

# Influence of cigarette smoke combined with different toothpastes on enamel erosion

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**Abstract:** This in vitro study aimed to evaluate the effect of different toothpastes on dental enamel subjected to an erosive cycle with and without exposure to cigarette smoke. Bovine enamel specimens were randomly allocated into 12 groups (n = 12). For the in vitro simulation of smoking, half the groups underwent an exposure cycle of 20 cigarettes per day for 5 days. Subsequently, all groups were subjected to a 5-day erosion cycle intercalating demineralization (1 min; 1% citric acid; pH = 3.5) and treatment with toothpaste slurries (2 min) of NaF, SnF<sub>2</sub>, F/Sn/Chitosan, F/CaSiO<sub>3</sub>/Na<sub>3</sub>PO<sub>4</sub>, and F/bioactive glass. The control group was immersed in distilled water. Surface microhardness (SMH) was measured initially, after exposure to smoke, and after the erosive cycle, and %SMH was calculated. At the end of the experimental cycle, surface roughness, profilometry, and atomic force microscopy (AFM) were performed. SMH increased after exposure to cigarette smoke (p < 0.05). After the erosive cycle, there were no differences between the presence and absence of cigarette smoke exposure in SMH and roughness (p > 0.05). Besides increasing enamel SMH, cigarette smoke did not prevent enamel loss after the erosion cycle (p < 0.05). In profilometry, roughness and surface loss had the lowest values in the groups treated with SnF<sub>2</sub> and F/Sn/Chitosan (p < 0.05). AFM showed lower mineral loss with F/CaSiO<sub>3</sub>/Na<sub>3</sub>PO<sub>4</sub> and F/Sn/Chitosan. For all groups, except F/CaSiO<sub>3</sub>/Na<sub>3</sub>PO<sub>4</sub>, cigarette smoke resulted in higher enamel wear. F/Sn/Chitosan showed the best results against erosion.

**Keywords:** Tooth Erosion; Toothpastes; Dental Enamel; Smoking.

## Introduction

Dental erosion is a condition in which non-bacterial acids cause demineralization of dental hard tissues.<sup>1</sup> Although acid contact is the main cause of erosion, its development involves a complex interaction among biological, chemical, and behavioral factors in each patient.<sup>1</sup> A common behavioral factor is smoking. The smoking by itself does not cause dental erosion, often associated with consumption of acidic beverages<sup>1,2</sup> that influences the development of dental erosion.<sup>1</sup>

Smoking cigarettes is often accompanied with prolonged consumption of acidic drinks, such as alcoholic beverages<sup>2</sup>. Excessive alcohol intake is associated with a high prevalence of erosion because of the direct effect



of alcohol and its association with regular vomiting or alcohol-induced gastroesophageal reflux.<sup>3</sup> Tobacco is commonly consumed by adolescents with eating disorders,<sup>3</sup> and it inhibits appetite in people with anorexia nervosa.<sup>3</sup> As smoking is frequently associated with episodes of low pH, studies evaluating this association are necessary to determine the effect of smoking on the severity of dental erosion.

During smoking, teeth are exposed to hot smoke from tobacco combustion. The temperature of tobacco pyrolysis and cigarette combustion is 300°C–1,000°C.<sup>4</sup> This hot smoke can heat the dental enamel, at high temperatures causing changes in the arrangement and morphology of the hydroxyapatite crystals.<sup>5</sup> In addition, the components of cigarette smoke result in accumulation of contaminants on the calcified tissues and restorative materials.<sup>6</sup> Although the effects of cigarette smoke on enamel are known, no studies have assessed the impact of these changes on the susceptibility to dental erosion.

As a therapeutic measure for eroding dental surfaces, their resistance to erosion can be increased, either by modifying the crystalline structure, making them less soluble, or by providing protective organic layers on dental tissues.<sup>7</sup> Toothpastes are the ideal therapy for eroded dental tissues.<sup>8</sup> The action of toothpastes is mainly associated with fluoride, which precipitates a  $\text{CaF}_2$  layer on demineralized dental tissues. Although partially, this can reduce enamel susceptibility to demineralization.<sup>9</sup>  $\text{CaF}_2$  precipitates may act as a barrier on dental structures, preventing their direct contact with acids.<sup>10</sup> However, under conditions promoting erosion, such as low pH, this barrier is not stable against erosive wear.<sup>7</sup> Due to the limited efficacy of fluoride in such cases, the treatment and prevention of dental erosion should combine it with other active agents.<sup>7</sup> The compounds of fluoride with metal cations, such as  $\text{SnF}_2$ , prevent or decrease tissue loss during acidic events.<sup>11</sup> Alternatively, biopolymers, such as chitosan,<sup>12</sup> combined with  $\text{SnF}_2$  can be used. Recently, commercially available bioactive glass (e.g.  $\text{CaNaO}_6\text{PSi}$ ) and the addition of calcium minerals (e.g.  $\text{CaSiO}_3$ ) to fluoride-containing toothpastes have prevented and treated dental erosion.<sup>7</sup>

There is no evidence that the effects of cigarette smoking on enamel may influence erosion and may

be treated with toothpastes. Therefore, this in vitro study aimed to evaluate the effect of different active compounds associated with fluoride on dental enamel with or without cigarette smoke, and submitted to cyclic episodes of enamel erosion.

