

DENTINAL TUBULES OBLITERATION USING A TOOTHPASTE WITH NANO-HYDROXYAPATITE OBTAINED FROM CHICKEN EGGSHELL

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ABSTRACT

Introduction: Dentin hypersensitivity is a common pathology, produced by exposed dental tubules.

Objectives: The aim of this study was the evaluation of the effectiveness of experimental toothpaste based on nano-hydroxyapatite (nHAP) obtained from chicken eggshell in the obliteration of dentinal tubules (DTs).

Material and methods: Toothpastes with different percentages of nHAP were formulated, including 3%, 7%, and 15% of nHAP, a commercial paste, and a toothpaste without nHAP. Assays were made using healthy premolar samples (n = 50). The pastes were applied and brushed for 7 days after a first erosive cycle, then a second erosive cycle was made at 15 days. Samples were analyzed using scanning electron microscopy and confocal Raman microscopy at three times: after the first erosive cycle (T0), after applications of the pastes (T7), and after the second erosive cycle (T15).

Results: The quantitative analysis of the micrographs showed a significant difference between the experimental toothpastes with respect to the controls. Moreover, there was no statistically significant difference between the experimental toothpastes at time T7. By mineral concentration analysis, the 3%-nHAP toothpaste presented a significant difference against conventional toothpaste, when evaluating mineral concentration at 7 days.

Conclusions: The experimental paste is effective in obliterating DTs at concentrations of 7 and 15%, in addition to being stable over time.

Key words: eggshell, durapatite, hypersensitivity, microscopy, toothpastes.

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INTRODUCTION

Dentin hypersensitivity (DH) is a common pathology mainly affecting people between 30 and 44 years old, with an estimated prevalence of 33.5% [1, 2]. This disease produces a pain on exposed dentinal tubules (DTs) due to thermal, mechanical, or chemical stimulus that provoke a reaction in the nerve endings when ingesting solid food, beverages, and even when brushing teeth [3].

In the current market, there are several alternatives to prevent DH, including toothpastes, mouthwashes, and varnishes, to block or inhibit nerve cells by acting

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in the polarity of their membranes as well as obliteration of DTs.

Moreover, there are products that include analgesic agents that offer a momentary relief of DH, but do not achieve the necessary stability due to a faster disintegration process [1, 4].

Recently, nano-hydroxyapatite (nHAP) obtained from synthetic salts to be used against DH due to their potential effect of obliteration of DTs was introduced [5-7]. Existing research indicate that DTs cannot withstand the continuous impact of nHAP and are easily eliminated; therefore, it is necessary to study new mechanisms of action that allow for a stable obliteration using bio-available resources for synthesis, such as eggshell or seashell, to achieve optimal adherence and stable remineralization, providing better results and easy production [8, 9].

There are different sources to produce nHAP of biological origin from natural residues, including animal tissues, aquatic, coral, eggshell, and plant origins. In this study, nHAP of natural origin extracted from eggshell was used, a waste that contained 94-98% calcium carbonate as a precursor compound [10]. The synthesis of hydroxyapatite from this bio-waste can be carried out using most widely used technique, the sol-gel method, with other components that contain phosphate ions [11].

In this context, the aim of this article was to study the effectiveness of experimental pastes based on nHAP in concentrations of 3%, 7%, and 15% obtained from chicken eggshell in the obliteration of DTs.

OBJECTIVES

This study was performed to determine the effectiveness of experimental toothpastes based on nHAP obtained from chicken eggshell in the obliteration of the dentinal tubules. Research hypothesis was that the experimental toothpastes would have a significance effect compared with a conventional toothpaste.

MATERIAL AND METHODS

This *in-vitro* study was reviewed and approved by the Institutional Ethics Committee of the Peruvian University Cayetano Heredia (No.: 124-07-19/102470-CIEI).

SYNTHESIS OF NANO-HYDROXYAPATITE FROM EGGSHELL

nHAP was obtained using the sol-gel method, and adapted the methodology presented by Ansari *et al.* [12]. Before process, chicken eggshells containing calcium carbonate ($CaCO_3$) were washed using sodium hypochlorite, dried, and grinded. The powder was calcined at 850°C for 3 hours to obtain calcium oxide (CaO). Next, this precursor suspended in distilled water was stirred for 1 hour at 40°C in order to obtain calcium hydroxide solution ($Ca(OH)_2$), which was prepared at a concentration of 0.5 M. Another suspension of phosphoric acid (H_3PO_4) at 0.3 M (Merck KGaA, Darmstadt, Germany) was also prepared, and mixed dropwise with $Ca(OH)_2$ in a magnetic stirring at 200 rpm. Immediately, the pH of the mixture was adjusted to 10.5 using ammonium hydroxide (Merck KGaA, Darmstadt, Germany) under stirring for 2 hours.

