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In Vitro morphological, optical and microbiological evaluation of nanosilver fluoride in the remineralization of deciduous teeth enamel

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Abstract

Background: Nanosilver fluoride (NSF) was developed as an alternative in the prevention of dental caries.

Purpose: The aim of this study was to test the remineralizing action of NSF on incipient enamel caries and its antimicrobial action on the acid production and adhesion of *Streptococcus mutans*.

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Methods: Deciduous enamel fragments were treated with sodium fluoride (NaF), NSF and deionized water. Microhardness, fluorescence spectroscopy and optical coherence tomography imaging were performed on each specimen before chemical caries induction, after caries induction and after 14 days of pH cycling. The treated enamel fragments were also placed into test tubes containing bacterial suspension and saliva. The pH readings and quantification of the adhered microorganisms to the dental enamel were determined. Analysis of variance, Kruskal-Wallis, Mann-Whitney, Tukey and mixed linear regression model were applied.

Results: NSF and NaF were effective in enamel remineralization, with a statistically significant difference (p < 0.001) to deionized water, and they had no statistically significant difference between themselves (p > 0.005). NSF had greater effectiveness compared to NaF in preventing decreases of pH and adhesion of *S. mutans* to the enamel surface, with statistically significant (p < 0.001) differences.

Conclusion: NSF may be more effective than conventional fluorides in treating incipient caries lesions due to its remineralization and antibacterial actions.

Keywords: cariostatic agents; dental caries; dental enamel; *S. mutans*; silver nanoparticles.

1 Introduction

Dental caries is one of the most prevalent and costly diseases in the world [1], and untreated dental caries in children remain a public health challenge in poor communities [2]. Control of dental caries is extremely difficult, especially because it has a multifactorial etiology. The progression or reversion of dental caries depends on the balance between demineralization and remineralization and can be visualized for clinical purposes as the caries

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balance. The knowledge of fluoride action on enamel demineralization and remineralization is an important advancement for the control of the disease [3]. Over the years, several fluoride preparations have been developed for topical application, with varying concentrations for dental caries control [4–10]. However, if the pathological factors, such as acid-producing bacteria, outweigh the protective factors, such as fluoride remineralization and antimicrobial use, the caries progresses [3]. Streptococcus mutans is considered the primary agent of dental caries and contributes significantly to the virulence of dental biofilms in the presence of sucrose. Silver nanoparticles may be effective for controlling *S. mutans* [9, 10], and the antimicrobial effect is better when the nanoparticle is only a few nanometers in diameter. Particles smaller than 10 nm are considered to have a high performance against pathogens of the dental biofilm [11].

Silver nanoparticles have shown high antibacterial properties against Gram-negative and Gram-positive bacteria [11] because of their ability to damage the cell wall, while silver nanoparticles accumulate in the bacterial membrane, which causes a significant increase in permeability, resulting in the death of the cell [11–13]. This antibacterial ability is especially important in deciduous teeth, as they have a thinner and more permeable enamel layer than permanent teeth and they are more vulnerable to the acid action of these bacteria and, therefore, more susceptible to dental caries [14].

Nanoparticles could control the formation of oral biofilm because of their biocidal and anti-adhesive microbial capabilities. As such, particles are reduced from micrometer to nanometer size, therefore altering active surface area, hardness, chemical reactivity and biological activity. For this reason, with respect to metallic nanoparticles, the biocidal effectiveness has been suggested to be due to both their size and high surfaceto-volume ratio [11].

Nanosilver fluoride (NSF) and sodium fluoride (NaF) with cariostatic action were described successfully in a controlled clinical trial that showed effectiveness in paralyzing dentine caries in children from poor communities. Unlike silver diamine fluoride (SDF), the NSF did not show distinctive tissue darkening due to the oxidation of silver ions when in contact with the teeth [9]. The effectiveness of NSF in the arrestment of caries is attributed to the synergistic actions of both silver nanoparticles and fluoride. However, the mechanism of arrestment is not clear.

Therefore, the mechanism of incorporating NSF in the enamel surface needs to be better studied, as well as its action on the healthy tissues of the tooth. To evaluate the effectiveness of dental enamel remineralization through the NaF and NSF, three different methods were used: microhardness, fluorescence spectroscopy and imaging by optical coherence tomography (OCT).

The microhardness test is an indirect test with excellent acceptance for experiments that test de-remineralization processes, as it has the ability to evaluate quantitatively the degree of mineralization through the measurement of the mineral level of the dental enamel [15, 16].

Fluorescence spectroscopy at the visible ultraviolet (UV-VIS) spectral range is a widely used tool for the evaluation of enamel demineralization due to its ability to detect minimal mineral changes with high sensitivity and specificity, providing an accurate identification of the carious lesion in the incipient stages [17, 18]. The dental enamel can fluoresce upon photon excitation, and mineralized and demineralized tissues can be differentiated. In general terms, it consists of a quantitative and noninvasive technique that captures the signal, resulting from the interaction of the tissue excited by monochromatic light, through a detector that converts the optical signal to digital signal, visualized in the spectral form, which allows differentiation between healthy and demineralized tissues when the ultraviolet excitation wavelength is used (405-nm led-induced fluorescence spectroscopy) [19-21]. This is relevant in dental practice because of the possibility of its clinical application.