## Methodology

### Experimental design

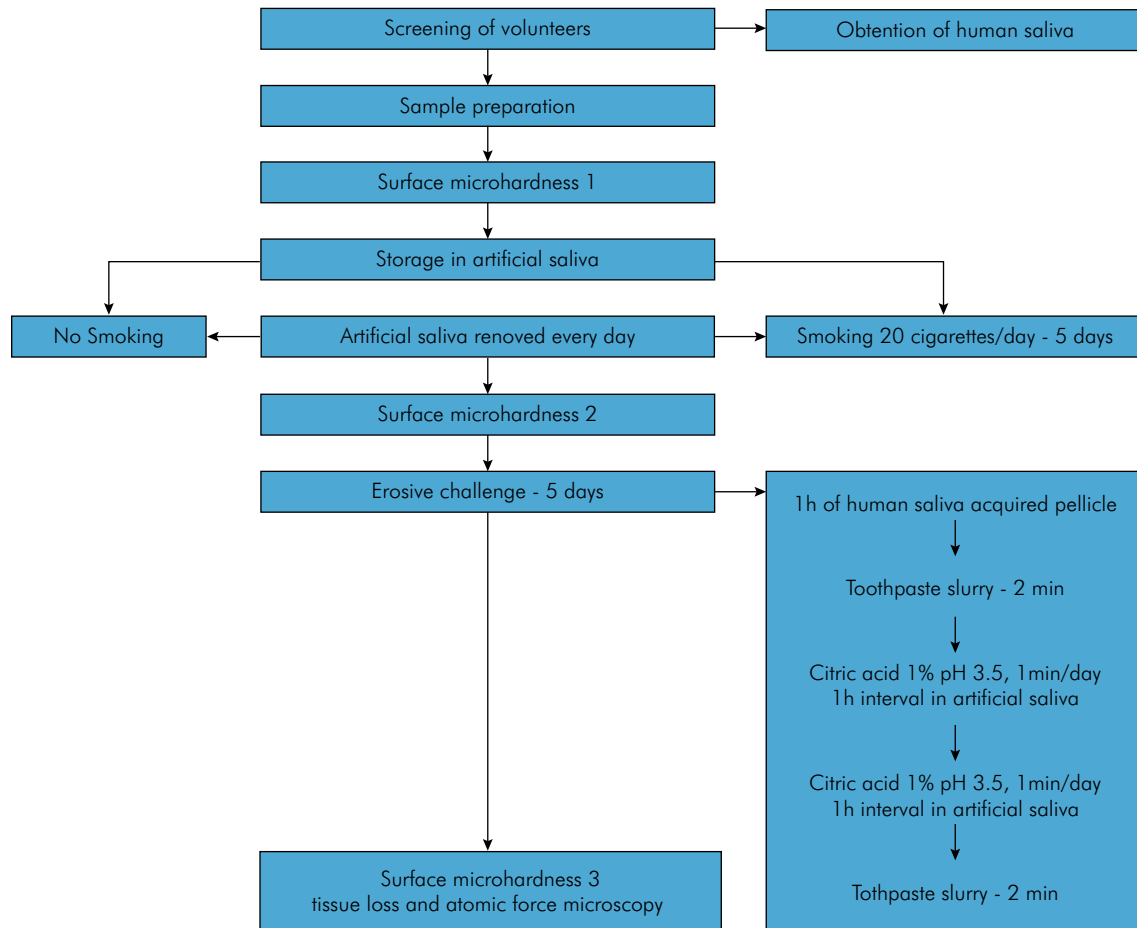
This in vitro study tested the factors cigarette smoke (2 levels: with or without cigarette smoke) and toothpastes (6 levels: Distilled Water, NaF,  $\text{SnF}_2$ , F/Sn/chitosan, F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$  and F/bioactive glass). Figure 1 shows the study design including experimental processes. Bovine enamel specimens were used and randomly allocated into 12 groups ( $n = 12$ ): distilled water, NaF,  $\text{SnF}_2$ , F/Sn/Chitosan, F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$ , and F/bioactive glass groups, each with and without cigarette smoke. The sample size was based on literature.<sup>13</sup> Human saliva was used for pellicle formation. The analyses were of surface microhardness (SMH), tested thrice (SMH1, baseline; SMH2, after exposure to cigarette smoke or not; SMH3, after erosive treatment), surface roughness (Ra), surface profilometry, and atomic force microscopy (AFM). Table 1 shows toothpaste information.

### Volunteers and ethical aspects

This study was conducted according to the Declaration of Helsinki and approved by the local ethics committee in research (process N<sup>o</sup>: 55288216.5). The participants were 12 volunteers (6 men and 6 women), 23–35 years old, who signed a consent form. The volunteers fulfilled the inclusion criteria (normal salivary flow, absence of caries and periodontal disease, healthy or sufficiently restored dentition, and adequate oral hygiene) without violating the exclusion criteria (use of orthodontic devices or prostheses and drugs interfering with salivary flow, smoking, pregnancy or lactation, allergy to oral hygiene products, dental materials, or therapeutic agents used in the study).