The formed gel was aged for 24 hours at room temperature, then filtered washing with distilled water, and dried at 120°C for 2 hours. Finally, the sample was subjected to heat treatment at 600°C for 3 hours to promote crystallization of the grains. The obtained powder was manually pulverized using an agate mortar, sieved using a 325-mesh to acquire particles smaller than 40 μm, and incorporated into the experimental toothpaste.

STRUCTURAL CHARACTERIZATION OF NANO-HYDROXYAPATITE BY X-RAY DIFFRACTION

Structural characterization of the obtained nHAP powder was carried out in a D8 Focus X-ray diffractometer (Bruker AXS, Karlsruhe, Germany), using a source of $CuK\alpha_1$ (λ = 1.5406 Å) at 40 kV and 40 mA and measured in an angular range 2θ from 15° to 90°. The qualitative analysis of the obtained diffractogram was carried out using Diffrac.Eva software (Bruker AXS, Karlsruhe, Germany) to identify the crystalline phase by comparing with the patterns from database of the International Center for Diffraction Data (ICDD). Additionally, in order to obtain crystallographic information, such as lattice parameters and average grain size of the modelled phase, a structural refinement procedure was performed using Rietveld method, employing Diffrac. TOPAS software (Bruker AXS, Karlsruhe, Germany) [13].

FORMULATION OF EXPERIMENTAL TOOTHPASTES BASED ON NANO-HYDROXYAPATITE

After synthesis and characterization of the nHAP, preparation of conventional toothpaste was carried out, following methodology proposed by Appel *et al.* [14]. This method uses a mixture of propylene glycol, xanthan gum, calcium carbonate, sodium lauryl sulfate, carboxymethylcellulose, sodium benzoate, sodium saccharin, and mint for formulation of a conventional toothpaste. To the above-mentioned experimental toothpaste, the nHAP was added in percentages, such as 3%, 7%, and 15% w/w under magnetic stirring. Moreover, an experimental toothpaste without nHAP was reserved as negative control. Biorepair commercial toothpaste (Coswell SPA, Bologna, Italy) was used in this study as a positive control.

SAMPLE PREPARATION AND EXPERIMENTAL DESIGN

The samples used in this study were 50 healthy human premolars, without caries, restorations, exposed dentin, erosion, abrasions, fissures, or micro-fractures. Before procedure, soft tissues or existing bone tissue remnants were removed from samples, washed with distilled water, and stored in 0.05% thymol solution to preserve permeability of DTs [6, 15].

The teeth were cut using a FX205m micromotor (NSK Dental Spain S.A., Madrid, Spain) and diamond discs, sectioning the clinical crown 5 mm below, and making a transverse cut corresponding to the dentin of the root cervical third (4 mm × 4 mm) [16]. Subsequently, DTs were exposed to wet sanding with SiC abrasive paper, with constant irrigation. After the dentinal tubules were exposed, the specimens received a first erosive cycle with 30 ml of Coca-Cola (The Coca-Cola Company, Lima, Peru) per specimen, with a pH = 2.3 at 25ºC, for 60 seconds [17]. Then the specimens were cleaned 3 times for 10 minutes by ultrasonic bath DA-968 (DADI, China) using distilled water to remove total excess of the debris. Finally, the specimens were stored in distilled water and were named as 'T0'.

APPLICATION OF TOOTHPASTES TO SPECIMENS

Experimental toothpastes were applied to 50 randomly assigned specimens, according to the defined study groups (n = 10): Group 1 (G1) – conventional paste with 3% nHAP; Group 2 (G2) – conventional paste with 7% nHAP; Group 3 (G3) – conventional paste with 15% nHAP; Group 4 (G4) – Biorepair commercial toothpaste; Group 5 (G5) – conventional toothpaste without nHAP.

