

Development of an Experimental Dentifrice with Hydroxyapatite Nanoparticles and High Fluoride Concentration to Manage Root Dentin Demineralization

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Background: High-fluoride dentifrice is used to manage root caries, but there is no evidence whether its association with nanohydroxyapatite could provide an additional protection for root caries. Therefore, this study aimed to develop and evaluate the effect of an experimental dentifrice with high fluoride (F^-) concentration and nanohydroxyapatite (nano-HA) on root dentin demineralization.

Materials and Methods: After formulation of dentifrices, root dentin specimens were randomly assigned to six groups ($n = 10$) using different dentifrice treatments: placebo; nano-HA without F^- ; 1,100 $\mu g F^-/g$; 1,100 $\mu g F^-/g$ + nano-HA; 5,000 $\mu g F^-/g$; and 5,000 $\mu g F^-/g$ + nano-HA. A pH cycling model was performed for 10 days, in which treatments were performed twice a day. After that period, the longitudinal hardness was evaluated and the area of demineralization (ΔS) was calculated. The formulated dentifrices were evaluated for primary stability, cytotoxicity, and other technical parameters. Two-way ANOVA and Tukey's test with p set at 5% were used for data analysis.

Results: The experimental dentifrices were stable and had no cytotoxicity. Regarding dentin demineralization, the placebo group significantly increased ΔS compared to all other treatment groups ($p < 0.001$). The dentifrices containing 5,000 $\mu g F^-/g$, regardless of the presence of nano-HA, led to a smaller lesion area in relation to the other treatments ($p < 0.001$).

Conclusion: The findings of this study suggest that nano-HA reduced dentin demineralization, and dentifrice with 5,000 $\mu g F^-/g$ dentifrices, regardless of the presence of nano-HA, showed a greater reduction in root dentin demineralization.

Keywords: material development, dentin, dentifrice, fluoride, nanohydroxyapatite

Introduction

Root caries is a global burden especially in the older population.¹ Therefore, several preventive methods have been studied to manage root caries and since root dentin is a more soluble tissue than enamel and would benefit more with Fluoride (F^-),² one of the most used strategies is the use of products with high F^- concentration.³ Indeed, evidence suggests that the use of high- F^- dentifrice is more effective than the use of conventional dentifrice, especially for root caries and patients with high caries risk.^{4,5} Moreover, it is important to address that F^- -dentifrices is the most rational mode of F^- use since it associates the mechanical disorganization of biofilm with the preventive and therapeutic action of F^- .⁶

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In addition to F^- , new materials have been developed to interfere in the development of dental caries, including those containing hydroxyapatite (HA). HA is the main constituent of the inorganic matrix of teeth and bones and this mineral would be able to provide calcium and phosphate ions for dealing with tooth demineralization and remineralization.⁷ Studies have evaluated the effect of HA in nanoparticles (nano-HA) on the tooth surface and showed positive results as a remineralizing and desensitizing material.^{8,9} These nano-HA have a high surface area and proportion of atomicity due to decrease of the particle, which makes this product able to act in the reorganization of calcium and phosphate ions lost during the demineralization process.^{10–12}

The main advantages of nano-HA are similar morphology, size, crystal structure, solubility, and biocompatibility compared to dental apatite.^{13,14} Furthermore, nano-HA could penetrate on tooth porosities and produce a protective layer on the tooth surface similar to the natural tooth.^{11,14} Therefore, we hypothesized that the association of high- F^- dentifrice and nano-HA could have a synergetic effect on root dentin demineralization, however, this product is not available in the market. Thus, the aim of this study was to develop and evaluate the effect of an experimental dentifrice containing high- F^- concentration associated with nano-HA on root dentin demineralization in vitro.

Materials and Methods

Ethics Statement

This study was approved by the Research Ethics Committee of the Federal University of Piauí (Opinion no. 1962181).

Experimental Dentifrice Development Synthesis and Characterization of Nano-HA

Synthesis of Nano-HA

Nano-HA synthesis was performed using the precipitation method, by the chemical reaction between dibasic ammonium phosphate $(NH_4)_2HPO_4$ and calcium hydroxide $Ca(OH)_2$. Quantities of each reagent were weighed on a precision analytical balance and separately stirred with distilled water until obtaining a homogeneous solution. This solution was centrifuged at 4000 rpm for 3 min and to the concentrated powder obtained in the tube, distilled water was added and mixed. This process was performed four times to remove impurities from the powder. The

solution was placed on a Petri dish and kept in an oven at 100 °C for 16 h. After drying, the obtained powder was macerated and stored in plastic tubes.

X-Ray Diffraction Characterization

X-Ray diffraction (XRD) was performed on a LabX-XDR 600, Shimadzu, $CuK\alpha$ ($\lambda=1.5406 \text{ \AA}$), with 2θ in the range of 5°–75°, a scanning rate of 2 °. min⁻¹ and 40 min of exposure time. The size of crystallites were calculated using the following Debye-Scherrer equation: $t_c = (0.9\lambda)/(\beta \cdot \cos \theta)$, where t_c is the size of the crystallite (nm), λ is the wavelength for the radiation $CuK\alpha$, β is the width at half height of the diffraction peak under consideration (rad) and θ is the angle of diffraction (degree).

