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Investigation of the effect of indirect pulp capping materials on dentin mineral density

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Aim: To evaluate the potential of inducing mineral density changes of indirect pulp capping materials applied to demineralized dentin. **Methods:** A total of 50 cavities were prepared, 5 in each tooth, in extracted ten molars without caries, impacted or semi-embedded. The cavities were scanned by microcomputed tomography (μ-CT) after creating artificial caries by microcosm method (pre-treatment). Each cavity was subjected to one of 5 different experimental conditions: control (dental wax), conventional glass ionomer cement (Fuji IX GP Extra), resin-modified calcium silicate (TheraCal LC), resin-modified calcium hydroxide (Ultra-Blend Plus), MTA (MM-MTA) and the samples were kept under intrapulpal pressure using simulated body fluid for 45 days. Then, the second μ-CT scan was performed (post-treatment), and the change in dentin mineral density was calculated. Afterward, elemental mapping was performed on the dentinal surfaces adjacent to the pulp capping agents of 5 randomly selected samples using energy dispersive X-ray spectroscopy (EDS) apparatus attached to a scanning electron microscope (SEM). The Ca/P ratio by weight was calculated. Friedman test and Wilcoxon Signed Ranks test were used to analyze the data. **Results:** There was a significant increase in mineral density values of demineralized dentin after treatment for all material groups ($p<0.05$). Resin-modified calcium silicate had similar efficacy to MTA and conventional glass ionomer cement, but was superior to resin-modified calcium hydroxide in increasing the mineral density values of demineralized dentin. **Conclusions:** Demineralized dentin tissue that is still repairable can be effectively preserved using materials with remineralization capability.

Keywords: Calcium hydroxide. Calcium compounds. Silicates. Spectrometry, X-ray emission. Glass ionomer cement. X-ray microtomography.

Introduction

The main theme of contemporary dentistry is the development of minimally invasive biological-based treatments aimed at preserving pulp vitality. A vital pulp is of great importance in keeping the tooth in the mouth, as it provides nutrition and defense of the tooth and acts as a biosensor detecting pathogenic stimuli[1]. Treatment of caries lesions includes removal of infected dentin and coating of the affected dentin tissue with a bioactive material with remineralization capacity to preserve pulp vitality[2,3]. The repair concept of demineralized dentin aims to promote the renucleation of mineral crystals into the hydroxyapatite structure[4]. Bioactive materials used for this purpose consist of calcium silicate, calcium hydroxide or hydroxyapatite-based materials, and glass ionomer cement.

Calcium hydroxide is a material with a long history of clinical success, considered the "gold standard" among pulp capping agents[5]. In recent years, calcium silicate-containing materials such as mineral trioxide aggregates (MTA) have attracted considerable attention. However, calcium hydroxide and calcium silicate show poor physical properties, such as high solubility and gradual resorption. By adding resin monomers to these materials, physical properties such as light polymerization and low solubility in water have been improved, so the use of resin-modified versions in clinical routine has become widespread[6]. However, this situation raises the concern that the moisture diffusion required for the ion release, which stimulates dentin formation, may be prevented, especially in calcium silicate-based materials[3]. In investigating the bioactive potential of these materials, non-destructive laboratory techniques that examine the mineral changes in dentin come to the fore. Many recent studies have proven that microcomputed tomography (μ-CT) can be successfully used to evaluate the mineralization dynamics of enamel and dentin tissues[7,8]. However, as a common result of previous studies evaluating the remineralization capacity of dentin, it can be said that the changes in mineral density vary due to the use of different experimental designs, such as the pH cycle or biofilm model, in simulating the caries formation process[2,9]. On the other hand, while μ-CT analysis does not provide information about the mineral content, energy dispersive X-ray spectroscopy (EDS) can be used to determine the percentages and distributions of elements on the adjacent dentin surface to which the material is applied[3].