The toothpastes were placed in syringes, and 0.1 ml per specimen were applied with a VITIS Sonic S20 electronic brush (Dentaid SL, Barcelona, Spain) for 3 minutes, every 24 hours, for 7 days. After this time, the samples were called 'T7'.

Subsequently, the specimens were stored in distilled water for an additional 7 days with daily change of medium. Finally, the resistance of therapeutic compound was tested by applying a second erosion cycle to each sample at the fifteenth day. This procedure was made using the commercially available drink Coca-Cola (The Coca-Cola Company, Lima, Peru) for 2 minutes. At this time, each sample was named as 'T15'.

SCANNING ELECTRON MICROSCOPY ANALYSIS

Morphological and elemental analysis were made using a Quanta 650 scanning electron microscope (FEI, Hillsboro, USA), with an electron beam voltage of 30 kV in high vacuum, and backscattered electron detector. During analysis, specimens were fixed with carbon tape on the microscope sample holder to avoid charge effect. Measurements were made at T0, T7, and T15 time-points described above. Finally, the micrographs were processed using ImageJ software (National Institute of Health, Bethesda, USA) to obtain the percentage of total tubule area (σ).

CONFOCAL RAMAN MICROSCOPY ANALYSIS

An alpha300 RA confocal Raman microscope (CRM) (WITec GmbH, Ulm, Germany), equipped with a laser source of 50 mW and 785 nm as the light source was applied. This equipment uses a spectrometer with a 300 lines/mm diffraction grating, and a CCD camera as the Raman spectra collection system. The analysis was made on 50 μm x 50 μm, divided into 75 pixels × 75 pixels, and recording each Raman spectrum using a 0.1 s/pixel. Chemical mapping of each study area was carried out with the phosphate Raman band intensity (960 cm^{-1}, υ_1-PO_4) of hydroxyapatite present in the dentin of each specimen. Furthermore, to obtain a representative spectrum per specimen, all spectra recorded for each image were averaged. In this analysis, the intensity of phosphate Raman band was used as an indicator of mineral amount present in a sample (AM), and therefore, of obliteration of DTs.

STATISTICAL ANALYSIS

The parameters studied to evaluate the obliteration of dentinal tubules were the percentage of total tubule area (σ) obtained by SEM, and the amount of mineral (AM) acquired by CRM. Those parameters were analyzed using Bartlett's test to verify their normality, and then subjected into non-parametric Kruskal-Wallis test for any significant differences between the groups. Subsequently, Bonferroni post-test was applied to compare the study groups at different evaluation moments (T0, T7, and T15) using STATA v. 16 software (StataCorp LLC, College Station, Texas, USA), with a significance level of 5% ($p < 0.05$).

RESULTS

CHARACTERIZATION OF NANO-HYDROXYAPATITE

Figure 1 corresponding to diffractogram pattern of nHAP was identified as hydroxyapatite phase (ICDD No. 09-0432). This diffractogram exhibited peaks with large full width at half maximum, which could be associated with presence of nano-scale grain sizes in the sample. The micro-structural analysis performed by the Rietveld refinement allowed to calculate the crystallographic information. The values of average grain size (D) and lattice parameters (a and c) are shown in Table 1.

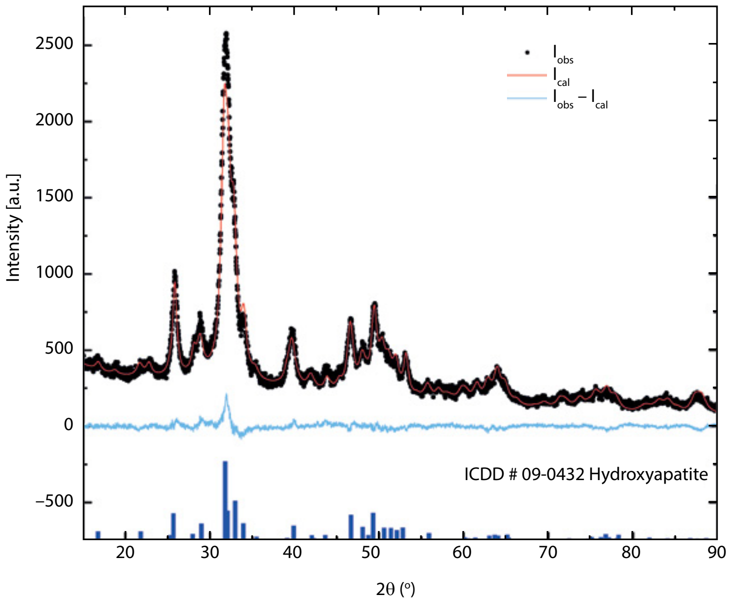

FIGURE 1. Experimental diffractogram of nano-hydroxyapatite sample (black points), calculated diffractogram by Rietveld refinement (red line), difference curve (light blue line), and hydroxyapatite pattern peaks (blue bars)