Fourier Transform Infrared Spectroscopy - FTIR

The powder was analyzed by Attenuated total reflectance/Fourier transform infrared spectroscopy (ATR/FTIR) using a Spectrometer (Brucker Optics—Vertex 70, Brucker, Billerica, MA, USA) with 64 scans for each sample, with a resolution of 4 cm⁻¹.

Transmission Electron Microscope Analysis

To determine the shape and size of particles obtained, the powder was analyzed by transmission electron microscope. For this analysis, the samples were diluted 1:100 in isopropyl alcohol, sonicated and a 25- μ L aliquot of the supernatant was placed on a thin carbon film previously deposited on a copper grid. A JEO JEM 1400 microscope (Akishima, Tokyo, Japan) was used with an acceleration voltage of 200 kV and a field emission gun (FEG).

Experimental Dentifrice Formulation

Formulation of Dentifrices and Determination of F^- Content

Six experimental dentifrices were produced and its composition is shown in Table 1. The source of F^- in dentifrices was Sodium Fluoride (NaF, Sigma-Aldrich, Saint Louis, MO, USA). Considering the dentifrices preparation technique, the NaF was previously added to heated distilled water to allow total solubilization. Subsequently, this fluoride solution was added to the other components of the formulation, such as saccharin, sodium lauryl sulfate, xanthan gum, titanium dioxide, and sorbitol, until it had a homogeneous appearance. Then, silica and nano-HAp (if present in the composition) were added previously levigated with glycerin. Finally, the flavoring agents were incorporated into the formulation. After dentifrices

Table I Composition of Formulated Experimental Dentifrices

Component	Dentifrices					
	Placebo*	Nano-HA	1,100 µg F ⁻ /g	1,100 µg F ⁻ /g + Nano-HA	5,000 µg F ⁻ /g	5,000 µg F ⁻ /g + Nano-HA
Sodium Fluoride	0	0	0,25%	0,25%	1,1%	1,1%
Nano-HA	0	20%	0	20%	0	20%
Sodium lauryl sulfate	1,5%	1,5%	1,5%	1,5%	1,5%	1,5%
Xanthan gum	0,8%	0,8%	0,8%	0,8%	0,8%	0,8%
Glycerol	20%	20%	20%	20%	20%	20%
Sorbitol	10%	10%	10%	10%	10%	10%
Saccharin	0,15%	0,15%	0,15%	0,15%	0,15%	0,15%
Titanium dioxide	1%	1%	1%	1%	1%	1%
Flavoring	1,1%	1,1%	1,1%	1,1%	1,1%	1,1%
Methylparaben	0,1%	0,1%	0,1%	0,1%	0,1%	0,1%
Silica	6%	6%	6%	6%	6%	6%
Purified water (q.s.p)	100%	100%	100%	100%	100%	100%

Note: *Placebo dentifrice regarding Fluoride and Nano-HA.

formulation, they were placed in stability for 48 h, protected from light and heat. F⁻ was quantified in formulations using an ion-specific F⁻ electrode (Orion model 96-09, Orion Research, Cambridge, MA, USA) coupled to an ion analyzer (Orion EA – 740). The calibration curve was performed prior to sample analysis, both performed in triplicate using F⁻ standards ranging from 0.5 to 64 µg F⁻/mL. F⁻ concentration was calculated by linear regression of the calibration curve and expressed in µg F⁻/mL.

Primary Stability of Dentifrice

All tests described above were performed according to the quality control guide for cosmetic products.¹⁵ Organoleptic (macroscopic analysis) and physico-chemical tests (pH and spreadability determinations) were performed before and after the freeze-thaw cycle, which consisted of 12 days of cycles of 24 h at 45 ± 2 °C and 24 h at - 5 ± 2 °C to accelerate possible reactions with the alternance of extreme temperature conditions.

Macroscopic Analysis

After 48 h of initial formulation and after the freeze-thaw cycle the dentifrices were analyzed for organoleptic characteristics to identify any signs of instability, color change, or phase separation. Also, samples of the initial formulated dentifrices were subjected to a thermal stress test, which consisted to gradual and significant temperature increases (30 min at 40 °C, 30 min at 50 °C, 30 min at 60 °C, 30 min at 70 °C, 30 min at 80 °C) to verify possible macroscopic changes.

pH Determination

The pH was measured directly on the formulations using a pH meter (PHS – 3B digital meter) calibrated with specific solutions. The pH measurement was performed in triplicate for each sample at 25.6 °C.

Determination of Spreadability

A base plate of known weight was placed on 1 g of sample. After 1 min, the diameters covered by the sample were read in two opposite directions, using the graph paper scale. Subsequently, the mean diameter was calculated. Results were expressed according to the following equation: $E_i = d^2 \times \pi/4$, where: E_i = sample spreadability for a given weight (mm²); d = mean diameter (mm).