Therefore, this study aimed to evaluate the effect of pulp capping agents with different contents, such as resin-modified calcium hydroxide/calcium silicate, MTA, and conventional glass ionomer cement on dentin tissue demineralized with an artificial caries model using human bacterial inoculum by μ-CT and EDS analysis. The first null hypothesis of the study was that, regardless of the mechanism of action of bioactive materials, when applied to demineralized dentin tissue, there would be no difference in mineral density between pre- and post-treatment. The second null hypothesis was that there would be no difference between the bioactive materials in terms of mineral density increase.

Materials and Methods

Sample preparation

For mineral density analysis (μ-CT imaging), sample size calculation was performed based on a previous study by Pires et al.[9] (2018), with $\alpha = 0.01$, power $(1-\beta) = 0.95$, effect

size = 1.719, using G*Power software (version 3.1, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). The resulting total sample size required was n = 10.

In the present study, ten non-carious, fully or partially erupted third molars, extracted for clinical reasons, belonging to individuals over the age of 18 were used under the approval of Çanakkale Onsekiz Mart University Clinical Research Ethics Committee (2011-KAEK-27/2019-E.1900029346). Written informed consent was obtained from all individual participants included in the study. All procedures performed in research involving human biological material were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The teeth were kept in a 0.1% thymol solution at 4°C for a period of 3 months, until they were used in the experimental phase. A flat dentin surface was obtained by removing the occlusal enamel of the samples, the apical two-thirds of the root was removed to reveal the pulp canal space. A total of 50 cavities were obtained by preparing five cavities with a depth of at least half the bur diameter (± 1mm) on the occlusal dentin surface of each sample using a diamond round bur.

Biofilm formation with a microcosm model

Samples were fixed on polystyrene tissue-culture plates with wax all around to ensure that the formed biofilm only came into contact with the occlusal surface. Plates were sterilized under UV light for 40 minutes before microbial inoculum. The artificial caries model used in the study was developed by Pires et al.[9] (2018) and was carried out in the same way. For this, after obtaining written informed consent from 3 volunteers who met the inclusion criteria, 1 ml of saliva sample was collected, mixed with 1 ml of synthetically prepared sterile saliva in a sterile tube and homogenized by vortex. A total of 9×10^7 CFU mL^{-1} microorganisms were counted in this suspension. In this microorganism community, 2.3×10^4 CFU mL^{-1} of *Streptococcus mutans*, 3.7×10^5 CFU mL^{-1} of *Lactobacilus ssp.* and 2.9×10^2 CFU mL^{-1} of *Candida ssp.* were determined in selective culture medium (Mitis Salivarius Bacitracin, Candida Chromagar) by conventional analysis method. 20 µl of homogeneous culture medium was seeded into each cavity on the tooth surface and incubated in a brain-heart infusion (BHI) containing 10% glucose. It was incubated at 37°C for 7 days under microaerophilic conditions for biofilm formation to occur. BHI and glucose were replenished every 24 hours (1000 µL per cavity). All procedures were performed in a laminar air flow chamber in an aseptic environment. Biofilm-forming tooth samples were washed three times with phosphate-buffered saline solution after the medium was removed.

µ-CT examination

After the biofilm growth was obtained on the tooth surfaces, the culture medium was removed, and the samples were scanned with µ-CT after clearing the biofilm by sonication, and initial images were obtained. A high-resolution, desktop µ-CT system (Bruker Skyscan 1275, Kontich, Belgium) was used to scan the samples. The scanning parameters were: 100 kVp, 100 mA, 0.5 mm Al/Cu filter, 10.2 µm pixel size, rotation in 0.5 steps. Air calibration of the detector was performed before each scan to minimize the rate of ring artifact. Each sample was rotated 360° in an integration

time of 5 minutes. Average scan time was around 2 hours. Other settings included beam hardening correction and entry of optimum contrast limits according to manufacturer's instructions based on pre-scanning and reconstructing teeth. Each tooth was scanned twice before and after the application of the experimental procedure, ensuring standardization with the same scanning parameters.