Another method to evaluate the action of fluoride in demineralizing/remineralizing on the tooth enamel is the OCT, which allows the accurate evaluation of enamel mineral losses. It is a non-invasive, non-destructive, non-ionizing, real-time diagnostic method with high sensitivity and specificity in detecting early lesions on smooth and occlusal surfaces and follow-up of lesion progression [22]. It is a well-accepted and exploited method in dentistry [22–24], and it was chosen to analyze the effective-ness of NSF in the remineralization treatment and ratify the findings obtained by the microhardness test and fluorescence spectroscopy.

The acid action of caries pathogens is more harmful in deciduous teeth due to the histological characteristic of this enamel [14, 25]. Among these pathogens, *Streptococcus mutans* stands out. For this reason, bacterial control is fundamental in the process of reversion of the caries at the initial stages, and microbiological assay was performed to complement this work.

The objective of this study was to test the remineralizing action of NSF on incipient enamel caries and its antimicrobial action in relation to enamel adhesion and acid production of *Streptococcus mutans*.

2 Methods

This study was approved by the Federal University of Paraíba Ethics Committee (Protocol No. 0469/15 and CAAE 48033215.0.0000.5188) in accordance with the World Medical Association Declaration of Helsinki.

This study was carried out in two stages. In the first stage, microhardness test, fluorescence spectroscopy and OCT image capture were performed to evaluate the performance of NSF on the enamel remineralization process. In the second stage, microbiological tests were carried out to evaluate the action of the NSF on *Streptococcus mutans*.

The assay solutions were as follows: NSF [400 ppm silver nanoparticles/5000 ppm NaF] (test solution), NaF [5000 ppm] (positive control) and deionized water (negative control).

2.1 NSF synthesis and characterization

To prepare the NSF, 1.0 g of chitosan, a carrier to stabilize the nanoparticles, was dissolved in 200 ml of 2% (v/v) acetic acid solution. Then, 4.0 ml of silver nitrate solution (AgNO₃) at a concentration of 0.012 mol/l was added to 60 ml of chitosan solution and left for 30 min before adding sodium borohydride (NaBH₄). NaF was added at a concentration of 5000 ppm and stirred continuously until it was completely dissolved. Soon after the synthesis, NSF was stored in a refrigerator [26, 27]. UV-VIS spectroscopy and transmission electron microscopy (TEM) were performed for the NSF characterization.

UV-VIS analysis showed a peak at the 400-nm wavelength, confirming the presence of silver nanoparticles in the colloid, and a TEM image showed monodispersal and spherical silver particles with a diameter of 8.7 ± 3.1 nm [28], as shown in Figure 1.

2.2 Specimen preparation and experimental design

All 33 samples were randomly distributed into one of three groups (n=11) according to the treatment applied and as described in Table 1, and then submitted to a demineralization process through pH cycling. The samples in the negative control group did not receive any remineralizing solution.

The samples were washed in deionized water and kept in phosphate-buffered saline (PBS) solution (8 g/l NaCl, 2 g/l KCl, 2 g/l Na₂HPO₄, 2 g/l KH₂PO₄, pH 7.0) for ionic enamel restoration until further microhardness test,

100 mm

Figure 1: NSF characterization.

Table 1: Description of the studied groups.

Groups	Туре	Remineralizing solution
G1	Positive control	NaF
G2	Experimental group	NSF
G3	Negative control	None

fluorescence spectroscopy and OCT. Measures and images were acquired at three different times: the initial stage (T0), the demineralization stage (after the chemical caries induction of caries – T1) and the remineralization stage (post-cariogenic challenge – T2).

At T1, the application of an acid pH solution removed the minerals from the enamel to induce the formation of a subsurface lesion. To avoid the erosive process in the adamantine tissue, which is characteristic of low-pH substances, it was necessary to saturate the solution in relation to the enamel. A 0.05 M acetate buffer solution containing 1.28 mM calcium, 0.74 mM phosphate and 0.03 µg fluorine/ml solution with a pH of 5.0 was used in the specimens, which were kept individually immersed in a 2 ml/mm² volume of this solution for 16 h at 37°C [29].

At T2, the pH cycling was carried out over 14 days as shown in Figure 2. The daily procedure included immersing the samples in demineralizing solution for 6 h, followed by 18 h of immersion in remineralizing solution. The demineralizing solution was composed of 2.0 mmol/l Ca, 2.0 mmol/l P and 75 mmol/l acetate buffer and had a pH of 4.4, while the remineralizing solution was composed of 1.5 mmol/l Ca, 0.9 mmol/l P, 130 mmol/l KCl and 20 mmol/l sodium cacodylate buffer and had a pH of 7.0. All samples were individually washed with deionized water for 1 min prior to the application of the tested substance (positive



Figure 2: Routine for 24-h pH cycling stages, repeated for 14 days.

control, experimental solution or negative control). All samples were washed again with deionized water before being immersed in the solution for the new cycle. The solutions were maintained at a temperature of 37°C in a biological stove [30]. All the solutions of this study were produced by the department of chemical engineering in the Federal University of Pernambuco (Brazil).

2.3 Enamel surface microhardness test

The microhardness test was performed using a Shimadzu tester and a Vickers indenter (Shimatzu Corporation, Kyoto, Japan) at a load of 50 g for 10 s on the sample. Three indentations were made on each sample, which were spaced 100 μ m apart [31].

2.4 Fluorescence spectroscopy

In this study, a 405-nm superluminescent light-emitting diode illuminated the samples in order to induce the fluorescence signal from the enamel specimens; beyond that, a spectrometer (BLUE-Wave, StellarNet Inc., USA) that operates in the visible and near-infrared spectrum (VIS/NIR, 350–1000 nm) was used to capture the fluorescence signal. A Y-shaped fiber optics cable completed the instrumentation just to guide the violet lighting to the specimens and collect its fluorescence signal and guide it to the spectrometer.

