThPT and ThLC, while cell viability continued in MTA. In addition, the materials differed from each other in terms of chemical properties and ion release. Therefore, the null hypothesis of the study was rejected.

A number of quantitative and qualitative in vitro methods are available to evaluate the cytotoxicity of biomaterials and their potential adverse effects on cell behavior [34]. Among the various cytotoxicity tests, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is widely used as an indicator of cell viability. MTT is reduced by a mitochondrial enzyme in metabolically active cells to form insoluble purple formazan crystals, and then, the crystals are dissolved by the addition of a detergent. However, insoluble purple formazan crystals may be cytotoxic, and inevitably added detergents may also affect cell viability which may complicate the analysis [35]. The WST-1 assay works similarly to the MTT method by reacting with mitochondrial succinate-tetrazolium reductase and forming the formazan dye, but the WST-1 reagent produces a water-soluble formazan that is stable in solution for more than 24 h and non-cytotoxic. In addition, the WST-1 assay is three times more sensitive than the MTT test [36]; hence, the WST-1 assay was preferred to detect cytotoxicity in this study.

There are two studies evaluating the cytotoxicity of the new ThPT in the literature. They reported an improved in vitro cytocompatibility of hDPSCs compared to ThLC, the precursor of ThPT [20, 21]. In those studies, cell viability was evaluated at 24, 48, and 72 h using MTT as a cytotoxicity test method. Those results, which are inconsistent with our results, may be due to our use of a more sensitive method, WST-1, and our evaluation on days 1, 3, and 7. The cell viability of ThPT and ThLC observed on day 3 ceased when followed up to day 7. Moreover, the materials were not evaluated in different concentration media which considers as being one of the limitations of the present study. In studies, it was revealed that ThLC negatively affected hDPSC viability in all extraction medium concentrations, whereas this effect was lower in ThPT and the concentration ratios had no effect [20, 21]. In previous studies comparing the cytotoxicity of ThLC, MTA, and a similar material (Biodentine), they reported that ThLC had a cytotoxic effect on hDPSC viability [9, 22, 37]. It was not surprising that MTA showed high cell viability for 7 days which has often been proven in previous studies [20, 21, 37]. However, in this study, the loss of cell viability by half in MTA when compared to the control group is likely due to the high alkaline pH observed in the initial setting. Bortoluzzi et al. [38] specified that all freshly set calcium silicate cements (resin-modified and resin-free) are initially cytotoxic, which may be due to their high alkalinity.

One of the methods used to determine the cytotoxicity of resin-structured materials is the measurement of the concentration of unreacted monomers released from the polymerized resin material [39]. The main reason for the cell cytotoxicity found in the TheraCal materials is that the main component (45%) contains resin monomers which can be toxic to dental pulp cells [40]. The safety data sheet shows that ThLC contains only BisGMA monomer, but UDMA and TEGDMA content was observed in studies and patients [41–43]. BisGMA, UDMA, and TEGDMA were tested by methods of cell culture, and it was reported that they might cause cytotoxic, genotoxic, mutagenic, and allergenic effects [44, 45]. Unreacted monomers and other degradation products can be analyzed using HPLC, gas chromatography, electrospray ionization, and mass spectrometry [46, 47]. HPLC is the most common analytical method for determining the quality and quantity of eluted monomers [46–48]. Studies on the release of monomers from resin materials are carried out using various extraction media. Solvent media are divided into two groups as aqueous mixtures (distilled water, cell culture media, artificial saliva, human saliva, and water-based buffer solutions) and different organic extraction media (ethanol, methanol, acetone, acetonitrile, tetrahydrofuran, and chloroform) [48–50]. These solvent environments are important as they penetrate the resin matrix and cause the release of unreacted monomers [48]. Ferracane reported that fluids in the oral cavity exhibited extraction properties intermediate between aggressive organic solvent and water [51]. Therefore, the US Food and Drug Administration recommends using a 75% ethanol/water solution as a liquid with properties corresponding to oral cavity conditions [39]. In this study, residual monomer releases of ThLC and ThPT were investigated by HPLC using 75% ethanol/water solution, and BisGMA and TEGDMA releases were observed while UDMA peaks were not observed. ThLC showed predominantly BisGMA release and tended to decrease after day 3, but TEGDMA releases for both materials revealed an increasing curve from day 1 to day 7. The decrease and loss of cell viability in the TheraCal materials may be due to the dramatic increase of TEGDMA release on day 7. Previous studies have suggested that TEGDMA may be more toxic than other monomers [35, 36]. This argument is in line with the results of this study.