Experimental procedure with bioactive cements

For indirect pulp capping, the most commonly used current materials in the clinical routine of today's dentistry were selected. In all tooth samples, capping materials were placed in the prepared cavities according to the manufacturer's instructions. The central cavity (no. 1) was filled with dental wax as the control group. The properties of the materials are shown in Table 1. Then, the samples were kept in simulated body fluid (SBF) with a pH of 7.4, where the intrapulpal pressure was simulated for 45 days at room temperature, in a 100% humidity environment.

Table 1. Pulp-capping materials used in the study

Cavity no.	Material type	Commercial name	Compositions	Manufacturer
C1	Dental wax (Control)	Cavex Set Up Regular	Paraffin waxes, micro-crystalline paraffin waxes, pigment	Cavex
C2	Glass ionomer cement (GIC)	Fuji IX GP Extra	Polyacrylic acid, fluoro-alumino-silicate glass, distilled water	GC
C3	Resin-modified calcium silicate (RMCS)	TheraCal LC	Portland cement (calcium silicates), fumed silica, Bis-GMA, polyglycol dimethacrylate	Bisco
C4	Resin-modified calcium hydroxide (RMCH)	Ultra-Blend Plus	Calcium hydroxide, tricalcium phosphate, diurethane, dimethacrylate, TEGDMA	Ultradent
C5	Mineral trioxide aggregate (MTA)	MM MTA	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, bismuth oxide, calcium sulphate dehydrate, magnesium oxide	MicroMega

Mineral density analysis

The modified algorithm described by Feldkamp et al.[10] (1989) was used to obtain axial, two-dimensional (2D), 1000×1000 pixel images for the visualization and quantitative measurements of the samples[10]. Ring artifact correction and smoothing for reconstruction parameters were fixed to zero and beam artifact correction was set to 40%. Contrast limits were applied following SkyScan's instructions. Using the NRecon software (version 1.6.10.4, Skyscan, Kontich, Belgium), the images acquired by the scanner were reconstructed to show 2D slices. Reconstructed images were further processed with Skyscan CTVox (version 3.3.0, SkyScan, Kontich, Belgium) for visualization. Skyscan CTAn software (version 1.17.7.2, SkyScan, Kontich, Belgium) provides an integrated calibration of datasets for these two density scales: Hounsfield unit (HU) and mineral density (MD). For this purpose, conical MD phantom (rods) with two different mineral concentrations of 0.25 and 0.75 g cm^{-3} were used for appropri-

ate calibration phantom scans and measurements. Thus, to aid calibrations, samples placed and scanned with MD phantom sticks were placed in an identical tube for the calibration scan, allowing the density value to be calibrated independently for each scan. After scanning, the grayscale values were converted to mineral density values with a linear calibration curve based on the grayscale values obtained from two different mineral concentration conical phantoms of 0.25 and 0.75 g cm^{-3}. Pixel size, rotation step, frame average, voltage, filter, etc. parameters were kept constant for all scans. A global thresholding was applied to separate dentin from other structures using CTAn software. To analyze dentin mineral density in 3D volumes, the original grayscale images were processed with a Gaussian low-pass filter for noise reduction and an automatic segmentation threshold was used. A thresholding (binary) process was used, which required processing of the gray level range to obtain the image of only black/white pixels. Next, a region of interest was selected for each slice individually to analyze the mineral density (Fig. 1).