Cytotoxicity of Dentifrices

To test cell viability after treatment with dentifrices, two cell types were used: immortalized gingival epithelial cells (OBA-9)¹⁶ and human gingival fibroblasts (FMM1).¹⁷ The use of the cell lines was approved by the review board of Institute of Biomedical Sciences, University of São Paulo (approval no. 2556792). OBA-9 was cultured in serum-free keratinocyte medium (K-SFM GIBCO™, Life Technologies, Carlsbad, CA, USA) supplied with human PenStrep GIBCO™ recombinant epidermal growth factor (EGF) (10,000 units.mL⁻¹ penicillin; 10,000 µg.mL⁻¹ de streptomycin) and 25 µg.mL⁻¹ amphotericin B at 37 °C in 5% CO₂. FMM1 were routinely cultured in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C and 5% CO₂. Cells were seeded at 6x10⁵ cells/in 96-well plates and incubated for 24 h at 37 °C. Next,

the cells were exposed to 200 μL of the two dentifrices tested, diluted 1:3 in the cell-specific medium. After 5 min and 24 h, the medium was removed. Cell survival was determined using the MTT assay. A 10 μL volume of MTT solution was added to each well, and cells were incubated for 1 h. The resulting formazan crystals were dissolved by removing the culture medium and adding 100 μL of dimethyl sulfoxide solvent to each well. The plates were stirred at room temperature for 10 min to dissolve the crystals and were then analyzed using a microplate reader. Quantitation of enzyme inhibition was performed using a spectrophotometer at 570 nm. Four replicated cell cultures were exposed to each of the dentifrices tested in two independent experiments.¹⁸ Absorbance value readings were normalized to untreated control cultures (100%).

Experimental Design of the in vitro Model

Collection and Selection of Dentin Specimens

Dentin specimens were obtained from bovine incisors previously sterilized in 0.5% thymol solution.⁸ Diamond disks separated by a 4-mm spacer were used to cut the cemento-enamel junction in order to separate crown and root. The roots obtained were sectioned in the mesiodistal direction, and the dentin specimens were sanded and polished, with approximately 4 x 4 x 4 mm in the end.¹⁹ To calculate initial hardness, three indentations in the center of the blocks' surface were made with a Knoop tip under a load of 5 g for 5 s with 100 μm spacing between them. The average of the three values of each block was calculated, and samples with variations up to 15% were selected. After selection, the blocks were randomized into six treatment groups. One-way ANOVA was performed to assess whether there was a statistically significant difference between the hardness of the allocated blocks in each group.

Treatments

Sixty dentin specimens were randomly distributed in the following treatments ($n = 10$): (1) placebo; (2) nano-HA dentifrice; (3) 1,100 $\mu\text{g F}^-/\text{g}$ dentifrice; (4) 1,100 $\mu\text{g F}^-/\text{g}$ + nano-HA dentifrice; (5) 5,000 $\mu\text{g F}^-/\text{g}$ dentifrice; (6) 5,000 $\mu\text{g F}^-/\text{g}$ + nano-HA dentifrice.

Preparation of Dental Blocks for pH Cycling

The selected blocks were isolated with acid-resistant varnish. Double-sided tape was placed on the central surface of each block, and the other surfaces were protected with

varnish. After using the varnish, the tape was removed, and an area of 8 mm^2 was left for the treatment.

pH Cycling Model

For this study, a pH cycling model developed by Queiroz²⁰ with modifications was used. The pH cycling used consisted of 10 cycles. The blocks were kept for 6 h in demineralizing solution (1.4 mM Ca, 0.91 mM P, 0.06 $\mu\text{g F}^-/\text{mL}$, 0.05 M acetate buffer, pH 5.0) and 18 h in remineralizing solution (1.5 mM Ca, 0.9 mM P, 150 mM KCl, 0.05 $\mu\text{g F}^-/\text{mL}$, 0.1 M Tris buffer, pH 7.0) each day. Phosphorus, calcium, and F^- concentrations were determined in these solutions to ensure the desired concentration. The volume of demineralizing and remineralizing solutions used per block area was 6.25 mL/mm^2 and 3.12 mL/mm^2 , respectively. Twice a day, before and after immersion in the demineralizing solution, the dental specimens were kept for 5 min under agitation (60 rpm) in the slurry with treatment solution, produced with one part of the dentifrice to three parts of water. This corresponds to the dilution caused by saliva during a regular toothbrushing. After the 10-day cycling period ended, the dentin specimens remained for an additional 24 h in the remineralizing solution.²¹

Determination of Longitudinal Hardness of Dental Blocks

After pH cycling, bovine root dentin specimens were fixed to acrylic plates and cut longitudinally. For this, a diamond disk coupled to the electric cutter was used. After cutting, the obtained hemiblock was embedded (Arotec Pre 30 inlayer) in colorless acrylic resin, so that the hardness of the inner part of the block could be evaluated. The sectioned surface of the dentin was sanded and polished with 320, 600, and 1200 grit sandpaper under cooling with purified water. Felt disks were also used for final polishing, and longitudinal hardness was determined. The indentations were made in the center of the block, at distances of 10, 20, 30, 40, 50, 60, 80, 100, 120, 140, 160, 180, and 200 μm from the dentin surface. Two more print lines were repeated 100 μm above and 100 μm below, all with 5 g loading and 5 s time²² and the demineralization area was then calculated. Lesion area (ΔS) was calculated by the difference between the area under the curve ($\text{kg}/\text{mm}^2 \times \mu\text{m}$) of sound and demineralized areas.^{23,24} The hardness was chosen as analysis because there is a correlation between hardness and mineral loss and gain.^{25,26}