2.5 OCT

A commercial OCT system model (Callisto, Spectral Domain [SD]-OCT System, Thorlabs Inc., New Jersey, USA) was used, as detailed by Mota et al. [32]. To provide a brief overview, the Callisto uses a superluminescent diode laser operating at a 930-nm central wavelength as a light source, with a 100-nm spectral bandwidth and a 3-mW maximum optical power. This model images samples with a 7-µm axial resolution when it is immersed in air and a 5.3-µm axial resolution when it is immersed in water. The transverse resolution does not depend on the background, being set at 8 µm. The axial scan rate is 1.2 kHz, which allows capturing two frames per second with a 105-dB sensitivity.

The Callisto SD-OCT captures data in a matrix of 512 lines \times 2000 columns. The A-scan mode projects *Y* axis data as dependent on the deep penetration of light, limited to 1.7 mm. The B-scan mode creates proper two-dimensional (2D)-OCT images, which are composed of all 2000 A-scans captured along a width of up to 6 mm, corresponding to 1.7-mm maximum depth penetration (in air). A complimentary 3D mode was composed of B-scans captured in a sequence of 250-µm steps, until the complete mapping of the surfaces and subsurfaces of the samples was achieved. Tridimensional images allow the user to visualize B-scans along the XY, XZ and YZ planes.

2.6 Statistical analysis and OCT imaging analysis

Data were processed through the STATA/SE 12.0 and Excel 2010 softwares. Statistical tests were applied at 95% confidence interval, and the numerical variables were represented by measures of central tendency and dispersion.

The Kolmogorov-Smirnov test was used to test the normality of the data, and analysis of variance (ANOVA) (because of the normal distribution) with post hoc Tukey's test was used for quantitative variables and comparison with more than two groups.

In the method for repeated measures, the mixed linear regression model was used, which takes into account the possible correlation between the values of the response variable that constitute the repeated measurements.

The OCT results were processed in the ImageJ software (National Institutes of Health, https://imagej.nih.gov/ij/) [23] with a computational routine that averaged 50 userselected A-scans. In fact, the choice of the region of interest (ROI) is important in that it ensures that the analysis is meaningful. For this, the central region of the OCT images of the specimens was chosen, where the surface was a plane. This allowed the depth of the OCT signal penetration to be similar among the 50 A-scans. This analysis qualitatively examines the changes that occurred in the optical properties of the enamel because the profile of the OCT signal at a certain depth into the sample will be affected by the demineralization and remineralization process. The integrity of the enamel surface and the measurement of the volume loss for the tissue after pH cycling in comparison to the initial images were obtained from the OCT images.

2.7 Evaluation of the antibacterial action of NSF

Twenty-seven autoclaved enamel 5×5 -mm fragments of deciduous teeth were selected and divided into three groups with nine units: test group (NSF), positive control group (NaF) and negative control group (deionized water), as shown in Table 2.

Groups	Туре	Solution test
G1	Experimental	Silver nanoparticle (NSF)
G2	Positive control	NaF
G3	Negative control	None

2.7.1 Bacterial adherence and acid production

The test and positive control solutions were filtered with $0.2\,\mu m$ pore diameter membrane, and the negative control solution was previously autoclaved.

A standard strain of *Streptococcus mutans* AU159 was used, and bacterial adherence and acidogenicity tests were carried out based on the method of Kim et al. [33] with variations. Initially, the inoculum was previously grown in brain heart infusion broth with 2% sucrose at 37° C for 24 h, and then standardization was performed spectrophotometrically until it reached 0.4 on the McFarland scale. After that, 200 µl of the bacterial inoculum was transferred to 27 test tubes containing 1 ml of sterile PBS with 10% sucrose and 1 ml of saliva.

Saliva used in the experiment was collected from volunteers aged 20–30 years, and it was centrifuged $(12,000 \times g)$ for 15 min and then heated at 60°C. The supernatant (treated saliva) was stored at 4°C for later use.

The 27 deciduous enamel fragments were placed into a 24-well microplate and immersed in 2 ml of test substances according to their group for 1 min. They were removed from the wells, washed with sterile deionized water and placed into test tubes containing the bacterial suspension and PBS-treated saliva and remained in the tubes for 24 h at 37°C. The pH was directly determined in the bacterial growth media using a pH meter. The pH readings were performed in triplicate at the initial time T_i (2 h) and at the final time T_f (24 h). After this phase, the fragments were autoclaved again for following assay.

For the bacterial adherence assay, the specimens were also placed into 24-well microplates and immersed in 2 ml of each solution according to their group for 1 min. After that, they were removed from the wells, washed with sterile deionized water and placed into test tubes containing the bacterial suspension and PBStreated saliva and remained in the tubes for 2 h at 37°C. They were transferred to Eppendorf tubes containing 1 ml of sterile saline (0.9% sodium chloride) and then sonicated for 30 s at 50 W to release the bacteria adhered to the enamel. Saline solution containing the bacterial suspension from the dental enamel was sequentially diluted (10⁻¹ to 10⁻⁴) and cultured in Petri plates with Mitis Salivarius agar at 37°C for 24 h to carry out counts for colony-forming units (CFU/ml). This step was performed in triplicate. Quantification of microorganisms that adhered to the dental enamel was determined by direct reading of the CFUs and subsequently calculating the adsorption inhibition percentage for each solution.