### Saliva collection

All the natural saliva was collected from the volunteers before the experiment. Oral hygiene



**Figure 1.** Flow diagram of the experimental process.

measures and breakfast were prohibited. Salivary flow was stimulated by chewing paraffin wax (Parafilm M, American National Can, Chicago, USA), and saliva was collected in falcon tubes inside a beaker filled with ice blocks. Subsequently, a salivary pool was made from the volunteers, which was clarified by centrifugation (JOUAN MR23i Benchtop High Speed Centrifuge Thermo Scientific MR23i, Waltham, USA) at 3.800 g for 10 min at 4°C. The precipitate was discarded, and the supernatant was stored in a freezer at -80°C. This was performed for 3 days to obtain 800 mL of saliva. For pellicle formation, aliquots of saliva were daily thawed at room temperature and mixed before use.

### Specimen preparation

A total of 144 enamel/dentin specimens (4 × 4 mm) were obtained from bovine incisors

stored in 0.1% thymol solution, using a precision saw (Isomet 1000; Buehler, IL, USA) and diamond disc (Buehler, USA). The enamel surface was planned and flattened using silicon carbide papers (500-, 1000-, and 2000- SiC; Buehler, USA) and felts (TCT, TWI, and FVC; Arotec, Cotia, Brazil) coupled with a diamond paste (6, 3, and 0.75 µm, Buehler, USA) in a polishing machine, under water cooling (Arotec, São Paulo, SP, Brazil). Subsequently, the specimens were placed in an ultrasonic machine for 10 min (Marconi, Piracicaba, Brazil) to remove residual particles and smear layers and obtain a standardized enamel surface. Finally, the specimens contained enamel and dentin, each 1 mm thick. The specimen surfaces, except the enamel surface, were protected with acid-resistant varnish (Risqué Colorless, Taboão da Serra, Brazil). Prior to (24 h) and during the experiment, the prepared specimens

**Table 1.** Products, manufacturers, and components of toothpaste.

Toothpaste	Manufacturer	Active agent	Fluoride agent	Other components
Colgate Total 12™ (NaF)	Colgate-Palmolive, São Bernardo do Campo, Brazil	-	Sodium fluoride (NaF) 1450 ppm	Water, Triclosan, Sorbitol, Hydrated Silica, Sodium Lauryl Sulfate, PVM/MA Copolymer, Flavor Carrageenan, Sodium Hydroxide, Sodium Saccharin, Titanium dioxide, and Dipentene.
Crest™ Pro Health™ Advanced (SnF <sub>2</sub> )	Procter & Gamble, Cincinnati, Ohio, United States	-	Stannous fluoride (SnF <sub>2</sub> ) 0,454%	Glycerin, Hydrated Silica, Sodium Hexametaphosphate, Propylene Glycol, PEG 6, Water, Zinc Lactate, Trisodium Phosphate, Flavor, Sodium Lauryl Sulfate, Sodium Gluconate, Carrageenan, Sodium Saccharin, Polyethylene, Xanthan Gum, Mica, Titanium Dioxide, Blue 1
Elmex™ Erosion Protection™ (F/Sn/chitosan)	GABA International AG, Grabatsmattweg, Switzerland	Chitosan (0.5%), Stannous Chloride	Amine Fluoride (AmF) 700 ppm, Sodium Fluoride (NaF) 700 ppm	Glycerin Hydrated, Silica, Sodium hexametaphosphate, Propylene glycol, PEG-6, Eau, Zinc lactate, trisodium phosphate, flavor, Sodium lauryl sulfate, Polyethylene, Sodium, Gluconate, Carrageenan, Sodium saccharin, Xanthan gum, Titanium dioxide, red 40 aluminium lake
Regenerate™ Enamel Science (F/CaSiO <sub>3</sub> /Na <sub>3</sub> PO <sub>4</sub> )	Unilever France HCL, Le Meux, France	Calcium Silicate, Sodium Phosphate	Sodium Monofluorophosphate (MFP) 1450 ppm	Glycerin, PEG-8, Hydrate Silica, Trisodium Phosphate, Water, PEG-60, Sodium Lauryl Sulfate, Aroma, Synthetic Fluorophlogopite, Sodium Saccharin, Polyacrylic Acid, Limonene, Tin Oxide
Sensodyne™ Repair & Protect Novamin™ Technology (F/bioactive glass)	SmithKline Beecham Consumer Healthcare, Berkshire, United Kingdom	5% Calcium Sodium Phosphosilicate	Sodium Monofluorophosphate (MFP) 1426 ppm	Glycerin, silica, PEG-8, Titanium Dioxide, Carbomer, Cocamidopropyl Betaine, Sodium Methyl Cocoyl Taurate, Sodium Saccharin, d-limonene

were stored in a 37°C incubator and artificial saliva, which was renewed every day. The artificial saliva contained 1.5 mM of Ca, 0.9 mM of PO<sub>4</sub>, and 150 mM of KCl, in 20 mM of Tris pH 7.0<sup>14</sup>.

