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Original Article

Investigation of the crystal formation from calcium silicate in human dentinal tubules and the effect of phosphate buffer saline concentration

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Abstract *Background/purpose:* Based on hydrodynamic theory, blocking the dentinal tubules can reduce discomfort caused by dentin hypersensitivity. This study identified the crystals formed in dentinal tubules from tricalcium silicate (TCS) in phosphate-buffered saline (PBS) and evaluated the effect of PBS concentration on crystal formation.
Materials and methods: Sixty-nine specimens were made by isolating the cervical part of extracted premolars. TCS was applied by brushing for 10,000 strokes on dentin surface simulating sensitive dentin. Specimens were stored in PBS or solutions with concentrations 1/100, 1/10, 10, and 100 times that of PBS for 1, 30, 60, or 90 days (n = 3). Another nine specimens applied TCS, were immersed in PBS for 3 months, and divided into three subgroups: no treatment, sonication for 10 min, and 1M acetic acid treatment for 3 min. Crystal formation was examined using a scanning electron microscope, assigned five grade scores (0–4) according to maturation, and analyzed by a nonparametric two-way ANOVA ($\alpha = 0.05$). Crystal components were analyzed using X-ray diffraction (XRD) and energy dispersion X-ray spectroscopy (EDS).
Results: The maturation of intratubular crystals was dependent on time and PBS concentration ($P < 0.05$). In all periods, the high-concentration group showed a higher maturation grade than

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the low-concentration group. Intratubular crystals were similar to hydroxyapatite according to XRD and EDS, and they withstood sonication and acid application.
Conclusion: TCS with nanosized particles formed hydroxyapatite-like crystals in the dentinal tubules, which were dependent on time and concentration of PBS and withstood sonication and acid application.

Introduction

Dentin hypersensitivity (DH) is a common oral clinical disease where abnormal pain is associated with various exogenous stimuli, such as cold, heat, acid, sweet, and mechanical stimulation.[1] DH is defined as "pain arising from exposed dentine in response to stimuli, typically thermal, evaporative, tactile, osmotic, or chemical, which cannot be ascribed to any other form of dental defect or pathology."[2] The hydrodynamic theory is the most widely accepted theoretical mechanism explaining DH,[3] stating that, external stimuli cause fluid movement within the dentinal tubules, which stimulates the end of the dentinal tubules or the nerve endings of the dentin–pulp complex, leading to pain. Therefore, DH is associated with opened dentinal tubules and pulpal patency, which provides a channel for stimulation transmission by fluid movement within the tubules.[4]

Several approaches have been used to reduce DH-related discomfort. To reduce intradental nerve fiber excitability, potassium salts have been used; however, no substantial evidence were found supporting their efficacy.[5] The physical occlusion of opened dentinal tubules is another strategy to reduce DH,[6] using ion- and salt-containing agents such as strontium, bioactive glass, amorphous calcium phosphate, and oxalate-containing agents.[7] Ideal desensitizing agents for dentinal tubule occlusion must easily penetrate the dentinal tubules, and penetration depth should be sufficient to ensure their longevity and resistance of being washed out. However, most desensitizing agents on the market do not achieve intratubular occlusion owing to large particle size and high solubility, or the effect is short-lived owing to poor resistance to acid attacks.[8,9] Effective occlusion of dentinal tubules is difficult owing to the small dimensions of dentinal tubules (0.5–4.0 μm in diameter),[10] the complex structure of the pulp–dentin system, and the outward hydraulic pressure of the dental pulp (0.15 kg/cm^2).[11]

Recently, various types of materials such as bioceramics,[12] synthetic polymers,[13] and peptides[14] have been tested as new desensitizers. However, they are unable to completely overcome the disadvantages of the existing desensitizers.[15]

This study aimed at producing an experimental material consisting of tricalcium silicate (TCS) with a particle size smaller than the diameter of the dentinal tubules and inducing crystal formation in dentinal tubules by applying experimental material to the exposed outer dentin surface in phosphate-buffered saline (PBS). In addition, the effect of PBS concentration on intratubular crystal formation was evaluated.