Variable	Groups		Deionized water group	p-Value ^a
	NaF group Media±SD	NSF group Media±SD	Media±SD	
Peak intensity ^b (fluorescence)				
ТО	447.37±85.03	463.26 ± 122.34	464.54 ± 157.62	0.938
T1	265.24 ± 74.70	319.23±75.63	299.67 ± 117.67	0.387
T2	$393.81 \pm 105.61^{\text{A}}$	497.74±99.53 ^A	295.72±96.03 ^B	< 0.001
Total intensity ^b				
ТО	42,403.27±6202.03	43,094.18±9913.77	43,748.36±12,523.15	0.950
T1	25,869.27±5995.10	29,200.27±6264.72	27,805.45±8951.75	0.558
T2	35,354.73±7449.12 ^A	43,336.64±7896.23 ^A	27,606.55±7324.63 ^B	< 0.001
Microhardness ^c				
ТО	327.66±66.43	321.12 ± 86.05	284.72±94.36	0.437
T1	286.15 ± 59.36	279.49 ± 59.40	244.39 ± 80.24	0.306
T2	335.88±56.65 ^A	331.31±71.86 ^A	215.09±88.24 ^B	0.001

Table 3: Description of the enamel remineralization performance (media \pm SD) of each group through the microhardness test andfluorescence spectroscopy at T0, T1 and T2.

^aANOVA post hoc Tukey. Means followed by different letters are significantly different from each other. ^bArbitrary units. ^cVickers hardness.

2.7.2 Statistical analysis

For each proposed test, all data were first converted to percentages and then analyzed by SPSS 13.0.

The pH values obtained for each sample were initially converted into percentages of pH variation (%pH=[100 $(pH_f - pH_i)/pH_i$]). Then, the data were statistically analyzed.

The inhibition percentage of bacterial adsorption for each assay solution was calculated [26]: 100 [(number of adsorbed cells to enamel in control group – number of adsorbed cells in test group)/number of adsorbed cells in control group)].

The percentage of pH variation was analyzed by ANOVA and Tukey's tests for a 5% significance level. The adsorption inhibition percentage was analyzed by Kruskal-Wallis and Mann-Whitney tests for a 5% significance level.

3 Results

Table 3 describes the results of the microhardness tests and fluorescence spectroscopy for the NSF, NaF and negative control groups at T0, T1 and T2. It was observed that there was a statistically significant difference for all variables in relation to the negative control group.

Figure 3 shows the performance of all the groups at T0, T1 and T2 through the fluorescence (intensity peak and total intensity) and microhardness tests: all the enamel samples were demineralized at T1 and only the NaF and NSF groups were remineralized at T2.

The principle of using a laser beam for diagnosis relies on the fact that an altered mineralized surface irradiated by a longitudinal light wave emits fluorescent radiation. If the emission of light occurs uniformly



Figure 3: Fluorescence spectroscopy in (A) intensity peak, (B) total intensity (in arbitrary units) and (C) microhardness measurements (in Vickers hardness) obtained from dental enamel at TO (before pH cycling), T1 (post-caries induction) and T2 (post-enamel treatment with NaF, NSF and deionized water).



Figure 4: Presentation of the fluorescence in sound enamel deciduous tooth (A) and diamond pyramid indentation for Vickers hardness measurement (B).

on the surface of the sound enamel, it is not possible to observe spots that denounce the presence of caries. In the microhardness test, a diamond pyramidal indentation for Vickers hardness measurement was observed on the enamel surface.

Figure 4 shows a representative image of a sound tooth sample before submitting it to pH cycling, during the fluorescence and microhardness tests.

Figure 5 shows a representative image of a sound tooth sample before submitting it to pH cycling. Note the enamel layer over the dentin and the dark line between them, which represents the dentin-enamel junction (DEJ). An A-scan (yellow curve) is shown, in which



Figure 5: OCT image showing the enamel and dentin layers and also the DEJ. These anatomical structures are confirmed by the A-scan of a selected point, presented in the form of yellow lines, showing the peaks of various tissue layers due to the differences in their refractive indexes.

the tissue interfaces are evidenced by the peaks in the graph.

The OCT analysis was also performed at T0, T1 and T2. As already described in the literature [22, 24], differences can be observed in the teeth submitted to a cariogenic challenge, as shown in Figure 6. For instance, in the T1 images (Figures 6B, E), it is not possible to visualize the DEJ: this is probably due to the higher backscattering of the demineralized enamel, which does not allow light to reach the dentin.

3.1 Acidogenicity

A value of p < 0.001 indicated a statistically significant difference between the groups (means followed by different letters in Table 4 are significantly different from each other). All assay solutions showed a statistically significant difference among them, indicating that both NaF (positive control) and NSF were successful in preventing pH decline when compared to the negative control (deionized water). However, NSF introduced even greater effectiveness in preventing pH decline compared to NaF.

3.2 Bacterial adhesion

Again, the NSF had a higher performance than the positive and negative control solutions to prevent the adhesion of *Streptococcus mutans* to the enamel surface (means followed by different letters in Table 5 are significantly different; p < 0.001). Additionally, no significant difference was observed between the NaF solution and water to prevent bacterial adhesion.



Figure 6: OCT images acquired from representative samples of the NSF group (A, B and C) and negative control (D, E and F) at T0, T1 and T2 phases.

Table 4: Percentage of pH variation (mean±standard deviation) of the samples treated with test and control solutions.