TABLE 1. Crystallographic parameters and standard deviation (SD) of hydroxyapatite phase obtained from Rietveld refinement. Cell parameter values are in good agreement with those of hexagonal phase of hydroxyapatite. Average grain size is in nano-metric range

Sample	a (Å) ± SD	c (Å) ± SD	D (nm) ± SD
nHAP	9.4190 ± 0.0002	6.8812 ± 0.0002	9.7 ± 0.1

nHAP – nano-hydroxyapatite

EFFECTIVENESS OF EXPERIMENTAL TOOTHPASTES BY SCANNING ELECTRON MICROSCOPY

Figure 2 shows total area percentage of open or partially open dentinal tubules σ at the three time-points of the study. For T0 samples, all the groups presented σ values without significant difference between them (p = 0.186). However, for T7 samples, a decrease in σ was seen in all the groups. For T15 samples, an increase in the value of σ was observed, which was statistically equivalent between all the groups (p = 0.075) and at the previous time. The mean values and standard deviation are shown in Table 2.

EFFECTIVENESS OF EXPERIMENTAL TOOTHPASTES BY CRM

Figure 3 shows the amount of mineral AM present in all the groups at the three time-points of the study. The results show that for T0 and T7, all the groups did not differ significantly (p = 1), while that for T15, the G2, G3, G4, and G5 groups were statistically equivalent to each other, with significant difference in the G1 group (p = 0.014). At different times, all the groups showed significant differences regarding remineralization (T7) and subsequent demineralization (T15) as seen in Table 3.

Figure 4 shows a comparative analysis of the techniques used at different time-points. It should be noted that SEM micrographs, CRM chemical mapping, and CRM spectra had similar results according to the time of evaluation, so the methodology followed in this work was validated, and it was ruled out that the instruments used influenced the obtained results.

DISCUSSION

The nHAP prepared using chicken eggshell as one of its precursor compounds is an eco-friendly alternative, since the eggshell is a bio-waste that contains mostly calcium carbonate as calcite structure [18]. However, since calcite is insoluble in water, it requires a calcination process to transform into calcium oxide, and therefore can be used by the sol-gel method, together with the phosphate precursor. In this way, the method chosen in this work was revealed as an eco-friendly alternative to produce nHAP compared with other methods, which use only synthetic salts, such as nitric acid, sulfuric acid, or hydrochloric acid [19, 20]. X-ray diffraction analysis revealed that nHAP crystallites reached an average size of 9.7 nm. This average grain size was expected according to the technique used for synthesis; it also allowed DTs obliteration by remineralizing dental tissues, such as enamel and dentin [21, 22]. The use of eggshell as a precursor for nHAP has been shown to have physiological advantages over nHAP obtained from synthetic salts, including stable bone regeneration [23].

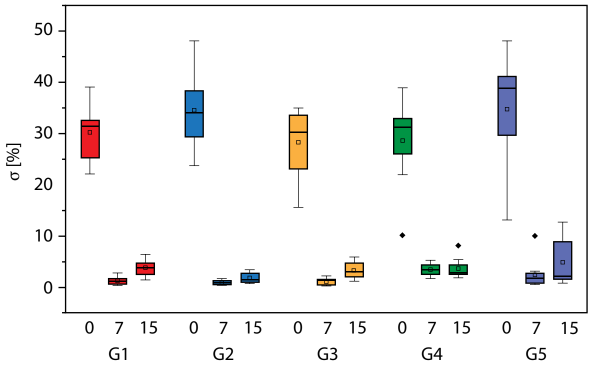

FIGURE 2. Percentage of total tubule area (σ) for each study group at 0 days (T0), 7 days (T7), and 15 days (T15) obtained by scanning electron microscopy