Materials and methods

Specimen preparation

This study was approved by the Ethics Committee of the Seoul National University, Graduate School of Dentistry (IRB number: S-D20190010). A total of 69 human premolars, recently extracted for orthodontic treatment, were prepared. Only teeth with intact crowns and roots, without caries or restorations, were included. All teeth were examined under microscope (200 ×) (Carl Zeiss Surgical GmbH, Oberkochen, Germany) to determine the absence of any crack lines. They were stored in 0.1% thymol solution for no longer than 3 months prior to use. Debris on the surface of all teeth was removed using periodontal curette.

A specimen with a height of 8 mm centered at the cementoenamel junction was isolated by horizontally sectioning the extracted premolar with a low-speed diamond saw (Isomet™; Buehler, Lake Bluff, IL, USA) under constant water cooling and flat and fresh dentin was exposed. Remaining pulp tissue was carefully removed with small forceps. Subsequently, the sectioned tooth was mounted in the ring-shaped acrylic mold with self-cured blue resin (Bosworth Fastray; Keystone Industries, Singen, Germany) (Fig. 1).

Each specimen's exposed upper dentin surface was immersed with 17% ethylenediaminetetraacetic acid (MD Cleanser; Meta Biomed, Chungju, Korea) for 1 min. This was followed by application of 2.5 mL of 5.25% sodium hypochlorite to simulate sensitive dentin, and rinsing with 10 mL of distilled water twice.

Tricalcium silicate preparation

The experimental material consisted of single-phase and high-purity TCS. Calcium carbonate [$CaCO_3$] (239,216; Sigma–Aldrich, St. Louis, MO) and silicon dioxide [SiO_2] (342,890; Sigma–Aldrich, St. Louis, MO, USA) were mixed at a molar ratio of 3:1. The mixed CaO–SiO_2-based compound was compressed into pellets using the high-temperature solid-state method.[16] The compound pellets were placed in an alumina crucible for solid chemical reaction synthesis and calcined in an electric furnace. During calcining, a temperature is fired at 1350 °C for 18 h. Further, the material was cooled and completely ground again. The process of pelleting and calcining was repeated several times to produce a single-phase and high-purity TCS. The particle size distribution of powders was measured using laser diffraction (Mastersizer S; Malvern Panalytical, Malvern,

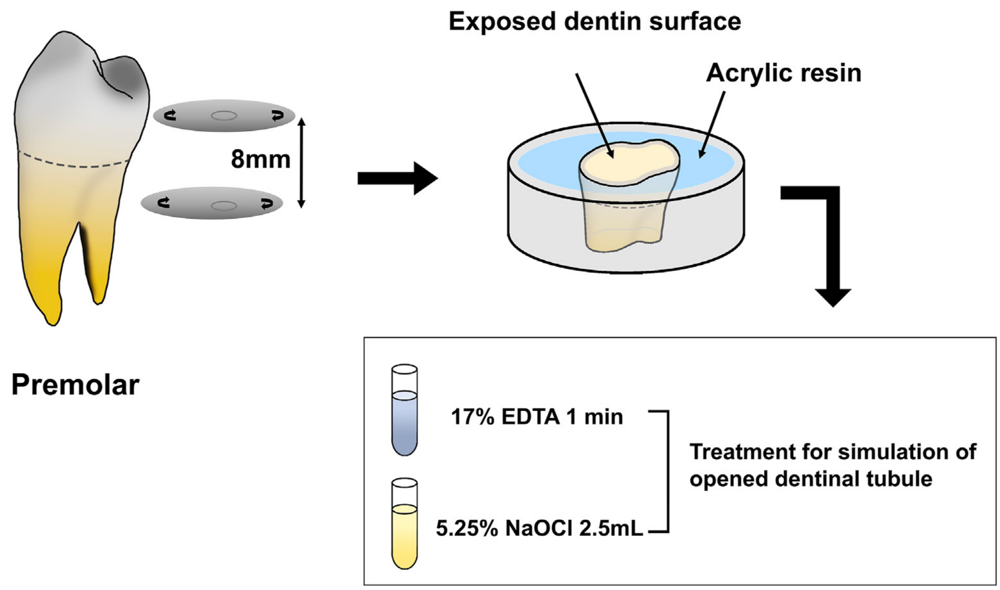

Figure 1 Schematic illustration of specimen preparation. Total of 8 mm of the cervical part of each tooth, centered at the cementoenamel junction, was isolated. Remaining pulp tissue was removed, and the sectioned tooth was mounted in the ring-shaped acrylic mold. The exposed upper dentin surface was treated with 17% EDTA for 1 min, followed by 2.5 mL of 5.25% NaOCl.