### Exposure to cigarette smoke

The specimens from 6 groups were exposed to cigarette smoke, while the others were stored in artificial saliva. The in vitro simulation of smoking was performed in a smoke machine (registration no. 01810012043 INPI; National Institute of Industrial Property). Each specimen was exposed to 20 cigarettes (Marlboro, Philip Morris Brazil Ind and Com, RS, Brazil) per day for 5 days<sup>15</sup>. The cycle was performed with intervals of 3 s, which simulates the aspiration usually performed by a smoker. A temporizer allowed ambient air to be inhaled every 10 s, simulating smoke exhaustion and subsequent disposal. In the interval between 2 simulations, the specimens were stored in artificial saliva at 37°C.

### Erosive challenge

This erosive cycle simulated the erosion process in the oral cavity, intercalating citric acid demineralization (1% citric acid; 3.5 pH)<sup>16</sup> and treatment with toothpaste slurry.<sup>17</sup> Half the surface area of the specimens were protected with a layer of acid-resistant varnish (Risque Colorless) for reference to allow the analysis of surface profilometry after the erosive cycle.

The erosion cycling model was of 5 days.<sup>17</sup> Before the 1<sup>st</sup> erosive challenge of each day, the specimens were immersed individually for 1 h in human saliva at 37°C under agitation (100 rpm) and subsequently in the respective erosive solutions for 1 min, 4 times daily,<sup>17</sup> with 1 h of interval. Citric acid was handled daily and renewed at each erosion episode. Before the 1<sup>st</sup> and after the last erosive challenge of each day, the specimens were immersed in the respective treatment slurries (1:3 ratio of toothpaste to distilled water) for 2 min. The treatments were performed

under agitation (100 rpm) at room temperature. The slurries were handled immediately before use. Between the erosive challenges (1 h) and after the daily cycle, the specimens were stored in artificial saliva at 37°C overnight (20 h). Before and after immersing the specimens in the erosive solution, slurries, and artificial saliva, they were washed with purified water for 30 s and dried. The amount of each solution (erosive, toothpaste slurry, and saliva) was the same per specimen (2.5 mL/mm<sup>2</sup> of exposed enamel). Artificial saliva was changed daily.

### Microhardness analysis

SMH was measured to assess the enamel characteristics after the challenges (smoking and erosion/treatment). It was analyzed at three times: baseline (SMH1), after exposure to cigarette smoke (SMH2) and after the end of the erosion cycle (SMH3). A Knoop indenter, loaded with 50 g, with indentation time of 5 s, was used in a microhardness tester (HMV-2000 Shimadzu, Tokyo, Japan). Three indentations, 100 µm apart, were made at the center of the enamel surface at different reading times. The mean values of the measuring points were determined (µm), and the SMH loss (%SMH) was calculated using the following formula: %SMH = 100 [(SMH<sub>t</sub> - SMH1)/SMH1], where "t" is the time taken to calculate %SMH. SMH2 was substituted for %SMH1 (loss after cigarette smoke) and SMH3 for %SMH2 (loss after the erosive cycle). Higher values of %SMH indicated higher enamel surface loss.

### Tissue loss measurement

After the last experimental day, the tissue loss was analyzed by surface profilometry (Veeco DEKTAK 150, Veeco, NY, USA). The roughness was measured in the area subjected to erosion and reference area, for which the protective coating was removed. At the center of each specimen, 3 traces were made at intervals of 0.2 mm, each 2 mm long (1 mm each in the reference and experimental areas). The minimum level detectable by the equipment is 4 Å. Two regression lines were constructed for each trait, 1 each in the reference and experimental areas. The vertical distance between the regression lines was

defined as tissue loss (µm). The value per specimen was the mean of the 3 measurements.

### Surface roughness (Ra) and Atomic Force Microscopy

Ra was evaluated using a rugosimeter (SV-3100S4 - Mitutoyo, Tokyo, Japan) coupled with an atomic force microscope (Easy Scan 2, Nano surf, Boston, USA). The precision was 0.01 µm, with a cut-off value of 0.25 mm, reading length of 5 times the cut-off value (1.25 mm), and mean velocity of 0.1 mm/s. The specimens were placed parallel to the equipment surface and marked with 3 equidistant points at the center to guide the reading. The readings were performed at 180°, 135°, and 90°. The atomic force microscope (Easy Scan 2, Nano surf, Boston, USA) was operated in the tapping mode, with a constant of 31–71 N/m, wavelength of 225 µm, and resonance frequency of 160–210 KHz. 3D topographic and lock-in phase images (15 × 15 µm) were obtained using a profilometer mounted on the microscope. Specimens from all groups were analyzed. For quantitative analysis of the surface, the mean of the 3 readings was the final Ra. For the qualitative analysis, 1 topographic image and representative lock-in phase images were randomly chosen for samples of each group. The images were processed using the Gwyddion software (Gwyddion 2.29, GNU General Public License).