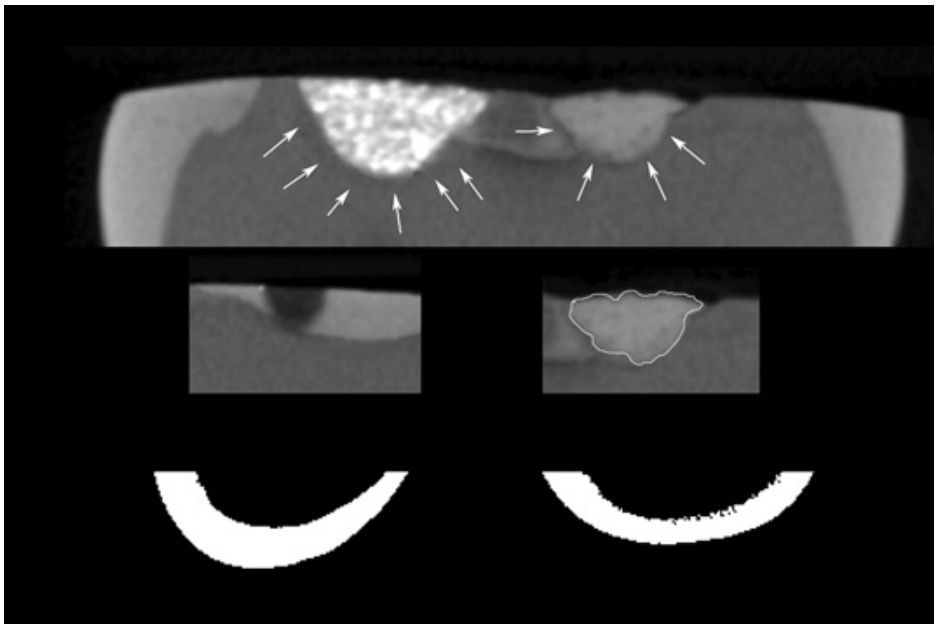

Figure 1. Processed images of pre- and post-treatment longitudinal sections of a representative sample.

Calculations of the difference in mineral loss (ΔZ: gHAp cm^{-3}) of each sample were made by calculating the MD values as an average loss of the entire demineralized area and the interior of the demineralized area (Fig. 2). Mineral loss from the sample was defined after subtracting the demineralized MD values from the baseline phantom rod MD values to correct for misalignment. Also, a mineral density threshold (1.2 g cm^{-3}) for dentin caries was used as the cut-off point for carious and intact dentin, as previously described[11]. The remineralization potential was measured as the percent change in mineral density values of demineralized dentin before and after the experimental procedures.

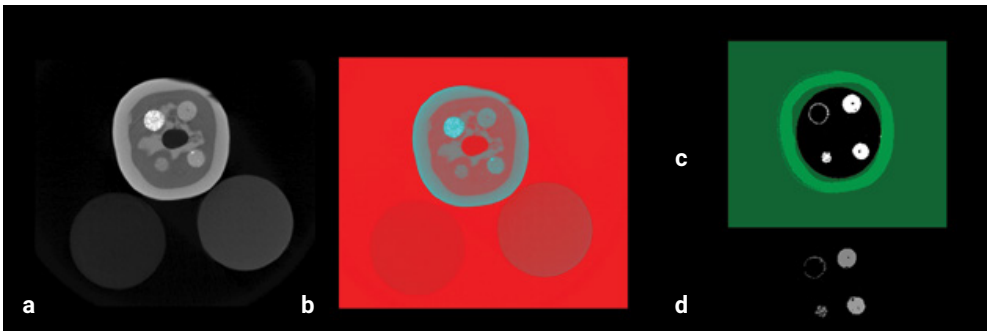

Figure 2. Pre- and post-treatment 2D representation of dentin demineralization areas of a representative sample.

Elemental composition analysis

Materials in the cavities of 5 randomly selected samples were removed under a dental microscope (Zumax OMS2360, Surgical Microscope, Suzhou, China) with the help of a slow speed micromotor and excavator, and cleaned of cement residues in an ultrasonic bath (Sonorex, Bandelin, Germany) for 10 minutes. Then, the samples were dehydrated in ethyl alcohol with 70% concentration for 30 minutes and at 95% concentration for 1 hour. Then, the elemental distribution was mapped using Energy Dispersive X-ray Spectroscopy (X-Max 80, Oxford Instruments, UK) on the images obtained by Scanning Electron Microscopy (SEM) (JSM-7001F, JEOL, Tokyo, Japan) from the dentin surfaces adjacent to the capping materials of each cavity, including the entire cavity area. Also, calcium and phosphorous contents (percentages by weight) were converted into Ca/P ratio for each material group.