Table 2 ANOVA Statistical Analysis (p values) for the Variables Used

Variables	Factors		Interaction
	F ⁻ Concentration on Dentifrice	Presence/Absence of Nano-HA	F ⁻ Concentration and Nano-HA
ΔS	< 0.001	< 0.001	< 0.001
pH (before freeze-thaw cycle)	< 0.001	< 0.001	< 0.001
pH (after freeze-thaw cycle)	< 0.001	< 0.001	< 0.001
Ei (before freeze-thaw cycle)	< 0.001	< 0.001	< 0.001
Ei (after freeze-thaw cycle)	< 0.001	< 0.001	< 0.001
Cell viability (OBA-9)	>0.05	>0.05	>0.05
Cell viability (FMMI)	>0.05	>0.05	>0.05

Statistical Analysis

The response variables were submitted to statistical analysis using the Statistical Analysis System Software (SAS, Cary, USA) version 9.0 for Windows, with a significance level of 5%. The Shapiro–Wilk and Levene's test were performed to confirm the normal distribution and homoscedasticity of data, respectively, which were analyzed by two-way ANOVA, considering the factors F concentration in dentifrices (0, 1100, or 5000 µg F⁻/g) and nano-HA (presence or absence), followed by Tukey's post hoc test. A paired *t*-test was performed to compare dentifrice formulation variables before and after the freeze–thaw cycle. The effect of F⁻ concentration in dentifrices on demineralization reduction was evaluated by linear regression analysis and Pearson correlation.

Results

Table 2 presents the summary of two-way ANOVA of the studied variables. For ΔS, pH and spreadability, there was statistical significance both for the isolated factors and for the interaction between them ($p < 0.001$), but not for cytotoxicity ($p > 0.05$).

Characterization of Nano-HA

XRD Analysis

Through XRD analysis, the atoms of a crystal build an interference pattern on the waves present in an X-ray beam. The analysis of the obtained pattern confirmed the formation of nano-HA crystals (Figure 1). The material produced was analyzed before and after sonication, and the comparison between the HA diffractogram synthesized with the corresponding crystallographic file showed that the desired phase of HA synthesis occurred. The Debye–Scherrer's equation was used to determine the size of

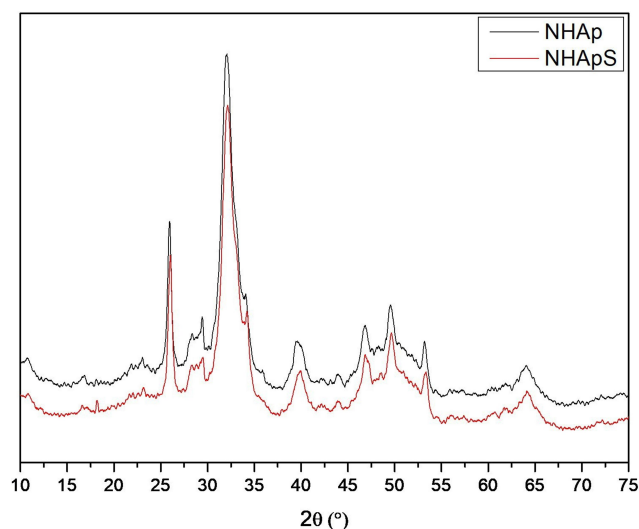
hydroxyapatite crystallites that showed a nanometric size, ranging from 0.83 to 3.85 nm.

Fourier Transform Infrared Spectroscopy – FTIR

FTIR spectrum analysis of both nano-HA and nano-HAs (Figure 2) showed the presence of a band in 3400 cm⁻¹ referring to the stretching vibrations of OH groups present in the HA. A band at 1649 cm⁻¹ was also observed, resulting from a deformation of the OH groups. The band present in the region of 1050 cm⁻¹ corresponded to the asymmetric deformation of the phosphate (PO₄³⁻) groups. The phosphate groups also appeared at 611 cm⁻¹. Therefore, FTIR analysis characterizes the successfully synthesized nano-HA.

Transmission Electron Microscope (TEM) Analysis

The TEM images showed that the biomaterial had cylindrical morphological particles with sizes varying in the

**Figure 1** XRD analysis of the synthesized nano-HA.

Abbreviations: NHAp, nano-HA; NHApS, sonicated nano-HA.

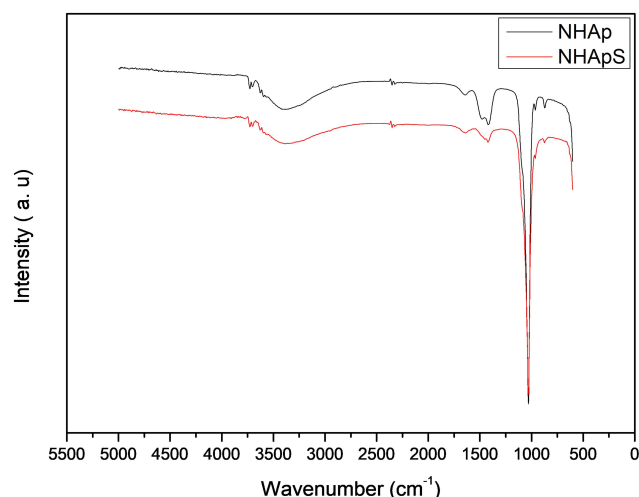


Figure 2 FTIR spectra of the synthesized nano-HA.