### Statistical analysis

SMH and Ra data were analyzed using mixed models for repeated measures analysis of variance (ANOVA) and the Tukey-Kramer test by the PROC MIXED procedure of the SAS program. %SMH1 data did not meet the ANOVA assumptions and were analyzed using generalized linear models. Contrarily, SMH2% data met the assumptions and were analyzed by two-way ANOVA and the Tukey's test. %SMH data were compared to Tukey's. Surface profilometry data indicated a logarithmic transformation to meet the assumptions of a parametric analysis. After the transformation, for these and Ra data were also submitted to ANOVA in 2 X 6 (smoke X treatment with toothpastes) factorial scheme and Tukey's test. The analyses had a significance level of 5%.



## Results

Regarding SMH (Table 2), triple interaction among the factors (treatment, smoke, and time) was significant ( $p = 0.0429$ ) and therefore deployed to compare the levels of the 3 factors. In SMH2, groups exposed to cigarette smoke were significantly different from those that were not ( $p < 0.05$ ). In the smoking groups, SMH2 was significantly higher than SMH1 ( $p < 0.05$ ). Irrespective of the treatment, all groups showed significant reduction from SMH1 and SMH2 to SMH3 ( $p < 0.05$ ). All toothpastes showed significant differences compared to distilled water ( $p < 0.05$ ). Among the toothpastes, NaF showed the lowest SMH values ( $p < 0.05$ ). No difference was found between  $\text{SnF}_2$  and F/bioactive glass ( $p > 0.05$ ). F/Sn/chitosan and F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$  showed the highest SMH, without significant differences between them ( $p > 0.05$ ). %SMH2 was statistically different from %SMH1, after higher enamel loss was found from erosive challenge ( $p < 0.05$ ). %SMH1 did not significantly differ among treatments ( $p > 0.05$ ) but did between presence and absence of cigarette smoke exposure ( $p < 0.05$ ). %SMH2 was the same as %SMH3 in toothpaste effectiveness and significantly differed between

presence and absence of cigarette smoke exposure for Sn-based toothpastes ( $p < 0.05$ ). When exposed to cigarette smoke,  $\text{SnF}_2$  showed lower %SMH and F/Sn/chitosan showed higher %SMH.

In profilometry (Table 3), interaction between the factors (treatment and smoke) was not significant ( $p = 0.1035$ ). Exposure to cigarette smoke resulted in higher tissue loss (~10%) irrespective of the toothpaste slurry ( $p < 0.05$ ), except for F/ $\text{CaCO}_3/\text{Na}_3\text{PO}_4$ , in which cigarette smoke resulted in less tissue loss. All treatment groups significantly differed from the distilled water group ( $p < 0.05$ ). NaF, F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$ , and F/bioactive glass had no differences ( $p > 0.05$ ), with 20–30% of tissue loss reduction, compared to distilled water.  $\text{SnF}_2$  and F/Sn/chitosan showed the lowest values of tissue loss, with a tissue loss reduction of 78% ( $p < 0.05$ ).

Regarding surface roughness interaction between the factors (treatment and smoke) was not significant ( $p = 0.3244$ ). The exposure to smoking did not affect the Ra values ( $p > 0.05$ ) (Table 3). All treatment groups were statistically different from the distilled water group ( $p < 0.05$ ). NaF and F/bioactive glass showed the lowest values ( $p < 0.05$ ). F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$  showed intermediate values, while  $\text{SnF}_2$  and F/Sn/chitosan showed the lowest mean Ra ( $p < 0.05$ ).

**Table 2.** Results (mean  $\pm$  standard deviation) of surface microhardness (SMH) and %SMH based on treatment and time.