Assay solutions	pH variation (%)	
NSF	$1.73 \pm 2.70^{\circ}$	
NaF	-14.70 ± 0^{h}	
Deionized water	-23.96 ± 1.4	

Means followed by different letters are significantly different from each other through the ANOVA post hoc Tukey's test.

Table 5: Percentage (mean ± standard deviation) of adsorption inhibition of *Streptococcus mutans* AU159 to enamel surface treated with the test and control solutions.

Assay solutions	Bacterial adsorption inhibition (%)	
NSF	88.89±33.33ª	
NaF	38.99±39.38 ^b	
Deionized water	11.11±33.33 ^b	

Means followed by different letters are significantly different from each other through the Kruskal-Wallis and Mann-Whitney tests.

4 Discussion

The mechanism of action of fluoride ions in the remineralization process occurs when there are interactions capable of decreasing the solubility of dental enamel [5, 34, 35]. Fluorine accelerates remineralization by adsorbing itself to the crystal surface attracting calcium and phosphate ions, resulting in a new mineral with a composition between hydroxyapatite and fluorapatite [36]. Fluorine acts as a catalyst by increasing the amount of hydroxyapatite and forming the fluorinated hydroxyapatite when the surface is exposed for at least 1 min to fluorine [34, 36]. To evaluate the mineral alterations, quantitative tests were performed in this study, which allowed the analysis of the deremineralization process in the enamel of the deciduous teeth tested.

NSF was tested as a dental enamel remineralizing agent. It was compared with NaF and a negative control through the microhardness test, fluorescence spectroscopy and OCT imaging.

The microhardness test and the fluorescence spectroscopy were used to evaluate dental enamel demineralization due to their high sensibility and specificity to the detection of minimal mineral changes [15]. Table 3 and Figure 3 show the performance of each group at T0, T1 and T2. It was observed that at phase T2, the NaF and NSF groups presented similar behavior with no significant difference between the remineralization pattern through fluorescence spectroscopy (total intensity) and microhardness test (p > 0.005). This can be explained by the ionic stability of the silver nanoparticles, which do not react when inserted in the various biomaterials [11] with no interference on the fluoride ability to reverse the de-remineralization process observed in the NSF group. Otherwise, the negative control group was not able to promote remineralization in the enamel and presented inferior performance compared to the NSF and NaF groups in both results of the presented tests (fluorescence spectroscopy p < 0.001 and microhardness p = 0.001), with the result expected due to the absence of remineralizing agent.

The OCT presents limitations for study of great depths, for example, in dentin, because a greater scattering can further reduce image quality [23, 37]. However, in this study, the thickness of the enamel was about 1–2 mm, which is quite appropriate for the analysis. Thus, parameters such as central wavelength, bandwidth and optical instrumentation must be adjusted so that the advantages of this system overcome the disadvantages [23].

The OCT images presented in Figure 6 show the light propagation from the enamel surface to the dentin at the phases for the NSF group and the negative control group. All the enamel layer, the enamel/dentin junction and the dentin, in phases T0 (Figure 6A, D) and T2 (Figure 6C), can be observed. In the Figure 6B, E and F, this distinction between layers can no longer be seen. This behavior can be explained by enamel demineralization. In this situation, there is an intense increase in backscattering in this region, so the light does not reach the deeper layers, where the dentine is found. This is justified by the induction of caries in the enamel at phase T1 for all the three groups. This fact corroborates the findings in the literature suggesting that in the presence of caries, there is a greater scattering due to the modification of the scattering properties in carious enamel, which can further reduce image quality at greater depths [23].

Whereas in the NSF and NaF groups, there was a reversal in that backscattering at T2 (Figure 6C), in the negative control group, a lesser reduction of backscattering (Figure 6F) was observed, precisely because this group was not able to remineralize the induced caries lesions.

The findings from the OCT images corroborate the data found from microhardness testing and fluorescence spectroscopy regarding the ability of NSF to remineralize primary tooth enamel. This result becomes especially important in the enamel surface of the deciduous tooth because it is more porous and permeable than the enamel surface of the permanent tooth, which implies a greater susceptibility to demineralization due to the acid action of caries pathogens [14]. However, this increased permeability of the deciduous tooth enamel favors the action of fluorides, which diffuse about 150 times more than in the permanent tooth [25].

In addition to the remineralization capacity, NSF is an antimicrobial agent. In this study, the antimicrobial action

of the silver nanoparticle colloid on Streptococcus mutans in relation to its enamel adhesion and its acid production was tested. Studies have reported the antimicrobial action of silver nanoparticles on Gram-positive and Gram-negative bacteria, showing bactericidal effects on microorganisms, such as Escherichia coli [33, 38, 39], Staphylococcus aureus [38], Enterococcus faecalis [39], and S. mutans [38, 40]. Furthermore, silver nanoparticles-fluoride colloids are potential options for the control of multidrug-resistant microorganisms and do not represent substantial risks to human health [40, 41]. S. mutans is considered the principal microorganism associated with dental caries, and for that reason, it was chosen in this assay. Spherical silver nanoparticles have bactericidal action on S. mutans, and that action depends on the particle size: the smaller the diameter, the lower the minimum inhibitory concentration for the microorganism [42]. Concentrations of 4.86±2.71 μ g/ml of silver nanoparticles, with an average size of 25 nm, are sufficient to inhibit the growth of *S. mutans* [43]. The action of NSF on the formation of biofilms containing S. mutans was assessed in vitro, and it was observed that, compared to SDF, NSF prevented biofilm formation on tooth enamel, confirming its antimicrobial potential even in lower concentrations than the SDF [9, 38].