Abbreviations: NHAp, nano-HA; NHApS, sonicated nano-HA.

order of nanometers confirming the proper format and size of the material (Figure 3)

Experimental Dentifrice Formulation

Determination of the Amount of F^-

Dentifrices containing nano-HA and F^- , irrespective of the F^- concentration, showed a slightly less total soluble F^- (FST) than the expected value, however without statistical significance and within of 15% variation accepted (Figure 4).

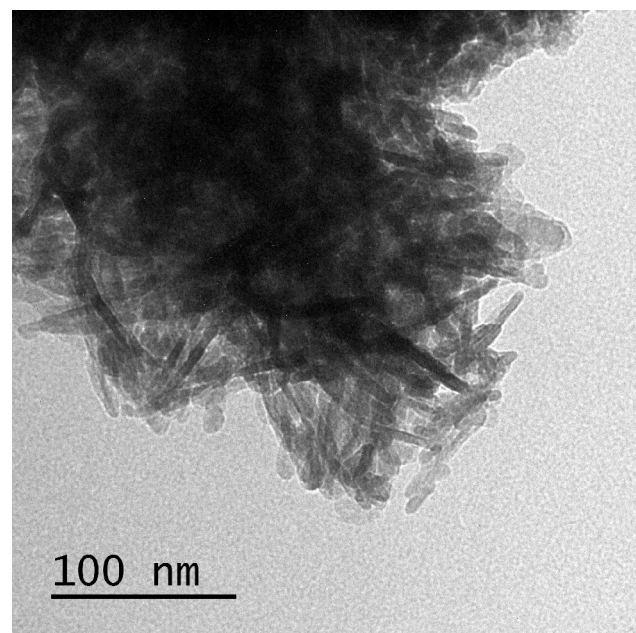


Figure 3 Image of synthesized nano-HA by transmission electron microscope (TEM).

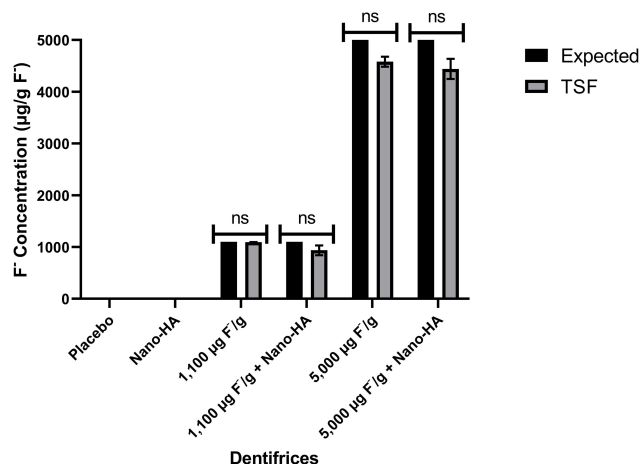


Figure 4 F^- concentration ($\mu F^-/g$) found in each dentifrice in relation to that expected ($n=6$).

Abbreviation: ns, no statistical significance ($p > 0.05$).

Macroscopic Analysis

There was no macroscopic alteration of the formulated dentifrices regarding color, smell, taste, segregation, compound precipitation, or phase separation neither before nor after the freeze–thaw cycle. Also, after the gradual increase in temperature (thermal stress test), there was no macroscopic alteration of the initial formulations.

pH Determination

Overall nano-HA containing dentifrices had a higher (more basic) pH than non-nano-HA dentifrices, and all showed statistical difference ($p < 0.001$). After the freeze–thaw cycle, all formulations reduced the pH value, except the 5000 $\mu g F^-/g$ + nano-HA group (Table 3).

Determination of Spreadability

The spreadability results suggest the resistance of the material to the movement induced by the weight of the overlapping plates. As can be seen in Table 3, nano-HA-containing toothpastes have lower spreadability compared to non-biomaterial ($p < 0.001$), and the freeze–thaw cycle did not change dentifrices' spreadability according to the paired t -test ($p > 0.05$).

Cytotoxicity of Dentifrices

None of the dentifrices showed considerable cytotoxicity, and there was no statistically significant difference between the formulations analyzed, nor was there any significant difference between the placebo and other formulations. The viability of OBA-9 and FMM1 was lower after 24 h of contact with the dentifrices (Figures 5 and 6).