Cigarette smoke	Treatment	Time			%SMH1	%SMH2
		SMH1	SMH2	SMH3		
Without	Distilled Water	316.07 $\pm$ 10.0 Aa	*320.55 $\pm$ 9.7 Aa	79.41 $\pm$ 3.9Bd	*1.42 (0.46) Ba	-74.87 (1.18) Ad
	NaF	316.19 $\pm$ 10.5 Aa	*318.98 $\pm$ 10.8 Aa	103.21 $\pm$ 4.4 Bc	*0.88 (0.78) Ba	-67.36 (0.81) Ac
	$\text{SnF}_2$	316.45 $\pm$ 10.5 Aa	*319.97 $\pm$ 10.4 Aa	119.67 $\pm$ 4.7 Bb	*1.12 (0.40) Ba	*-62.17 (1.46) Ab
	F/Sn/chitosan	316.07 $\pm$ 9.3 Aa	*319.86 $\pm$ 8.8 Aa	152.71 $\pm$ 8.7 Ba	*1.21 (0.56) Ba	*-51.68 (2.60) Aa
	F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$	316.01 $\pm$ 10.5 Aa	*319.10 $\pm$ 9.8 Aa	156.14 $\pm$ 8.7 Ba	*0.99 (0.63) Ba	-50.60 (2.08) Aa
	F/bioactive glass	316.28 $\pm$ 10.3 Aa	*318.95 $\pm$ 9.6 Aa	122.26 $\pm$ 9.3 Bb	*0.85 (0.43) Ba	-61.37 (2.17) Ab
With	Distilled Water	316.00 $\pm$ 10.3 Ba	347.32 $\pm$ 11.2 Aa	84.04 $\pm$ 4.0 Cd	9.92 (1.64) Ba	-73.38 (1.48) Ad
	NaF	316.31 $\pm$ 9.7 Ba	345.41 $\pm$ 9.0 Aa	107.16 $\pm$ 3.6 Cc	9.24 (2.44) Ba	-66.10 (1.30) Ac
	$\text{SnF}_2$	316.27 $\pm$ 12.4 Ba	343.55 $\pm$ 9.4 Aa	127.54 $\pm$ 5.3 Cb	8.69 (2.44) Ba	-59.64 (1.73) Ab
	F/Sn/chitosan	316.26 $\pm$ 12.2 Ba	346.27 $\pm$ 8.5 Aa	145.02 $\pm$ 3.4 Ca	9.58 (3.31) Ba	-54.10 (1.70) Aa
	F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$	316.13 $\pm$ 12.3 Ba	345.61 $\pm$ 8.8 Aa	151.26 $\pm$ 3.9 Ca	9.42 (3.53) Ba	-52.07 (2.50) Aa
	F/bioactive glass	316.13 $\pm$ 9.5 Ba	344.22 $\pm$ 7.6 Aa	124.81 $\pm$ 4.6 Cb	8.92 (1.65) Ba	-60.50 (1.60) Ab

p-values: treatment,  $< 0.0001$ ; smoke,  $< 0.0001$ ; time,  $< 0.0001$ ; treatment and smoke, = 0.8931; treatment and time,  $< 0.0001$ ; smoke and time,  $< 0.0001$ ; treatment, smoke, and time, = 0.0429. \*differs from the group with cigarette smoke exposure at the same treatment conditions and time ( $p \leq 0.05$ ). Means followed by distinct letters (horizontal uppercase and vertical lower case comparing treatment within each smoking level) indicate statistically significant differences ( $p \leq 0.05$ ). SMH1, baseline; SMH2, after exposure to cigarette smoke; SMH3, after the end of the erosion cycle; %SMH1, percentage of surface hardness loss after exposure to cigarette smoke; %SMH2, percentage of surface hardness loss after erosion cycle.

**Table 3.** Results (mean  $\pm$  standard deviation) of tissue loss ( $\mu\text{m}$ ) and surface roughness (nm) based on treatment with toothpastes and exposure to cigarette smoke.

Treatment	Surface profilometry		Surface roughness	
	Cigarette smoke		Cigarette smoke	
	Without	With	Without	With
Distilled water	2.92 $\pm$ 0.8 Ba	3.6 $\pm$ 0.7 Aa	177.75 $\pm$ 19.2 Aa	175.33 $\pm$ 26.5 Aa
NaF	2.54 $\pm$ 0.2 Bb	2.8 $\pm$ 0.4 Ab	141.58 $\pm$ 20.1 Ab	156.08 $\pm$ 17.5 Ab
SnF <sub>2</sub>	0.7 $\pm$ 0.1 Bc	0.79 $\pm$ 0.1 Ac	58.42 $\pm$ 15.6 Ad	58.25 $\pm$ 15.5 Ad
F/Sn/chitosan	0.66 $\pm$ 0.1 Bc	0.73 $\pm$ 0.1 Ac	49.42 $\pm$ 11.8 Ad	57.75 $\pm$ 16.5 Ad
F/CaSiO <sub>3</sub> /Na <sub>3</sub> PO <sub>4</sub>	2.79 $\pm$ 0.5 Bb	2.48 $\pm$ 0.5 Ab	100.50 $\pm$ 16.9 Ac	109.33 $\pm$ 18.7 Ac
F/bioactive glass	2.51 $\pm$ 0.5 Bb	2.85 $\pm$ 0.5 Ab	161.33 $\pm$ 16.8 Ab	154.33 $\pm$ 21.1 Ab

Surface profilometry, p-values: treatment,  $< 0.0001$ ; smoke,  $= 0.0083$ ; treatment and smoke,  $= 0.1035$ . Surface Roughness, p-values: treatment,  $< 0.0001$ ; smoke,  $= 0.2319$ ; treatment and smoke,  $= 0.3244$ . Different letters (upper case for exposure to cigarette smoke and lower case for treatment) indicate statistically significant differences ( $p \leq 0.05$ ).