The results corroborated those findings. The concentration of NSF was 400 ppm with a particle size of 8.7 ± 3.1 nm, which was capable of showing antimicrobial action. It is suggested that the inhibitory power of the silver ions is due to their interaction with thiol groups of the protein and phospholipid portion of bacterial membrane [44], since silver ions have a large chemical affinity for compounds containing nitrogen, sulfur and phosphorus. When formed into nanoparticles, silver ions interact more strongly with other organic and inorganic molecules due to their larger surface area [45]. Thus, silver nanoparticles also act on bacterial membranes by altering their permeability and causing rupture, and, inside the cell, interact with nucleic acids, which likely prevents the replication process [41-43]. Therefore, these mechanisms of action may interfere with bacterial metabolism, including adhesion and acidogenicity.

The NSF prevented bacterial adhesion to the sound enamel, and its effectiveness was statically significantly higher than NaF. The percentage of adsorption inhibition of NSF adhesion was more than double that presented by NaF, indicating its greater power in preventing bacterial adhesion. This fact may be related to the ability of silver ions to present bacterial biofilm formation [39] and because the synthesis of glucans by the bacteria is responsible for their adhesion to the enamel surface [10], perhaps that synthesis could be impaired by NSF. Furthermore, chitosan used as a stabilizing agent of the colloid [28, 46] also has antimicrobial properties [28, 47, 48], including inhibition of *Streptococcus mutans* adhesion to the enamel [49, 50], which may confer a synergistic effect.

Fluoride causes inhibition of bacterial activity [51] and acid production of *Streptococcus mutans* [40]. In this study, acid production of *S. mutans* AU159 was inhibited by NSF, but NaF did not show efficacy by inhibiting bacterial acid production. Samples treated with NaF showed a negative percentage of pH variation, meaning that all the samples showed a drop in the pH of the medium because the NaF was not able to inhibit bacterial acid production. The samples treated with NSF showed a positive percentage of pH variation, increasing on average the pH of the medium from 6.6 to 6.8, indicating that the colloid could prevent acid production by *S. mutans*.

Clinical development of dental caries involves the simultaneous demineralization and remineralization, and the effect of fluorides in those processes is well known. Zhi et al. [51] reported that silver ions (non-nanoparticles) may increase mineral density during the remineralization of enamel caries. However, silver ions have little effect on the prevention of enamel demineralization, unlike fluorides, which are deposited on the enamel surface as CaF, which is inclined for remineralization [41]. This study focused on investigating silver nanoparticles and the effect of fluoride-containing colloids on preventing the formation of enamel demineralization damage, which also differs from the studies mentioned above because the enamel blocks were submitted to a pH cycling challenge. Hence, the results showed a similar effect (nonstatistically significant difference) of both NSF and NaF on enamel demineralization even though the NaF presented a lower percentage of microhardness variation. All enamel blocks were submitted to a pH challenge for 14 days, and all the samples had some demineralization, but NSF and NaF avoided higher demineralization, such as the negative control-treated samples. Furthermore, NSF-treated samples did not show black stains like SDF does [9]. Since silver particles were reduced to nanometer size, their chemical reactivity was altered and the colloid was not able to stain the teeth black. Chitosan may have acted as an adjuvant in preventing enamel mineral loss during pH cycling because it can positively affect the in vitro enamel demineralization, preventing the loss of ions, especially P, from hydroxyapatite [50]. These results are a satisfactory outcome because NaF is considered the reference standard for the prevention of enamel demineralization. NSF is a potential agent to be used in public health programs because it has great advantages over SDF and

better action than NaF on the virulence factors of *Streptococcus mutans* as analyzed in this study.

5 Conclusions

Microhardness, fluorescence spectroscopy and OCT tests indicated that NSF is as efficient as NaF for decidous enamel remineralization. Besides that, NSF presented higher interference on *Streptococcus mutans* AU159 adhesion to the enamel surface and higher inhibition on *S. mutans* AU159 acidogenicity than NaF. Therefore, NSF may be more effective than conventional fluorides for incipient caries lesion treatment due to its remineralization and antibacterial actions.

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References

- Nakornchai S, Atsawasuwanb P, Kitamurac E, Surarit R, Yamauchi M. Partial biochemical characterisation of collagen in carious dentin of human primary teeth. *Arch. Oral. Biol.* 2004, 49, 267–273.
- [2] World Health Organization. Available in: http://www.who.int/ oral_health/policy/en/. Access on July, 23, 2017.
- [3] Ten Cate JM, Duijsters PPE. Alternating demineralisation and remineralisation of artificial enamel lesions. *Caries Res.* 1982, 16, 201–210.
- [4] Cury JA, Tenuta LMA. Enamel remineralization: controlling the caries disease or treating early caries lesions? *Braz. Oral. Res.* 2009, 23, 23–30.