UK). The particle size of the TCS ranged from 0.737 μm (D0.1) to 3.415 μm (D0.9), and the median value (D0.5) was 2.012 μm.

Tricalcium silicate application

After the preparation of the specimens, 0.5 g of TCS was mixed with 5 mL distilled water and applied to the exposed dentin surface by tooth brushing motion. According to the ISO 11609 standards for the dentin wear test, the abrasive in the dentifrice should be 10% of dentifrice and water mixture, and the concentration of TCS applied followed this standard. A total of 10,000 strokes (1 stroke/s) were applied onto each specimen using the toothbrush under a 150 g load, with continuous contact between TCS and the exposed dentin surface. Subsequently, the excess TCS was removed with two rinses of 15 mL of distilled water each.

Storage media preparation

The composition (in g/L) of PBS (Dulbecco's phosphate-buffered saline, D8662; Sigma—Aldrich, St. Louis, MO, USA) was $CaCl_2 \bullet 2H_2O$ 0.133, $MgCl_2 \bullet 6H_2O$ 0.1, KCl 0.2, KH_2PO_4 0.2, NaCl 8.0, and Na_2HPO_4 (anhydrous) 1.15, and the pH was 7.2. The 60 specimens were randomly divided into five groups ($n = 12$). The control group was stored in standard PBS. Two experimental groups (low-concentration groups) were immersed in PBS media diluted to 1/100- and 1/10-times concentrations of standard PBS using distilled water. The remaining two experimental groups (high-concentration groups) were immersed in solutions with concentrations 10 and 100 times that of standard PBS, prepared by adding an equivalent amount of $CaCl_2 \bullet 2H_2O$, KH_2PO_4, and Na_2HPO_4 (anhydrous). Each group was divided into four subgroups ($n = 3$) according to the period of immersion: 1, 30, 60, or 90 days at 37 °C. The immersion media were replaced every 7 days.

Crystal formation on occlusal surface and effect of environmental change

The remaining nine specimens had TCS applied and were then immersed in standard PBS for 3 months to analyze the occlusal surface. Specimens were randomly divided into three groups: an untreated group, a group sonicated for 10 min in an ultrasonic bath (WUC-A03H; Daihan Scientific, Seoul, Korea) at 50 kHz and 296 W, and a group treated with 1M acetic acid for 3 min.

Intratubular crystal formation and crystal maturation grade

Longitudinally sectioned surfaces of specimens ($n = 3$) in each group were examined to assess crystal formation in dentinal tubules after TCS application on exposed dentin surfaces in various concentrations of PBS. All specimens were mounted on aluminum stubs, sputter-coated with a 30-nm layer of gold, and examined using field emission scanning electron microscopy (FE-SEM, Apreo S; Thermo Fisher Scientific, Waltham, MA, USA).

Specimens were divided into five grades according to the maturity of intratubular crystals in the SEM images. The average grade of each group was derived from the grades of the four images representing the group.

Grade 0: no change.

Grade 1: confirmed start of partial crystal formation in dentinal tubules.

Grade 2: confirmed crystal growth in all areas of the dentinal tubules.

Grade 3: confirmed petal-shaped crystal maturation.

Grade 4: confirmed densely filled petal-shaped crystal in the dentinal tubules.

X-ray diffraction and energy dispersive spectroscopy analysis

To characterize the crystal formed by TCS in PBS, the substance generated on the dentin surface after 3 months of immersion in standard PBS was analyzed using an X-ray diffractometer (Aeris 600w; PANalytical, Almelo, Netherlands) with the following parameters: 40 KeV, 15 mA, 2 theta range of 20–50°, and scan rate 4°/min.