The 3D images of the enamel surface topography (Figure 2) showed no differences between the presence and absence of cigarette smoke exposure for the same treatment. The distilled water group had greater demineralization, showing a rough, irregular, and peaked surface with small, shallow depressions and irregular margins. The depths of tissue loss were 3.5 and 3.7 with distilled water, 2.5 and 2.7 with NaF, 2.1 and 2.1 with F/bioactive glass, 1.2 and 1.6 with SnF<sub>2</sub>, 0.56 and 0.59 with F/Sn/chitosan, and 1.20 and 1.40 with F/CaSiO<sub>3</sub>/Na<sub>3</sub>PO<sub>4</sub>, with and without cigarette smoke exposure, respectively. NaF and F/bioactive glass showed a characteristic honeycomb appearance resulting from the dissolution of nuclei and sheath areas of the prism, but with relatively sound interprismatic areas. SnF<sub>2</sub> showed a fairly defined demineralization pattern, but with a low difference between the heights of the surface peaks and valleys, which may have resulted in the low Ra values. The presence of surface precipitates was verified. F/Sn/chitosan and F/CaSiO<sub>3</sub>/Na<sub>3</sub>PO<sub>4</sub> had the same performance. They promoted more regular surface demineralization and showed no honeycomb pattern and a thicker layer of surface precipitates.

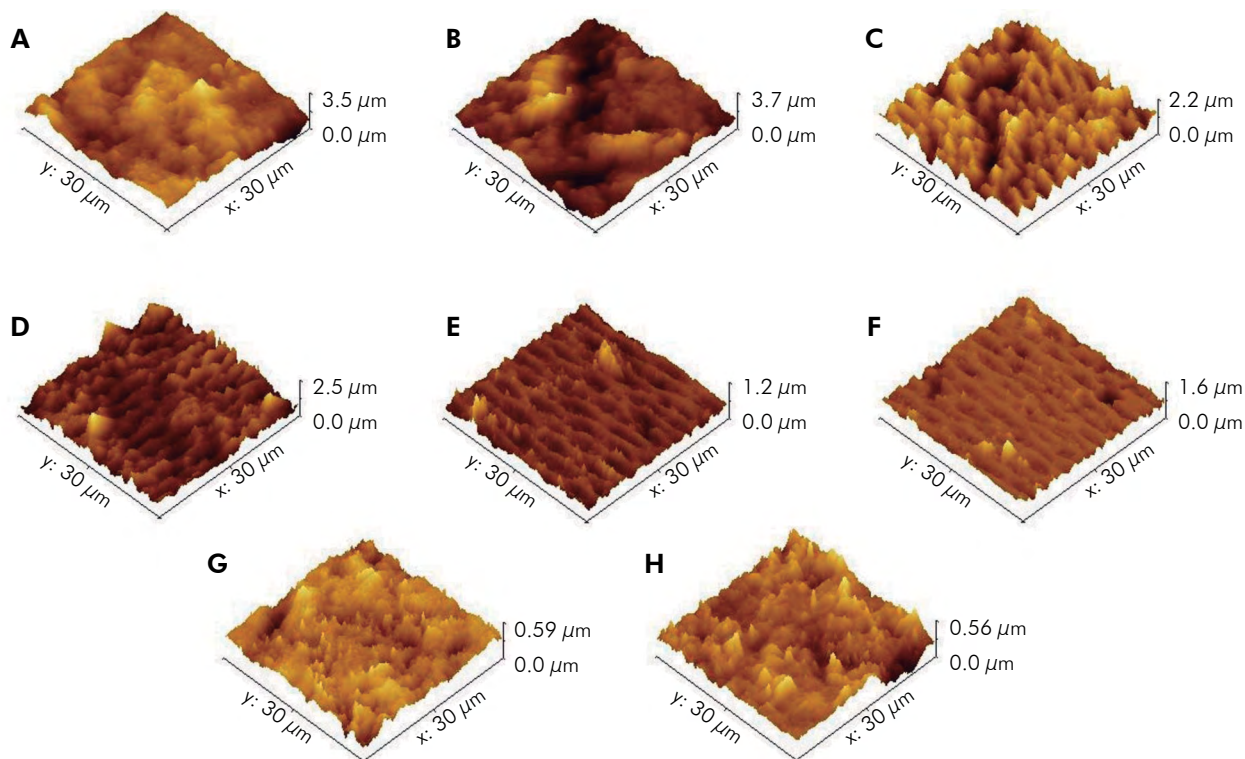
## Discussion

Cigarette smoking promoted changes in dental enamel (Table 2) after exposure to 20 cigarettes per day for 5 days increased SMH, as previous reported,<sup>15</sup> probably because of the incorporation of heavy metals,

such as lead, cadmium and arsenic.<sup>6</sup> Indeed, heat can alter the hydroxyapatite crystal morphology.<sup>5</sup>