- [5] Robinson C, Shore RC, Brookes SJ, Strafford S, Wood SR, Kirkham J. The chemistry of enamel caries. *Crit. Rev. Oral Biol. Med.* 2000, 11, 481–495.
- [6] Rosin-Grget K, Peros K, Sutej I, Basic K. The cariostatic mechanisms of fluoride. Acta Med. Acad. 2013, 42, 179–188.
- [7] Tenuta LM, Cury JA. Fluoride: its role in dentistry. Braz. Oral Res. 2010, 24, 9–13.
- [8] Ijaz S, Croucher RE, Marinho VC. Systematic reviews of topical fluorides for dental caries: a review of reporting practice. *Caries Res.* 2010, 44, 579–592.
- [9] Santos Jr VE, Vasconcelos Filho A, Targino AGR, Flores MAP, Galembeck A, Caldas Jr AF, Rosenblatt A. A new "silver-bullet" to treat caries in children – nano silver fluoride: a randomised clinical trial. J. Dent. 2014, 42, 945–951.
- [10] Targino AGR, Flores MAP, Santos Jr VE, Bezerra FGB, Freire HL, Galembeck A, Rosemblatt A. An innovative approach to treating dental decay in children. A new anti-caries agent. J. Mater. Sci. Mater. Med. 2014, 25, 2041–2047.
- [11] Noronha VT, Amauri JP, Durán G, Galembeck A, Cogo-Müller K, Franz-Montan M, Durán N. Silver nanoparticles in dentistry. *Dent. Mater.* 2017, 33, 1110–1126.
- [12] Singh R, Shedbalkar UU, Wadhwani SA, Chopade BA. Bacteriagenic silver nanoparticles: synthesis, mechanism, and applications. *Appl. Microbiol. Biotechnol.* 2015, 99, 4579–4593.
- [13] Le Ouay B, Stellacci F. Antibacterial activity of silver nanoparticles: a surface science insight. *Nano Today* 2015, 10, 339–354.
- [14] Oliveira, MAHM, Torres, CP, Gomes-Silva, JM, Chinelatti, MA, Menezes, FCH, Palma-Dibb, RG, Borsatto, MC. Microestruture and mineral composition of. dental enamel of permanent and deciduous teeth. *Microsc. Res. Tech.* 2010, 73, 572–577.
- [15] Gutiérrez-Salazar MP, Reyes-Gasga J. Microhardness and chemical composition of human tooth. *Mat. Res.* 2003, 6, 367–373.
- [16] Cara ACB, Zezell DM, Ana PA, Maldonado EP, Freitas AZ. Evaluation of two quantitative analysis methods of optical coherence tomography for detection of enamel demineralization and comparison with microhardness. *Lasers Surg. Med. Sci.* 2014, 46, 666–671.
- [17] Rodrigues JA, Diniz MB, Josgrilberg EB, Cordeiro RL. *In vitro* comparison of laser fluorescence performance with visual examination for detection of occlusal caries in permanent and primary molars. *Lasers Med. Sci.* 2009, 24, 501–506.
- [18] Mualla. SK. Fluorescence and dentistry. IOSR J. Dent. Med. Sci. 2016, 15, 65–75.
- [19] Maia AMA, Freitas AZ, Campello SL, Gomes ASL, Karlsson L. Evaluation of dental enamel caries assessment using quantitative light induced fluorescence and optical coherence tomography. J. Biophotonics 2016, 9, 596–602.
- [20] Yong-Keun L. Fluorescence properties of human teeth and dental calculus for clinical applications. J. Biomed. Opt. 2015, 20. doi: 10.1117/1.JBO.20.4.040901.
- [21] Carvalho FB, Barbosa AFS, Zanin FAA, Brugnera Jr A, Silveira Jr L, Pinheiro ALB. Use of laser fluorescence in dental caries diagnosis: a fluorescence x biomolecular vibrational spectroscopic comparative study. *Braz. Dent. J.* 2013, 24, 59–63.
- [22] Amaechi BT. Emerging technologies for diagnosis of dental caries: the road so far. J. Appl. Phys. 2009, 105, 102–104.
- [23] Schneider H, Park KJ, Häfer M, Rüger C, Schmalz G, Krause F, Ziebolz JSD, Haak R. Dental applications of optical coherence tomography (OCT) in cariology. *Appl. Sci.* 2017, 7, 472.