TABLE 2. Mean value of percentage of total tubule area (σ) and standard deviation (SD) for G1 (conventional paste with 3% nano-hydroxyapatite [nHAP]), G2 (conventional paste with 7% nHAP), G3 (conventional paste with 15% nHAP), G4 (Biorepair, commercial toothpaste), and G5 (conventional toothpaste without nHAP) groups by scanning electron microscopy at each evaluated time

Time	Percentage of total tubule area σ (%) ± SD				
	G1	G2	G3	G4	G5
T0	30.24 ± 5.32^{aA}	34.59 ± 6.91^{aA}	28.26 ± 6.57^{aA}	28.64 ± 8.10^{aA}	34.79 ± 10.34^{aA}
T7	1.18 ± 0.69^{bA}	0.83 ± 0.49^{bA}	1.03 ± 0.70^{bA}	3.39 ± 1.23^{bB}	2.52 ± 2.78^{bB}
T15	3.78 ± 1.52^{bA}	1.82 ± 1.07^{bA}	3.20 ± 1.66^{bA}	3.59 ± 1.90^{bA}	4.81 ± 4.37^{bA}

Lowercase letters indicate significant differences between rows (times), uppercase letters show significant differences between columns (groups).

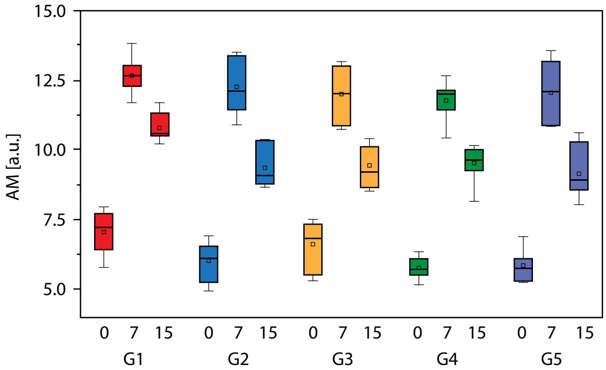

FIGURE 3. Amount of mineral (AM) for each study group at 0 days (T0), 7 days (T7), and 15 days (T15) obtained by CRM

TABLE 3. Mean value of amount of mineral (AM) and standard deviation (SD) for G1 (conventional paste with 3% nano-hydroxyapatite [nHAP]), G2 (conventional paste with 7% nHAP), G3 (conventional paste with 15% nHAP), G4 (Biorepair, commercial toothpaste), and G5 (conventional toothpaste without nHAP) groups by confocal Raman microscopy (CRM) at each evaluated time

Time	Amount of mineral AM (a.u.) ± SD				
	G1	G2	G3	G4	G5
T0	$7.05 \pm 0.76^{a,A}$	$6.02 \pm 0.70^{a,A}$	$6.61 \pm 0.88^{a,A}$	$5.77 \pm 0.40^{a,A}$	$5.85 \pm 0.53^{a,A}$
T7	$12.63 \pm 0.66^{b,A}$	$12.23 \pm 0.96^{b,A}$	$11.98 \pm 0.96^{b,A}$	$11.77 \pm 0.70^{b,A}$	$12.04 \pm 1.08^{b,A}$
T15	$10.77 \pm 0.52^{c,A}$	$9.32 \pm 0.71^{c,B}$	$9.42 \pm 0.76^{c,B}$	$9.51 \pm 0.67^{c,B}$	$9.13 \pm 0.94^{c,B}$

Lowercase letters indicate significant differences between rows (times), uppercase letters show significant differences between columns (groups).