The change in calcium:phosphorus (Ca/P) ratio of crystals on the dentin surface after immersion in PBS for 1, 30, 60, and 90 days was analyzed by energy dispersive spectroscopy (EDS, XFlash 6160; Bruker, Karlsruhe, Germany).

Statistical analysis

The grades of SEM images were statistically analyzed to determine the relationship between crystal maturation in the dentinal tubules over time and PBS concentration. To account for the non-normal distribution of values, a nonparametric two-way ANOVA based on the Scheirer–Ray–Hare extension of the Kruskal–Wallis test was conducted, and Mann Whitney U post-hoc test was used at the 95% confidence level. The statistical analysis was conducted using R version 4.1.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Crystal formation on occlusal surface

Fig. 2 shows the SEM images and X-ray diffraction (XRD) pattern of specimens immersed for 3 months in standard PBS after applying TCS. The petal-shaped crystals were formed on occlusal surface, and the XRD pattern of the crystals showed peaks at approximately 26° and 32° in a 2-theta position, indicating the presence of hydroxyapatite.

The crystals on the occlusal surface were removed via sonication for 10 min in an ultrasonic bath and treatment with 1M acetic acid for 3 min, but occluding materials in dentinal tubules remained (Fig. 2c and d).

In longitudinal section of a longitudinally sectioned specimen immersed for 3 months in PBS after applying the experimental material, there were plug shape precipitates below the dentinal tubule orifice and plate-shaped intratubular crystals were formed below the occluding plug. The intratubular crystals were formed at a depth of more than 50 μm from the surface of the exposed dentin surface (Fig. 2e white arrows).

Intratubular crystal formation and maturation grade analysis

Fig. 3 shows SEM images of longitudinally sectioned specimens that illustrate the intratubular crystal formation according to the immersion period and media concentration within a range of 20–100 μm from the dentin surface. Small-sized and petal-shaped crystals were formed in dentinal tubules in standard PBS group after 1 day. Intratubular crystals inside the dentinal tubules enlarged over time, densely filling the dentinal tubules. The average maturation grades of the four representing images per group are shown in Table 1. No crystal formation was apparent in the low-concentration groups for all experimental periods (Grade ≤ 2). In the high-concentration groups, partial crystal formation was observed from Day 1, and a denser crystal complex was observed than in the low-concentration groups. The higher the concentration of PBS and the longer the immersion period, higher was the maturation grade ($P < 0.05$).

The Ca/P ratio of the crystals on the exposed dentin surface after immersion in standard PBS was evaluated using EDS (Table 2). The Ca/P ratio was 2.121 after 1 day, and it reduced to 1.687 after 3 months.

Discussion

Physically blocking opened dentinal tubules can help reduce DH by reducing fluid flow and blocking pain signals from the odontoblast processes.[17] In this study, TCS with a particle size smaller than the diameter of the dentinal tubules was used to occlude opened dentinal tubules. Changes in intratubular crystal formation according to the concentration of media and resistance of occluding materials to physical and chemical challenges with ultrasonic and acid treatment were investigated.

The plug-shaped precipitate near the dentinal tubule orifice and the petal-shaped intratubular crystals below the occluding plug were observed after TCS application (Fig. 2). Unlike endodontic sealers in previous studies,[18] which formed tag-like structures within dentinal tubules during root canal treatment, TCS is designed for application on the exposed dentin surface, which is not in continuously contact with the dentin due to physical and chemical factors in the oral cavity. Therefore, efficient penetration of dentinal tubules by desensitizers is crucial for improved intratubular occlusion.[19]

TCS consisted of particles with a diameter smaller than those in other studies using calcium silicate for DH (85 μm[20] or 0.5 mm[21]). can enhance penetration into dentinal tubules and facilitate the formation of occluding plugs.[22,23]

The occluding plug and deep penetrated particles from TCS initially form calcium silicate hydrate and act as a nucleation site and calcium reservoir in dentinal tubules.[24] When TCS comes in contact with water, it is hydroxylated, and calcium and hydroxyl ions are released, resulting in a highly alkaline environment.[25] Subsequently, calcium hydroxide is attracted by the hydroxyl groups, forming calcium silicate hydrate serving as a nucleation site for hydroxyapatite formation upon contact with PBS.[26,27] Hydroxyapatite crystals are formed on the surface of nucleation sites in a supersaturated environment, and crystals gradually grow over time through a process of continuous calcium dissolution from the experimental material and continuous provision of phosphate ions from PBS[24,28] to create a petal-like structure.[23]