Table 3 pH and Spreadability (Average $\pm$ SD) of Dentifrices Before and After the Freeze-Thaw Cycle

Variables	Condition	Dentifrices					
		Placebo	Nano-HA	1100 $\mu\text{g F}^-/\text{g}$	1100 $\mu\text{g F}^-/\text{g}$ + Nano-HA	5000 $\mu\text{g F}^-/\text{g}$	5000 $\mu\text{g F}^-/\text{g}$ + Nano-HA
pH	Before the cycle	6.01 $\pm$ 0.04 ^A	8.68 $\pm$ 0.03 ^B	7.77 $\pm$ 0.06 ^C	8.90 $\pm$ 0.05 ^D	7.95 $\pm$ 0.02 ^E	9.44 $\pm$ 0.06 ^F
	After the cycle	5.66 $\pm$ 0.07 ^{A*}	8.35 $\pm$ 0.01 ^{B*}	7.52 $\pm$ 0.02 ^{C*}	8.67 $\pm$ 0.07 ^{D*}	7.73 $\pm$ 0.02 ^{E*}	9.43 $\pm$ 0.05 ^F
Ei (mm ²)	Before the cycle	53.94 $\pm$ 1.41 ^A	10.52 $\pm$ 0.52 ^B	54.09 $\pm$ 2.06 ^A	13.65 $\pm$ 0.68 ^C	49.76 $\pm$ 2.32 ^D	27.43 $\pm$ 1.41 ^E
	After the cycle	52.02 $\pm$ 1.46 ^A	11.88 $\pm$ 0.59 ^B	45.24 $\pm$ 1.81 ^C	13.33 $\pm$ 0.78 ^B	49.62 $\pm$ 1.59 ^A	28.65 $\pm$ 1.59 ^D

Notes: Superscript letters represent statistical difference between the groups ($p < 0.001$); *Represents a difference in each group before and after the freeze-thaw cycle using the *t*-test ($p < 0.001$).

pH Cycling

Dentin Demineralization After pH Cycling

The placebo treatment group presented a significant larger lesion when compared to the other treatments ($p < 0.001$), as can be observed in the Figure 7. There was no difference among the nano-HA, 1,100 $\mu\text{g F}^-/\text{g}$, and 11,00 $\mu\text{g F}^-/\text{g}$ + nano-HA groups ($p > 0.05$). These presented a larger lesion area than the groups with 5,000 $\mu\text{g F}^-/\text{g}$, regardless of the presence of nano-HA ($p < 0.001$), which did not present a significant difference between them ($p > 0.05$). There was a strong negative correlation between F^- concentration in dentifrices and lesion area, regardless of the presence of nano-HA, showing that increased F^- concentration leads to decreased lesion area (Figure 8).

Discussion

Despite advances in studies on restorative techniques and dental materials, effective and less invasive therapies

remain a challenge in dentistry. Nanotechnology is indicated as a means of effective alternatives for prevention and less invasive treatments.²⁷ The use of nanotechnology is investigated in several biomaterials to repair biological structures and functions, including HA-based materials, which possess biocompatibility and biofunctionality as important properties.²⁸

In the present study, it was possible to observe that nano-HA decreased dentin demineralization, even in the absence of F^- in the dentifrice. Evidence shows great similarity in morphology and crystalline structure of nanometer-sized particles to dental apatite.²⁹ The similarity of the particles and their deposition in the demineralized structure may justify the effect promoted by the nano-HA dentifrice. Furthermore, the nanometer size of the particle, which promotes an increase in their contact surface with the dental surface and may promote the reorganization of HA-calcium and phosphate ions on demineralized tissue, is also responsible for this result. Indeed, XRD and FIRT techniques confirmed the synthesis of nano-HA, while TEM revealed the morphology and the reduction of particles to a nanometer scale. Furthermore, particle size also influences the intrinsic properties of this material, such as solubility and biocompatibility.^{13,28}

In dentistry, nano-HA is currently used for the treatment of dentin hypersensitivity,^{9,12,30} remineralization of carious lesion,^{11,31} repair and prevention of initial erosion lesions, and formulation of resin adhesives.^{7,32} In vitro and in situ studies have also been performed on the effect of nano-HA on biofilm formation,²⁷ dentin demineralization,¹⁴ and obliteration of dentinal tubules.³¹ The applicability of nano-HA and the positive results of its use may be related to the effect of the material on the dental demineralization and remineralization process. The demineralization process occurs with dental substrate solubilization, and there are indications that calcium and phosphate ions present in

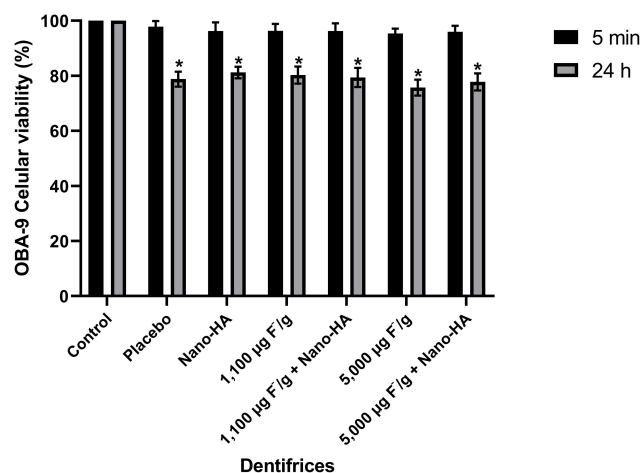


Figure 5 Mean ($\pm$ SD) of the percentage of OBA-9 cell viability after 5 min and 24 h of contact with the dentifrices tested by the MTT assay ($n=4$). *Statistical difference between 5 min and 24 h by paired *t*-test ($p < 0.001$). No statistical difference was observed among the dentifrices in each time evaluated ($p > 0.05$).