Resin-based materials should be highly polymerized to minimize the amount of residual monomer elution [52]. The DC of monomers in resin materials is a measure of the percentage of carbon double bonds converted into single-carbon bonds to form a polymer chain, depending on the polymerization process. In a material, relatively 70% of the average monomers are converted to polymers, and a relative 30% remain unpolymerized. While most of these unconverted monomers remain in the polymer matrix, 9% of them remain free and may diffuse into the dentinal tubules and reach the

pulp [9]. Various methods are used to determine the DC of resin materials. FTIR spectroscopy is a powerful and reliable method based on molecular vibrations that able to be used to directly detect the amount of unreacted $C=C$ bond in the resin matrix, as opposed to indirect techniques that rely on measuring changes in the mechanical performance of the material to assess relative DC [53, 54]. In this study, DC values of ThLC and ThPT were compared using the FTIR spectroscopy. It was determined that ThPT showed higher DC. ThPT was introduced to the market as a dual-cure with the improved formulation of ThLC. The purpose of dual-cure materials is able to provide complete curing in the absence of a visible light source by combining chemical polymerization and photo-polymerization. DC is generally considered to correlate with the release of unreacted monomers; however, a specific DC limit that occurs proportionally to the DC of a material for a significant amount of monomer release is unknown [55]. However, this result contradicts the data of the highest TEGDMA monomer release observed in ThPT. Similar to our study, there are studies reporting no correlation between the DC and monomer release [56–58]. This was attributed to the imperfect match of the data of HPLC analysis with the data of FTIR analysis, the different sensitivity of these two analytical techniques, and the possibility that unpolymerized monomers may not be released into the surrounding environment by remaining trapped in the polymeric network. Therefore, the highest DC values may not always correlate with the release of the lowest monomer amounts [56].

The ability to release biologically active ions is a prerequisite for a material to be bioactive and induce apatite formation. Ca^{2+} is considered a bioactive component in pulp capping materials [59]. Ca^{2+} is essential for the differentiation and mineralization of pulp cells, and a Ca-rich environment stimulates both proliferation and differentiation into odontoblast-like cells [60]. The released Ca^{2+} increases the growth of hDPSCs in a dose-dependent manner and induces the activity of pyrophosphatase, which helps maintain dentin mineralization and develop a dentin bridge [38, 61]. In light of this knowledge, the sustained release of Ca^{2+} from a pulp capping material is probably the main cause of material-induced proliferation and differentiation of hDPSCs.

ICP-OES, XRD, and SEM–EDS results of all pulp capping materials tested in this study proved to be formulations showing calcium content and ion release in all materials as previously reported [62, 63]. In the EDS analysis, MTA was found to have higher Ca content than ThLC and ThPT. The ICP-OES analysis showed that the highest release of MTA was observed on day 1, and Ca^{2+} release tended to decrease until day 14. This steep Ca peak observed on day 1 confirms the decrease in cell viability with high alkalinization. The EDS analysis revealed a higher ratio of Ca of ThLC than ThPT, which supports ThLC with more Ca^{2+} release than ThPT. Also, these results are supported by previous studies showing that MTA releases higher Ca^{2+} than TheraCal materials [62, 63].

MTA particularly showed Ca and calcium silicate peaks in XRD analysis. Calcium silicate peak was not observed in ThPT compared to MTA and ThLC. This result is similar to the study reporting that the calcium silicate phase is weak in ThPT [62]. Also, ThLC and ThPT both showed peaks for barium strontium zirconium oxide, similar to a previous study [63]. This study has limitations of the in vitro test setup. Further studies are recommended, including in vivo animal tests and clinical studies, as the behavior of tested materials can be potentially affected by a number of external factors in the clinical setting.

Conclusion

MTA emerged as a superior material with low cytotoxicity compared to ThLC and ThPT. After 7 days, no viable hDPSCs were detected in the ThLC and ThPT groups. All materials had Ca content in the certain ratios and exhibited time-dependent Ca^{2+} release. Although the ThPT had a better DC with its dual-cure feature, the residual monomer release of ThPT was higher than ThLC.

Acknowledgements The authors thank Ahmet Öztürk and Kocaeli University, Stem Cell and Gene Therapies Research, and Application Center (KÖGEM) for the support of this study.

Author contribution Conceptualization: C.K, S.N.K., and S.G.B.; methodology: C.K, S.N.K., and S.G.B.; formal analysis and investigation: S.N.K. and S.G.B.; writing—original draft preparation: C.K, S.N.K., and S.G.B.; writing—review and editing: S.N.K. and C.K.; funding acquisition: S.N.K.; resources: C.K, S.N.K., and S.G.B; supervision: S.N.K. and C.K.

Funding This research was supported by the Scientific Research Projects of Cukurova University (project number TSA-2022–14441).

Declarations

Competing interests The authors declare no competing interests.

Ethics approval All methods performed in the present study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later modifications or comparable ethical standards. The present study and the protocol were found medically appropriate with the ethics committee report numbered 2021/117.65 of Cukurova University Faculty of Medicine Clinical Research Ethics Committee.

Consent to participate The written informed consent was obtained from all individual participants included in the study.

Conflict of interest The authors declare no competing interests.

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