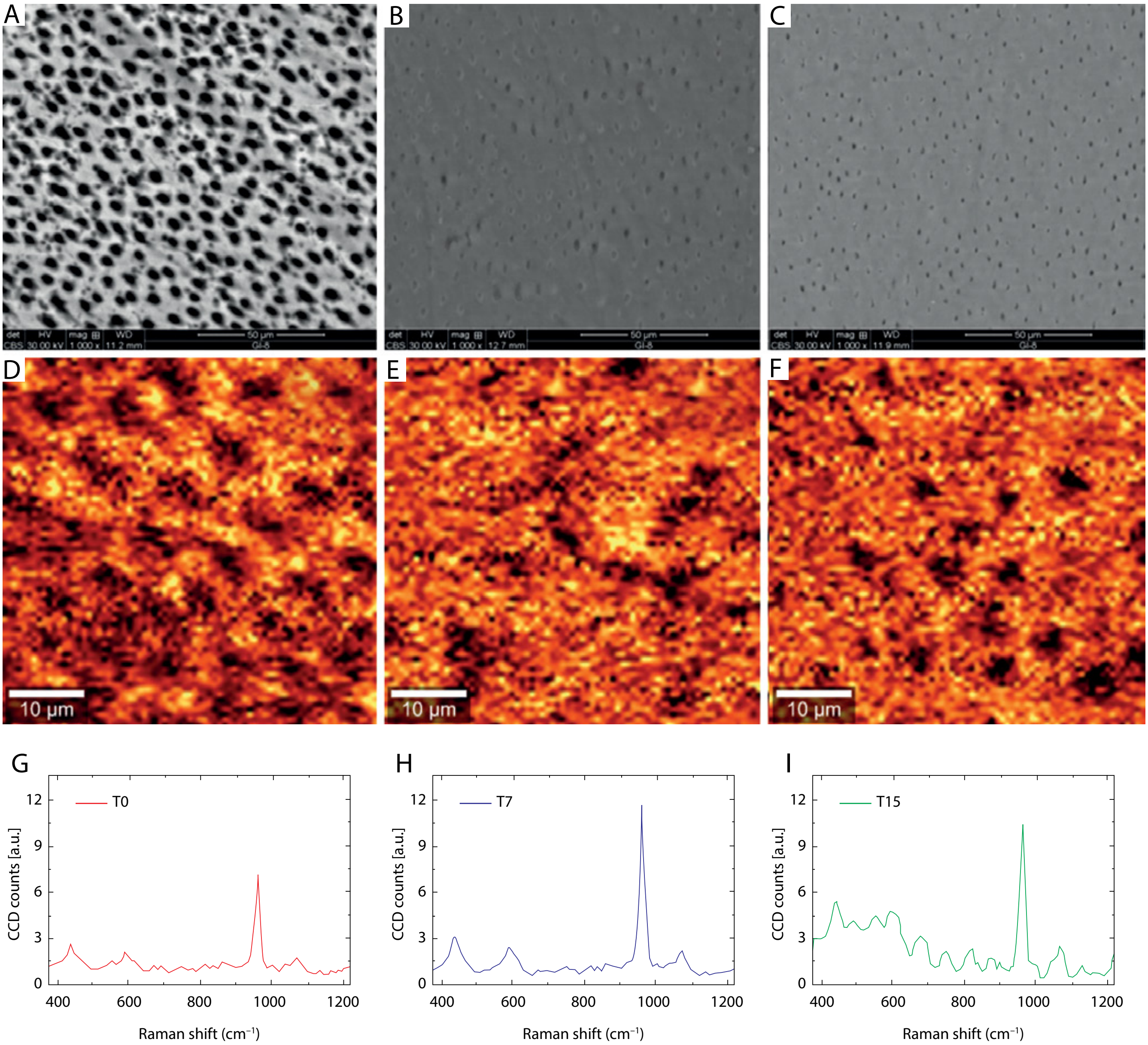

FIGURE 4. Scanning electron microscopy images (**A**, **B**, and **C**), CRM mapping (**D**, **E**, and **F**), and averaged Raman spectra (**G**, **H**, and **I**) of samples of G1 group for T0, T7, and T15 times, respectively. Dentinal tubules exposed in an initial stage and obliterated in the later moments are observed according to the surface morphology. Characteristic bands of hydroxyapatite are observed, where the υ_1 band (960 cm^{-1}) varies in its intensity for different measurement times

The concentrations of nHAP in the formulated toothpastes (3%, 7%, and 15%) were selected considering the maximum limit of active ingredient for oral care (20%) [24]. Huang *et al.* evaluated the effect of 1%, 5%, 10%, and 15% nHAP concentrations on the initial enamel lesions under dynamic pH cycling conditions, reporting that the mineral amount deposition would eventually reach a stable level, despite increases in concentration. Therefore, a non-significant difference between groups containing 10% and 15% nHAP were reported [25]. It still remains to be investigated whether these results could differ in an oral environment, with a variable pH and salivary buffering capacity.