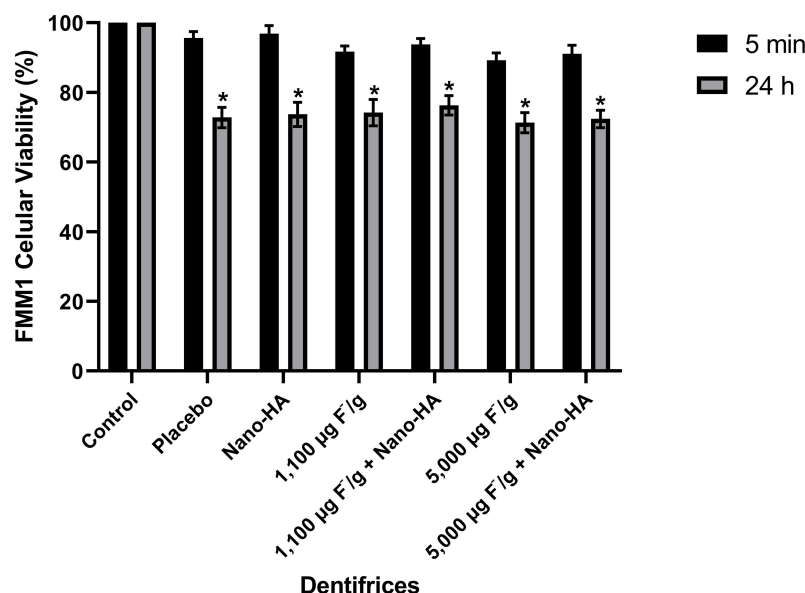


Figure 6 Mean ($\pm$ SD) of the percentage of FMM1 cell viability after 5 min and 24h of contact with the dentifrices tested by the MTT assay (n=4). *Statistical difference between 5 min and 24 h by paired t-test ($p < 0.001$). No statistical difference was observed among the dentifrices in each time evaluated ($p > 0.05$).

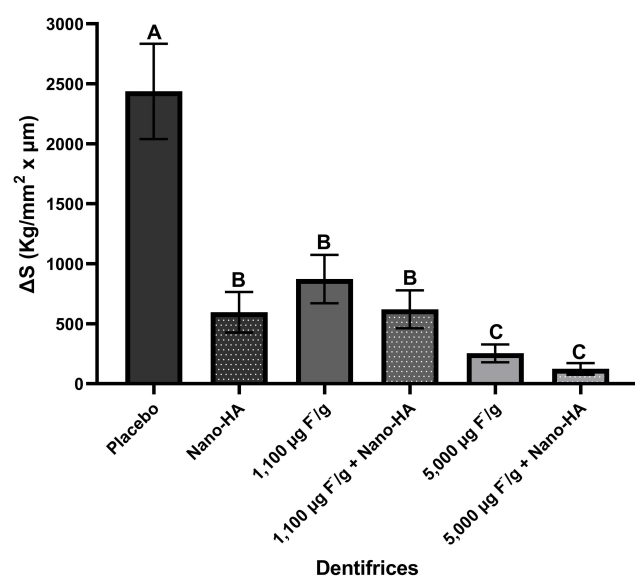


Figure 7 Comparison of the average lesion area (ΔS) for the interaction between factors (concentration of F^- and the presence of nano-HA) by Tukey's post hoc test. Different letters mean statistical difference ($p < 0.001$) and vertical bars denote standard deviation (n=10).

nano-HA can replace lost ions and form a protective layer of HA in the dental structure,²⁹ which may justify the results of the present study, in which dentifrices containing nano-HA had lowest scores of dentin demineralization.

Several studies associate the increase of F^- concentration in dentifrices with the effectiveness in the dental demineralization.^{33–35} F^- acts by incorporating the ion into hydroxyapatite, which results in the formation of fluorapatite during the demineralization and remineralization

process. The critical pH, which is the pH at which tooth dissolution begins to occur, is lower for fluorapatite. Therefore, this compound is more resistant to acid dissolution. The constant presence of F^- ions promotes inhibition of demineralization and remineralization activation.³⁶ In the present study, apart from the better results of high-fluoride dentifrice in reducing dentin demineralization, it was possible to observe a dose-response effect on F^- dentifrices, regardless of the presence of nano-HA. Therefore, the higher the F concentration in dentifrice the lower dentin demineralization, which corroborates with a study showing that dentin benefits from higher F concentrations since is a more soluble substrate than enamel.^{2,5}

After the dentifrices formulation, a primary stability test was performed through the freeze-thaw cycle, and parameters such as pH and spreadability were evaluated. The rise and fall in temperature may have altered the chemical properties of the formulations, which caused changes in the pH and the spreadability. Nevertheless, the pH of dentifrices containing nano-HA was overall higher. According to international norms, the dentifrices pH should be within a range considered safe (5.5–10.5),³⁷ which was observed in all formulated dentifrices. The enamel and dentin solubilization process is caused by a drop in pH beyond the critical value for the solubilization of these structures. In the oral environment, saliva (pH = 7.0) acts as a buffer and helps to resist tooth decay, as there is a neutralization of acids. Dentifrices with more