For a long-term desensitization, the material blocking the dentinal tubules should be deep enough.[29,30] Compared

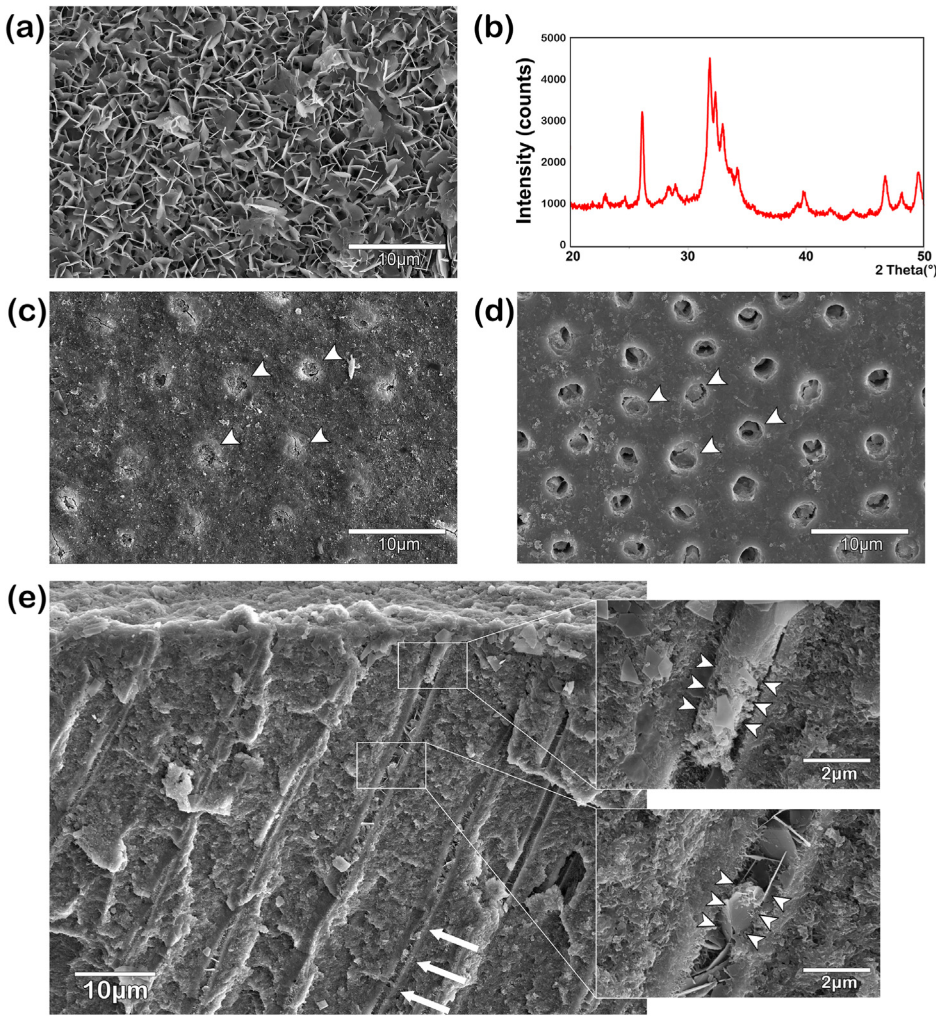

Figure 2 Scanning electron microscopy and X-ray diffraction results of a specimen immersed for 3 months in standard PBS after applying TCS. (a) The petal-shaped crystals were formed on the occlusal surface (× 10,000). (b) X-ray diffraction pattern of the crystal showing peaks at approximately 26 and 32°, indicating the presence of hydroxyapatite. (c—d) The crystals were removed by sonication (c) and acid treatment (d), but occluding materials in dentinal tubules remained. (e) In longitudinal section, plug-shaped precipitates (white arrowheads in upper right image) were observed below the dentinal tubule orifice, and petal-shaped intratubular crystals (white arrowheads in lower right image) were formed below the occluding plug; × 5000 (left) and × 30,000 (right) magnification. The intratubular crystals were formed at a depth of more than 50 μm from the surface of the exposed dentin.