- [24] Mota CCBO, Gueiros LA, Maia AMA, Santos-Silva AR, Gomes ASL, Alves FA, Leao JC, Freitas AZ, Goes M, Lopes MA. Optical coherence tomography as an auxiliary tool for the screening of radiation-related caries. *Photomed. Laser Surg.* 2013, 31, 301–306.
- [25] Low LM, Duraman N, Mahmood U. Mapping the structure, composition and mechanical properties of human teeth. *Mater. Sci. Eng. C* 2008, 28, 243–247.
- [26] Bin Ahmad M, Lim JJ, Shameli K, Ibrahim NA, Tay MY. Synthesis of silver nanoparticles in chitosan, gelatin and chitosan/gelatin bionanocomposites by a chemical reducing agent and their characterization. *Molecules* 2011, 16, 7237–7248.
- [27] Wei D, Sun W, Qian W, Ye Y, Ma X. The synthesis of chitosanbased silver nanoparticles and their antibacterial activity. *Carbohydr. Res.* 2009, 344, 2375–2382.
- [28] Freire PLL, Albuquerque AJR, Farias IAP, Silva TG, Aguiar JS, Galembeck A, Flores MAP, Sampaio FC, Stamford TCM, Rosemblatt A. Antimicrobial and cytotoxicity evaluation of colloidal chitosan – silver nanoparticles – fluoride nanocomposites. *Int. J. Biol. Macromol.* 2016, 93(A), 896–903.
- [29] Alexandria AKF, Valença AMG, Lima SJG, Nóbrega CBC, Lima AL, Claudino LV. In vitro evaluation of the demineralization of bovine enamel subjected to pH and immersion time variations in caries-inducing solution. *Pesq. Bras. Odontoped. Clin. Integr. João Pessoa, maio/ago* 2008, 8(2), 233–238.
- [30] Stookey GK, Featherstone JDB, Rapozo-Hilo M, Schemehorn BR, Williams RA, Baker RA, Braker ML, Kaminski MA, McQueen CM, Amburgey JS, Casey K, Faller RV. The Featherstone laboratory pH cycling model: a prospective, multisite validation exercise. Am. J. Dent. 2011, 24, 322–328.
- [31] Vieira AEM, Delben ACB, Sassaki KT, Rodrigues E, Cury JA, Cunha RF. Fluoride dose response in pH-cycling models using bovine enamel. *Caries Res.* 2005, 39, 514–520.
- [32] Mota CCBO, Fernandes LO, Cimões R, Gomes ASL. Non-invasive periodontal probing through Fourier-domain optical coherence tomography. J. Periodontol. 2015, 86, 1087–1094.
- [33] Kim B, Park S, Kim M, Kim Y, Lee S, Lee K, Choi N, Lee Y, Lee Y, You Y. Inhibitory effects of Chrysanthemum boreale essencial oil on biofilm formation and virulence factor expression of Streptococcus mutans. *Evid. Based. Complement. Alternat. Med.* 2015, 2015, 1–11.
- [34] Garcia-Godoy F, Hicks MJ. Maintaining the integrity of the enamel surface: the role of dental biofilm, saliva and preventive agents in enamel demineralization and remineralization. J. Am. Dent. Assoc. 2008, 139(Suppl), 25s–34s.
- [35] Gao SS, Zhang S, Mei ML, Lo ECM, Chu CH. Caries remineralisation and arresting effect in children by professionally applied fluoride treatment – a systematic review. *BMC Oral Health* 2016, 16, 12.
- [36] Featherstone JD. The science and practice of caries prevention. J. Am. Dent. Assoc. 2000, 131, 887–899.
- [37] Ishida S, Nishizawa N. Quantitative comparison of contrast and imaging depth of ultrahigh-resolution optical coherence tomography images in 800–1700 nm wavelength region. *Biomed. Opt. Express* 2012, 3, 282–294.
- [38] Stamford TCM, Pereira DMS, Alcântara LC, Couto GBL. Microbiological and biochemical parameters and their relationship with carie in healthy teenagers. *Rev. Bras. Saúde Matern. Infant.* 2005, 5, 71–76.

- [39] Pandit S, Cai JN, Jung JE, Jeon JG. Effect of 1-minute fluoride treatment on potencial virulence and viability of a cariogenic biofilm. *Caries Res.* 2015, 49, 449–457.
- [40] Guillen AJP, Tapia-Perez H, Castanon GM. The antimicrobial sensitivity of Streptococcus mutans to nanoparticles of silver, zinc oxide, and gold. *Nanomed. Nanotechnol. Biol. Med.* 2008, 4, 237–240.
- [41] Allaker RP. The use of nanoparticles to control oral biofilm formation. *J. Dent. Res.* 2010, 89, 1175–1186.
- [42] Martinez-Castañon GA, Nino-Martinez N, Martinez-Guierrez F, Martinez-Mendoza JR, Ruiz F. Synthesis and antibacterial activity of silver nanoparticles with different sizes. J. Nanopart. Res. 2008, 10, 1343–1348.
- [43] Eby DM, Schaeublin NM, Farrington KE, Hussain SM, Johnson GR. Lysozyme catalyzes the formation of antimicrobial silver nanoparticles. ACS Nano 2009, 3, 984–994.
- [44] Panacek A, Kvítek L, Prucek R, Kolar M, Vecerova R, Pizúrova N, Sharma VK, Nevecna T, Zboril R. Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J. Phys. Chem. B 2006, 110, 16248–16253.
- [45] Duran N, Marcato PD, De Conti R, Alves OL, Costa FTM, Brocchi M. Potential use of silver nanoparticles on pathogenic bacteria,

their toxicity and possible mechanisms of action. J. Braz. Chem. Soc. 2010, 21, 949–959.

- [46] Wang B. Microwave-assisted synthesis of silver nanoparticles in alkalic carboxymethyl chitosan solution. *Engineering* 2010, 2, 387–390.
- [47] Chavez de Paz LE, Resin A, Howard KA, Sutherland DS, Wejse PL. Antimicrobial effect of chitosan nanoparticles on Streptococcus mutans biofilms. *Appl. Environ. Microbiol.* 2011, 77, 3892–3895.
- [48] Dash M, Chiellini F, Ottenbrite RM, Chiellini E. Chitosan a versatile semi-synthetic polymer in biomedical applications. *Prog. Polym. Sci.* 2011, 36, 981–1014.
- [49] Arnaud TMS, Barros Neto B, Diniz FB. Chitosan effect on dental enamel de-remineralization: an in vitro evaluation. J. Dent. 2010, 38, 848–852.
- [50] Costa EM, Silva S, Madureira AR, Cardelle-Cobas A, Tavaria FK, Pintado MM. A comprehensive study into the impact of a chitosan mouthwash upon oral microorganism's biofilm formation in vitro. *Carbohydr. Polym.* 2014, 10, 1081–1086.
- [51] Zhi QH, Lo ECM, Kwok ACY. An in vitro study of silver and fluoride ions on remineralization of demineralized enamel and dentine. *Australian Dent. J.* 2013, 58, 50–56.