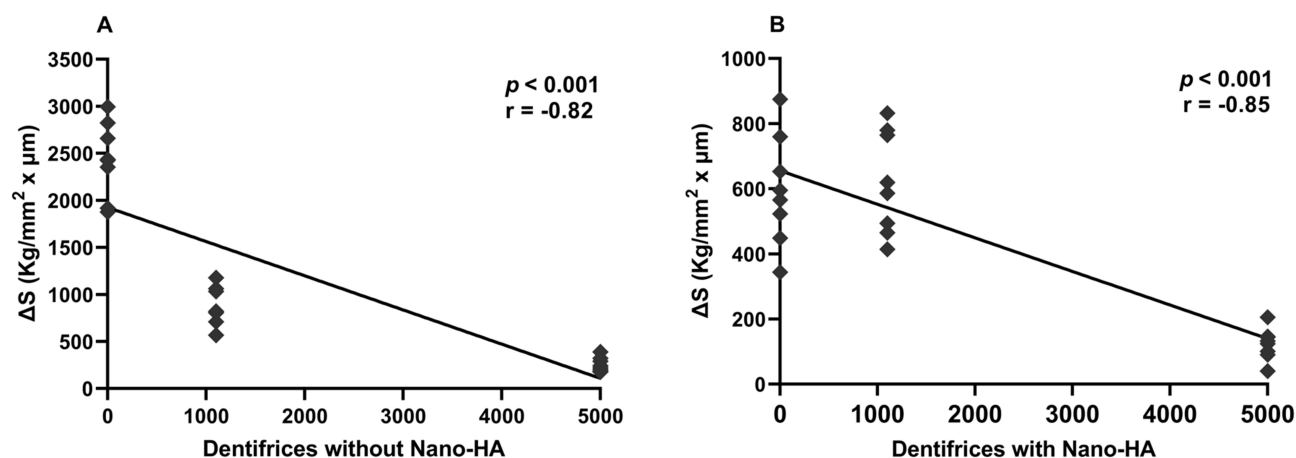


Figure 8 Linear regression fits of the lesion area as a function of the concentration of F⁻ in dentifrices without nano-HA (A) and with nano-HA (B). The statistical significance and the correlation coefficient (r) for this association are described in the figure.

basic pH may have a similar effect, which is considered another factor associated with the effect of nano-HA on demineralization³⁸ and could help to explain the lower dentin demineralization in nano-HA dentifrices found in this study. On other hand, the spreading capacity reflects the consistency of a dentifrice, as a large spreading area is equivalent to better consistency.³⁹ Regarding spreadability, the dentifrices containing nano-HA showed a higher consistency, meaning that the material is more viscous and therefore more difficult to apply to the toothbrush for instance.

Cytotoxicity of dentifrices was evaluated by cell viability after contact with the formulation used. For this, the MTT trial was performed and there was no statistical difference between the different formulations. The formulations were placed in contact with OBA (gingival epithelial cells) and FMM1 (gingival fibroblasts) for 5 min and 24h. Those cells form the gingival mucosa and are exposed to the dentifrices during the tooth brushing. The formulations were produced with the constituents commonly present in dentifrices and the absence of significant difference after nano-HA addition is justified by the biocompatibility of this substance, which corroborates the safety of hydroxyapatite nanoparticles for oral care products.⁴⁰

A pH cycling model was used to simulate the mineral loss and gain that occur in the oral cavity. This model is able to mimic the dynamic conditions of the oral cavity and, therefore, provides important data on in vitro models.²¹ When dentin is used as a substrate in pH cycling, it is important to address that the diffusion process occurs easily since this substrate is more porous and

therefore the remineralization is also facilitated.² The pH cycling model used in the present study allowed us to notice differences in dentin demineralization induced by the different dentifrice formulations. The statistically significant difference observed between 1,100 and 5,000 μg F/g dentifrices in dentin demineralization was also found in other studies.^{41,42}

There are few studies on the effect of F dentifrice associated with nano-HA. Most of them compare nano-HA and F⁻-free dentifrice with F⁻ dentifrice, without investigating the possible synergistic effect of nano-HA and F⁻. To the best of our knowledge, this is the first study that addressed the synergistic effect of nano-HA and high-fluoride dentifrice. Although, the greater reduction in dentin demineralization in dentifrice with 5,000 μg F/g associated with nano-HA, no statistical significance was observed when compared to 5,000 μg F/g dentifrice without nano-HA. Therefore, we reject our hypotheses that the association of high-fluoride dentifrice and nano-HA would have an additional protection to root dentin caries, i.e., a synergistic effect. However, this is an in vitro study that are unable to completely simulate the complex intraoral conditions leading to caries development as the presence of saliva and plaque fluid. Furthermore, while dentifrices are typically slurried to simulate dilution during brushing, the uptake and reactivity of fluoride are consistently lower in vivo than in vitro, which could lead to an overestimation of F⁻ treatment.⁴³ Nevertheless, the results found in the present study highlights the importance of further investigations using in situ and clinical designs to provide more realistic data.

Conclusion

The structural characterizations, XRD, FTIR, and TEM showed the success of obtaining the nano-HA. The experimental dentifrice presented stability and no cytotoxicity. Moreover, the findings suggest that nano-HA reduces dentine demineralization and that 5,000 µg F/g dentifrices, regardless of the presence of nano-HA, showed a greater reduction in root dentin demineralization.

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Disclosure

The authors declare that they have no conflicts of interest for this work.

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