Statistical analysis

Statistical package program (SPSS 19, IBM-SPSS Inc, Chicago, IL) was used for data analysis. Shapiro-Wilk test was used to determine whether the data was normally distributed. Since the data were obtained from dependent samples, the Friedman Test followed by the Wilcoxon Signed Ranks Test was used to analyze the differences between groups. Wilcoxon Signed Ranks Test was used to analyze pre- and post-treatment differences of the same sample. The results were considered significant for $p<0.05$.

Results

The values of mineral density in intact and demineralized dentin and the percentage of decrease in mineral density in the cavity floor before treatment are shown in Table 2. No statistically significant difference was found within the intact and demineralized dentin tissues in terms of mineral density ($p>0.05$). Also, there was no significant difference between the groups in terms of the percentage of decrease in mineral density after demineralization ($p>0.05$).

Table 2. Mean (SD) mineral density values (g cm^{-3}) of intact and demineralized dentin and decrease in mineral density (%) at the cavity floor

Cavity no.	n	Intact dentine (mean ± sd)	Demineralized dentine (mean ± sd)	Decrease in mineral density (%)
C1 (Control)	10	2.22 ± 0.07[a]	0.17 ± 0.02[a]	88.82 ± 4.07[a]
C2	10	2.29 ± 0.06[a]	0.18 ± 0.02[a]	91.82 ± 1.65[a]
C3	10	2.28 ± 0.08[a]	0.18 ± 0.03[a]	89.56 ± 2.33[a]
C4	10	2.29 ± 0.05[a]	0.18 ± 0.03[a]	88.87 ± 2.55[a]
C5	10	2.32 ± 0.07[a]	0.19 ± 0.04[a]	89.33 ± 2.28[a]

Different superscripts indicate statistical differences between groups in the same column. *p<0.05*

Table 3 shows the mean mineral density values for the entire volume of demineralized dentin for each group before and after treatment. A significant increase was observed in the mineral density values after treatment in all cement groups, except for the control group in which dental wax was applied. When the groups were compared among themselves, the group with the highest increase after treatment was RMCS, followed by the GIC and MTA groups.

Table 3. Mean (SD) mineral density values (g cm^{-3}) for the entire volume of demineralized dentin before and after treatment

Cavity no.	Material Group	n	Pre-treatment (mean ± sd)	Post-treatment (mean ± sd)	Effect size
C1	Control	10	0.26 ± 0.11[A,a]	0.26 ± 0.10[A,a]	-
C2	GIC	10	0.20 ± 0.06[A,a]	0.35 ± 0.06[B,ab]	2.5
C3	RMCS	10	0.16 ± 0.07[A,a]	0.42 ± 0.08[B,b]	3.44
C4	RMCH	10	0.20 ± 0.05[A,a]	0.33 ± 0.02[B,a]	2.98
C5	MTA	10	0.15 ± 0.06[A,a]	0.34 ± 0.03[B,ab]	3.66

Different capital letters indicate pre- and post-treatment statistical differences in the same row. Different lowercase letters indicate statistical differences between groups in the same column. *p<0.05*

EDS mapping images of the dentin surfaces adjacent to the post-treatment material of a representative sample are shown in Figures 3a-e, and the Ca and P weight percentages and Ca/P ratios of the groups are listed in Table 4. The analysis revealed that the different capping materials made no statistically significant difference in the Ca/P ratio, with a relatively high ratio in the MTA group (p>0.05).