with the precipitates from commercial dental care products for daily use (such as Sensodyne Prophylaxis Paste with NovaMin and Colgate Sensitive Pro-Relief Toothpaste) that only penetrate the dentinal tubules 2—10 μm,[31,32] the intratubular crystals produced in this study do not easily wash out; thus, they can be expected to have a long-term effect. To determine the durability of occluding materials in the dentinal tubules, sonication (a physical challenge) or 1M acetic acid (a chemical challenge) was applied. The crystals formed on the occlusal surface were separated because they were loosely attached to the smear layer formed by the tooth brushing. In contrast, the occluding materials in this study had durability against environmental changes in the oral cavity, unlike desensitizers on the market.[29,33]

Crystal formation occurred in dentinal tubules forming denser crystal complexes with prolonged storage in PBS (Fig. 3). The denser crystal complex could prevent the movement of the pulpal fluid through the dentinal tubules and effectively reduce discomfort from DH.[19,34] The PBS used as a media in this study was a synthetic tissue fluid with a composition similar to that of body fluids[35] and allowed crystal formation in the dentinal tubules under the dentin surface after calcium silicate application. Because the oral cavity is dynamic, the ion concentration of the tissue fluid can change, which affects intratubular crystal formation.

Thus, the higher the concentration of PBS and the longer the immersion period, higher is the maturation grade (Table 1), which can be explained by an equilibrium shift in the chemical reaction. Chemical reactions occurring in nature are in equilibrium, and when the concentration of reactants changes, the equilibrium shifts. In this study, the occluding plug and deeply-penetrated particles from the experimental material served as a nucleation site and calcium reservoir while PBS was the major