In this work, two methodologies were used to compare the effect of formulated experimental toothpastes. The first one was the analysis of micrographs obtained by SEM, through quantification of exposed DTs. The study of the effectiveness of experimental toothpastes was not directed to the number of open or partially open DTs, but to the percentage of area that these represent on the studied surface, with the purpose of a greater precision in the relevant information unlike other investigations [26]. The second one included analyzing the spectra obtained by CRM, a technique widely used to investigate the remineralization capacity of different dental materials [27]. The υ_1 vibrational mode of phosphate (960 cm^{-1} Raman band), which is the characteristic band of hydroxyapatite in dental tissue, was examined. This analysis was performed in a similar way as other investigations [28, 29], considering the intensity of Raman band as an indicator of the amount of mineral. The data obtained were related inversely to the area percentage of DTs measured from SEM.

The T7 samples evaluated by the two methodologies showed conformational and structural changes. The SEM measurements showed statistically significant differences in the degree of obliteration of DTs. These results demonstrated that nHAP-based experimental toothpastes used with different concentrations achieve a similar obliteration of DTs compared to commercial toothpaste and the control group. The SEM micrographs revealed fully mineralized areas, according to CRM, which also showed a considerable increase in remineralization.

When nHAP is integrated into the tooth surface due to tooth brushing, the surface acquires appropriate physical properties to promote particle incorporation into DTs, resulting in their obliteration. An explanation of this result is associated with generation of bioavailable ions supply, helping to maintain a supersaturated topical state of ions related to tooth remineralizations [30]. Although this theory is still poorly understood, the effectiveness increases when its dimensions are reduced to nano-metric regime [31], promoting the formation of organized apatite crystals [32]. This physical interaction comes from a molecular similarity, where grain boundaries can dissociate to produce a larger grain, which enter by contact, pressure, or electrostatic adhesion to form a crystalline structure within DTs. This bio-mimetic system would enhance the tooth surface, so that a lesion is not appreciated [33, 34].

In the case of Biorepair, the total protective repair toothpaste used in this study contained a patented formula consisting mainly of carbonated hydroxyapatite doped with zinc without fluoride content, with a particle size that varied from 50 to 100 nm at a concentration of 15% [35]. Previously, this toothpaste was studied for its properties, demonstrating its ability to adhere to dental tissue forming a protective surface layer of calcium phosphate mineral resistant to dietary acids, while doping with zinc ions showed an anti-bacterial effect [36].

In the control group (treated with toothpaste without nHAP), calcium carbonate was included as a therapeutic agent, which was found to obliterate DTs, but its resistance and stability were variable, since it could occlude but not efficiently resistant to buccal acids [37].

The storage of the samples without the application of toothpastes and the erosive cycle carried out after 15 days, partially eliminated the precipitated phases on the dentin surface observed by SEM, which can be corroborated with a decrease in mineral concentration by CRM. However, the mineral loss corresponds only to 15-20% less than the gain without statistically significant differences between the groups. Therefore, the supply of zinc ions to the commercial Biorepair toothpaste did not reduce the protective effect against an erosive challenge.

The dynamic and gradual process of demineralization of dental surfaces, caused mainly by the consumption of acidic foods or drinks, and bacteria present in the mouth ($pH < 5.5$), creates an environment conducive to the disintegration of hydroxyapatite crystals. With the findings obtained, we can indicate that the nHAP present in the experimental toothpastes managed to integrate as a biological apatite to the dentin surface, creating chemical bonds between the new and the natural enamel crystals, and thus be resistant to acid attack [38].

By presenting with a wide variety of treatments for DH, and without having a gold standard agent [39, 40], we suggest that future studies evaluate and compare the efficacy of toothpastes formulated with nHAP synthesized from eggshell, but with a longer follow-up time and a larger sample size. With the information obtained by SEM and CRM, we can confirm the stability and resistance properties of nHAP-based experimental toothpastes.

CONCLUSIONS

The experimental toothpaste based on nano-hydroxyapatite in the studied concentrations evaluated obliteration of the dentinal tubules. By SEM, obliteration of tubules is observed in the samples treated with the toothpastes with concentrations of 3%, 7%, and 15% after 7 days of brushing. According to CRM, remineralization of the tooth structure is demonstrated from the application of toothpastes; however, major remineralization occurs with the experimental paste with 3% nHAP at 15 days. There are no differences in the groups studied after the erosive effect at 15 days, which partially demineralized the precipitated phases on the dentin surface observed by SEM and CRM.

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CONFLICT OF INTEREST

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

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