Table 4. Mean (SD) of elemental levels (wt%) for EDS analysis of post-treatment dentin surface (Friedman's test)

Groups	Elements (wt %)		
	Ca	P	Ca/P
Control	12.28 ± 1.78	11.46 ± 0.81	1.07 ± 0.11
GIC	10.04 ± 2.58	10.32 ± 0.48	0.97 ± 0.22
RMCS	11.03 ± 5.51	9.86 ± 2.52	1.06 ± 0.28
RMCH	8.05 ± 2.25	8.19 ± 1.35	0.97 ± 0.13
MTA	5.8 ± 1.96	3.67 ± 0.75	1.55 ± 0.28
	p value		***0.308***

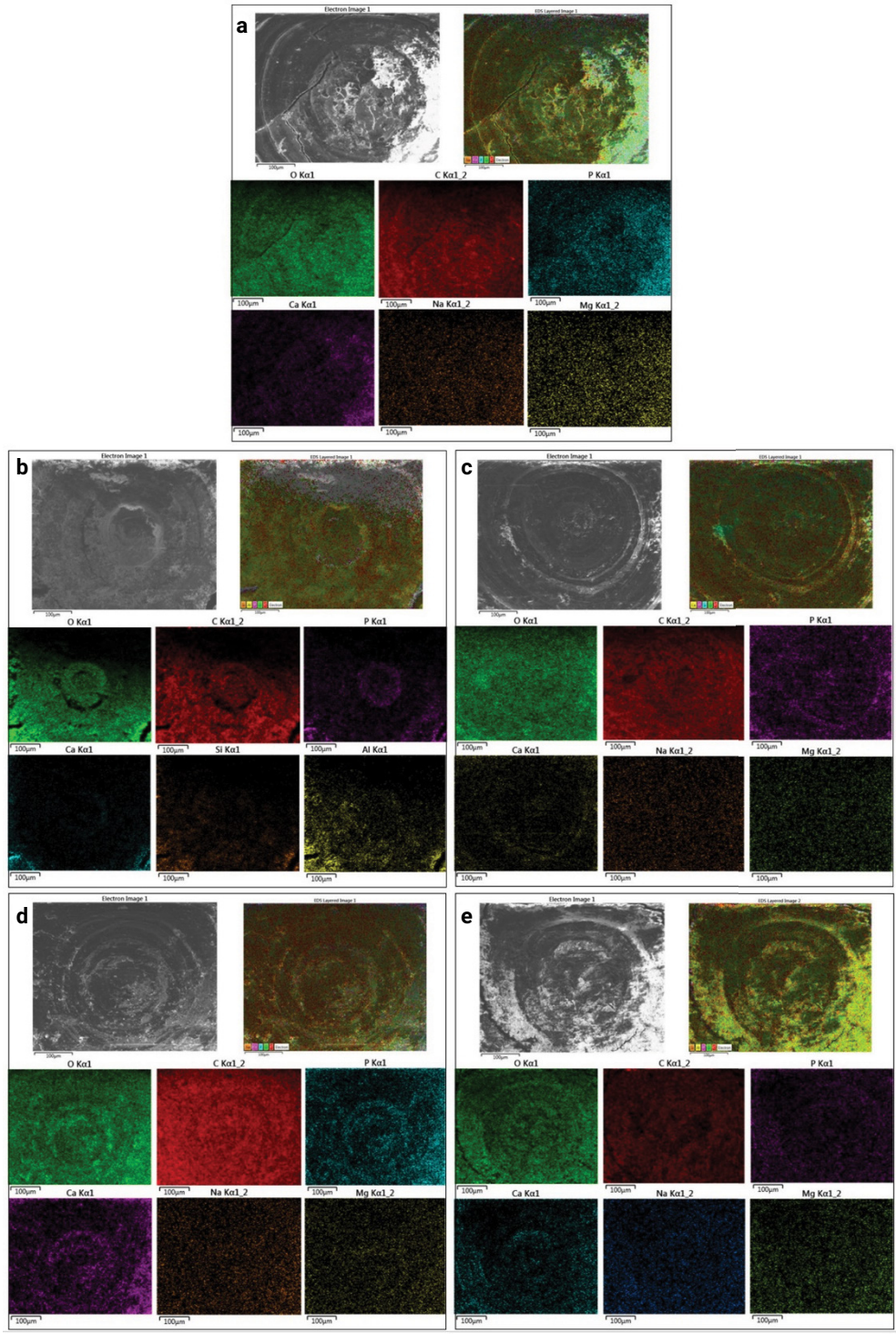

Figure 3. Representative EDS mapping images of the dentin surfaces adjacent to the test materials (a) control (b) GIC (c) RMCS (d) RMCH (e) MTA.