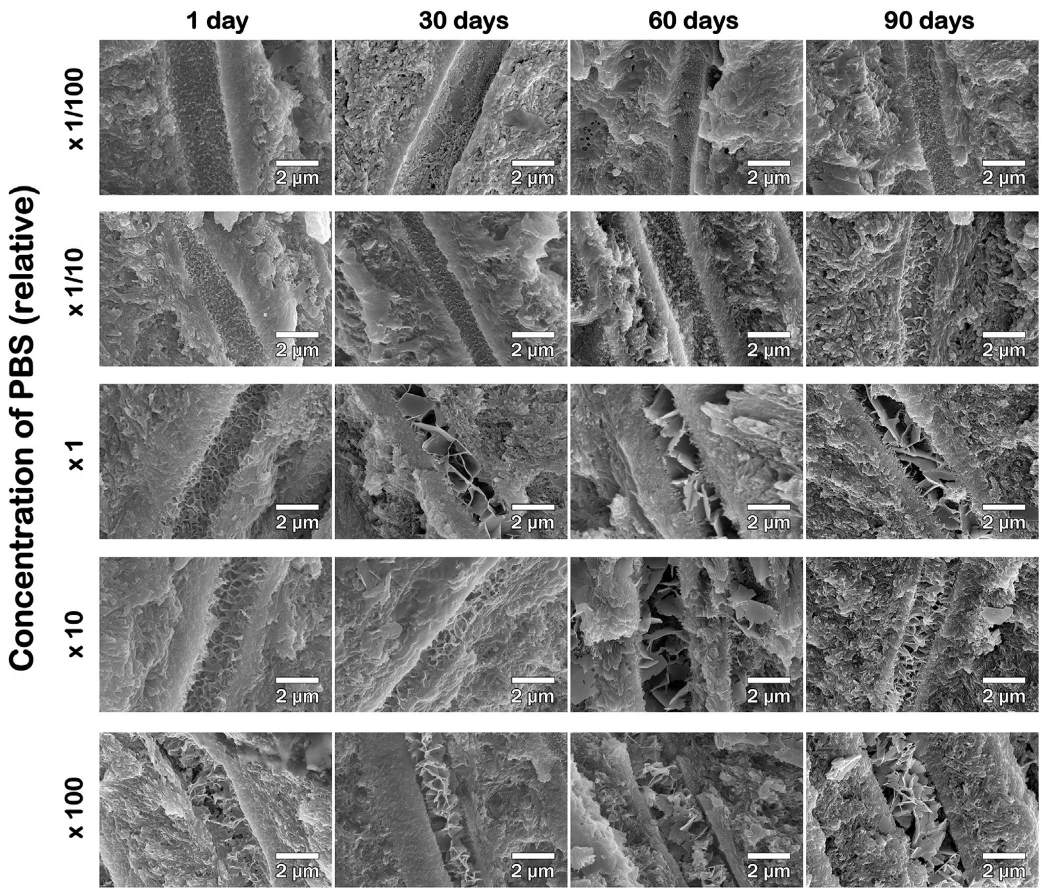

Figure 3 Scanning electron microscopy images of the intratubular crystals in longitudinally sectioned specimens (× 50,000). With the increase in immersion time, denser crystals were observed in dentinal tubules. The formation of intratubular crystals occurred faster as the media concentration increased.

Table 1 Maturation grades of intratubular crystals in scanning electron microscopy images.

PBS Concentration	Time			
	1 day^{A}	30 days^{B}	60 days^{C}	90 days^{D}
1/100^{A}	0.00 ± 0.00	0.50 ± 1.00	1.00 ± 0.00	1.00 ± 0.00
1/10^{B}	1.00 ± 0.00	1.33 ± 1.00	1.75 ± 1.00	2.00 ± 0.00
1 (standard)^{C}	2.00 ± 0.00	2.50 ± 1.00	3.00 ± 0.00	3.50 ± 1.00
10^{D}	2.00 ± 0.00	3.00 ± 0.00	3.75 ± 1.00	3.75 ± 1.00
100^{D}	2.25 ± 1.00	3.50 ± 1.00	4.00 ± 0.00	4.00 ± 0.00

Significant differences are represented by different uppercase letters within the same row and column ($P < 0.05$).

phosphate source.[36] In the precipitation equilibrium, an increase in PBS reactants could facilitate intratubular crystal formation.[37]

The XRD pattern of the precipitates showed peaks at approximately 26° and 32° in a 2-theta position, indicating the presence of hydroxyapatite, forming the most dominant peak, despite the presence of other peaks and suboptimal crystallinity, corroborating previous studies[24,28,38] reporting that calcium silicate in PBS could form hydroxyapatites after hydration. EDS analysis conducted to predict the growth pattern of crystals over time showed that the Ca/P ratio of crystals formed on the occlusal surface was 2.122 after immersion in standard PBS for 1 day and 1.687 after immersion for 3 months (Table 2). It was because the amount of calcium supplied from the dissolved calcium silicate was greater than the amount of phosphate supplied by PBS after 1 day. Phosphate ions were provided continuously by PBS replacement, and the Ca/P ratio gradually decreased as the crystal grew.

Table 2 Ca/P ratio of crystals on the dentin surface of a specimen immersed in standard PBS.

	1 day	30 days	60 days	90 days
Ca/P	2.122	1.988	1.869	1.687

This study had some limitations. TCS was applied to the extracted teeth by brushing, which did not accurately represent teeth with vital pulp. As vital teeth experience outward hydrostatic pressure, reproducing this condition is crucial to accurately assess the desensitizing effect of the experimental material. No comparison was made with commercially available toothpaste regarding its efficacy in treating DH. Moreover, the pH of the surrounding environment tends to decrease as the concentration of PBS buffer increases (pH 7.09 at 100 times concentration and pH 7.15 at 10 times concentration). The hydration reaction of calcium silicate creates an alkaline environment, and under these conditions, a low pH environment resulting from an increasing PBS concentration can promote the crystallization reaction. However, the pH change was only about 0.11 at 100 times or less of PBS, so this analysis was not performed in this study. Further research is needed to address these limitations and provide additional insights.

This study demonstrated that TCS composed of nano-sized particles formed hydroxyapatite-like crystals within dentinal tubules at a depth >50 μm from the dentin surface in PBS, and the crystals filled the dentinal tubules more densely over time. Intratubular crystal formation proceeded more reliably as the media concentration increased, and these crystals were resistant to physical and chemical challenges.

Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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