Discussion

For endodontic and restorative materials, bioactivity is explained by the ability to induce specific and deliberate mineral loading to the dentin substrate. These materials are expected to release significant amounts of ions to enable specific biomineralization in the clinical setting[4]. This study evaluated the effectiveness of different bioactive materials in restoring the mineral structure of demineralized dentin tissue using the indirect pulp capping technique by μ-CT and EDS analysis. Regardless of the bioactive material used, the first null hypothesis was rejected because a significant increase in mineral density was observed in the demineralized dentin tissue after treatment compared with before. The second null hypothesis was rejected because there was a difference between the materials in terms of the change in mineral density.

Recently, μ-CT has been widely used as a three-dimensional analytical technique in studies of changes in mineral density of dental hard tissues[2,7,8,12]. The method allows three-dimensional visualization of the internal structure of an object, analyzing the entire mass and obtaining volumetric results. Microradiography, which is used to evaluate the remineralization capacity of dental hard tissues, is a non-destructive method, unlike microhardness and traditional microscopic methods[13]. Therefore, in this study, the μ-CT method was used to evaluate the samples before and after the experimental procedures. The μ-CT evaluation after demineralization showed that the mineral density values of the cavities were below the threshold value determined for dentin caries (1.2 g cm^{-3} of hydroxyapatite). This indicated that biofilm formation with the microcosm model could lead to mineral loss on the dentin surface, similar to natural caries lesions.

In the artificial caries model called microcosm, it is possible to mimic an actual dental biofilm, which is a complex structure, more accurately *in vitro*, thanks to the biofilm model created by the saliva pool[14]. Thus, in contrast to methods that only deal with the physicochemical aspects of demineralization and cannot simulate the primary factors of the natural process, such as collagen degradation, saliva, and biofilm, *in vivo* caries lesion progression can be more accurately mimicked[15]. Using this model, it can be said that the decrease in the mineral density of the intact dentin tissue was very high in all the cavity samples in the present study. Similar results were obtained in dentin demineralization with this model used in previous studies by Pires et al.[9,15] (2018).

Dentin is naturally moist, and induction of hydroxyapatite formation occurs as a result of the reaction of calcium ions released from the material with phosphates in the dentinal fluid[5,16]. For this reason, intrapulpal pressure was simulated in this study, and the materials were contacted with simulated body fluid, a metastable solution supersaturated with apatite and containing ions, mainly calcium and phosphate ions, in concentrations similar to human blood plasma. The components of the simulated body fluid were prepared as described by Kokubo and Takadama[17] (2006). The purpose of simulating intrapulpal pressure was to reproduce what occurs in the oral environment when these materials come into contact with dentin, thus obtaining results closer to a clinical scenario. A constant positive pressure value of approximately 15 cm H_2O reported in previous studies was used to simulate normal

pulp pressure in vital teeth[18]. The experimental setup was prepared as described by Scheffel et al.[19] (2014). In addition, cavities were prepared on the dentin surface of the same tooth for five different materials to be tested so that the dentin substrate structure had similar mineralization properties.

All indirect pulp capping materials significantly increased the mineral density values of demineralized dentin after the experimental conditions. Among the materials, the most significant increase was observed in the resin-modified calcium silicate group (TheraCal LC), while the least effective material group was resin-modified calcium hydroxide (Ultrablend Plus). Besides the superior physical properties of MTA/calcium silicate-based materials, such as better sealing ability and biocompatibility than calcium hydroxide, their mechanism of action is similar. Calcium hydroxide is released as a by-product of the hydration reaction of MTA/calcium silicates with water[6].

In this study, resin-added versions were tested for both material types, which were developed to improve their weak physical properties and increase bond strength by facilitating immediate permanent restoration (thanks to their light-curing feature)[5]. A previous study reported that the presence of a resin matrix in resin-modified calcium silicate cement (TheraCal LC) restricts moisture diffusion into the material so that hydration cannot be completed[20]. It has been thought that this situation may alter calcium ion kinetics and result in a lower remineralization potential with a reduced ability to release calcium ions[5]. In contrast, in this study, the increase in mineral density in the resin-modified calcium silicate group was similar to that in the MTA group, while it was higher than that in the resin-modified calcium hydroxide group. This can be explained by the differences in the resin content of the calcium hydroxide and calcium silicate material groups in which the resin-modified forms were investigated. The resin-modified calcium hydroxide (Ultra-Blend Plus) used in the study contains a hydrophobic monomer/polymer matrix, while the resin-modified calcium silicate (TheraCal LC) contains a hydrophilic monomer/polymer matrix. The hydrophilic matrix preserves its ability to stimulate dentin formation by allowing higher calcium and hydroxide release[21,22].

Another material group tested in the study is glass ionomer cement. When freshly mixed glass ionomer cement is placed on the wet dentin surface, an ion exchange interaction occurs between the material and the dentin substrate. The release of fluoride and calcium/strontium caused by the dissolution of the fluoro-aluminosilicate glass particles in these cements promotes the balance to be reversed in favor of apatite formation by ion supplementation in demineralized tissues, and the process is considered bioactive[4,23]. In the glass ionomer cement (Fuji IX GP) tested in this study, strontium was added instead of calcium due to its radiopaque properties[23]. However, it has been reported that this change does not have any effect on the remineralization ability of the cement[24]. The findings of the present study showed that the tested glass ionomer cement caused a similar increase in mineral density as the resin-modified calcium silicate and MTA groups. This is in line with the results of previous μ-CT studies exhibiting that glass ionomer cement has similar bioactive potential to calcium silicate-based materials[2,9].

Chemical analysis can be used to determine the composition and distribution of elements on the sample surface. With EDS analysis, it is possible to detect the elemental composition and obtain semi-quantitative data from the SEM images of the sample surface[25]. The weight-based Ca/P ratio determines the amount of hydroxyapatite mineralization[26]. Considering the Ca/P ratios obtained as a result of the EDS analysis of the dentin surfaces on which the capping material was placed in this study, it can be said that the tested materials did not induce any significant difference in the apatite structures. Although the MTA group had lower Ca and P levels, it showed a higher Ca/P ratio, which was not statistically significant. This result can be explained by the relative decrease in the proportions of the investigated minerals (Ca and P) since different minerals are released depending on the material placed on the cavity. The higher Ca/P ratio of the MTA group in this study was in line with a previous study reporting that calcium silicate-based material compositions immersed in simulated body fluid for 6 weeks showed a higher Ca/P ratio[3]. On the other hand, the lower Ca/P ratio in the resin-modified calcium silicate group, another calcium silicate-based material group, may be due to the lower solubility of this material due to the shorter curing time resulting from its resin content.

As a limitation of the current study, pure material properties were evaluated independently of stem cells and biochemical and hormonal stimuli that have proven to be effective in their activity in previous studies[27,28]. Further investigation that also considers the activation mechanisms of these cells is needed to assess the role of hybrid, composite, or mineral molecules in inducing controlled cell differentiation.

In conclusion, within the limitations of this study, it can be concluded that the resin-modified calcium silicate material has similar potential to MTA and conventional glass ionomer cement, but higher potential than resin-modified calcium hydroxide cement to induce mineral density changes as an indirect pulp capping agent.

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Data availability

Datasets related to this article will be available to the corresponding author upon request.

Author Contribution

All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by [Tuğba Misilli], [Gülşah Uslu], [Kaan Orhan], [Demet Erdönmez]. The first draft of the manuscript was written by [Tuğba Misilli], [Demet Erdönmez], [İbrahim Şevki Bayrakdar] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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