Despite increased SMH, smoking did not improve enamel resistance to erosive challenges, as measured by SMH and profilometry. Dental enamel is highly mineralized.<sup>7</sup> The crystals consist of a Ca-deficient CO<sub>3</sub><sup>2-</sup>-rich hydroxyapatite with a Ca/P ratio of 1.61 instead of 1.66, as in hydroxyapatite.<sup>19</sup> The simplified formula for tooth mineral is Ca<sub>10x</sub>Na<sub>n</sub>(PO<sub>4</sub>)<sub>6-y</sub>(CO<sub>3</sub>)<sub>z</sub>(OH)<sub>2-u</sub>F<sub>u</sub>, which is different from the Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub> of hydroxyapatite.<sup>19</sup> During an acid attack, H<sup>+</sup> ions are released from the acid and adhere to different sites on hard dental-tissue surfaces.<sup>7</sup> Additionally, PO<sub>4</sub><sup>3-</sup> and OH<sup>-</sup> concentrations decrease at lower pH.<sup>7</sup> These ions dissolve from teeth to maintain the equilibrium of the solution around the surface.<sup>7</sup> In this study, changes in SMH after exposure to cigarette smoke might not have interfered with the amount of Ca<sup>2+</sup>, PO<sub>4</sub><sup>3-</sup>, OH<sup>-</sup>, or F<sup>-</sup>. Moreover, the erosive process depends not only on crystal solubility at the tooth surface but also on the liquid surrounding the tooth.<sup>20</sup> In this study, all groups were eroded with the same solution.

Exposure to cigarette smoke resulted in greater enamel loss, as shown by surface profilometry (Table 3). This may have occurred because of the enamel heating caused by the smoke.<sup>5</sup> The change in hydroxyapatite crystal morphology may have decreased its resistance to low pH. The greatest loss of dental tissue may also be associated with the incorporation of heavy metals.<sup>6</sup> Until recently, studies did not investigate which enamel region the metals were adsorbed in or how they bond with hydroxyapatite crystals. The metals



**Figure 2.** 3D images of enamel surface topography ( $30 \times 30 \mu\text{m}$ ) under the atomic force microscope. (A) enamel eroded and treated with distilled water, (B) enamel exposed to cigarette smoke and eroded and treated with distilled water, (C) enamel eroded and treated with NaF, (D) enamel exposed to cigarette smoke and eroded and treated with NaF, (E) enamel eroded and treated with  $\text{SnF}_2$ , (F) enamel exposed to cigarette smoke and eroded and treated with  $\text{SnF}_2$ , (G) enamel eroded and treated with F/Sn/chitosan, (H) enamel exposed to cigarette smoke and eroded and treated with F/Sn/chitosan.

may be occupying a susceptible area in the enamel prism, making it more soluble. Further studies are needed to better elucidate the interactions between cigarette smoke and dental enamel.

In this study, the acquired pellicle was produced using human saliva, with and without exposure to cigarette smoke, even in the presence of remineralizing agents. The pellicle can interact with some of the anti-erosive agents used, such as F, Sn, and Chitosan, modifying their protective effects.<sup>11</sup> The acquired film proteins can influence the formation and stabilization of  $\text{CaF}_2$  precipitates on the enamel surface after exposure to fluoride because of the adsorption of  $\text{PO}_4^{3-}$  and proteins, increasing the efficacy of fluoride treatment.<sup>21</sup> The presence of Sn ions is associated with the retention of abundant protein from the acquired pellicle,<sup>11</sup> which may explain the efficacy of Sn-based compounds in this study. Additionally, the pellicle acts as a

barrier with selective permeability that prevents direct contact between erosive acids and the tooth surface, thereby reducing demineralization.<sup>22</sup> Most laboratory studies used artificial saliva, thereby preventing adequate formation of the acquired film, which might impact the erosion.<sup>23</sup> Ideally, in vitro erosion models should incorporate exposure to natural human saliva<sup>23</sup> because all of these factors and modulators of dental erosion.

Natural saliva has some limitations with in vitro models, such as rapid decomposition and possibility of contamination.<sup>24</sup> Therefore, several studies used artificial saliva.<sup>24</sup> An in vitro study compared the remineralizing capacities of human saliva, artificial saliva, and remineralizing solution, and microradiography showed no significant differences in mineral loss among them.<sup>25</sup> The use of natural saliva for storage in this study would require the collection of abundant saliva for the many days of erosive cycle



and number of study groups, which was not feasible, and artificial saliva was chosen for storage.

Enamel repair and protection may be enhanced by the use of fluoride-containing products.<sup>7</sup> Toothpaste is a vehicle for supplying fluoride in the oral cavity, with good availability. However, brushing promotes dental wear by removing the soft enamel layer, especially after an erosive challenge.<sup>26</sup> Enamel abrasion by brushing is influenced mainly by toothpaste abrasiveness,<sup>27</sup> which varies between the marketed toothpastes in relation to enamel and dentin.<sup>27</sup> In this study, toothbrushing was not performed, which may be a limitation. However, as several toothpastes were used, brushing was not designed because the abrasiveness could not be standardized. Therefore, the effects of active agents from toothpastes were assessed, excluding their potential of abrasiveness. In clinical situations, these results may be different because depending on the toothpaste type, erosion can be greater than the protective effect of the active ingredients.

The action of fluoride seems to be limited in erosion and does not provide total protection.<sup>9</sup> Therefore, it is necessary to combine fluoride with other agents.<sup>7</sup> In this study, NaF had more anti-erosive effects only in relation to the negative control treated to distilled water, as previously reported<sup>28</sup>. Under acidic conditions, the NaF action of forming a  $\text{CaF}_2$  layer on demineralized surfaces is reduced at low pH.<sup>9</sup> This impairs the effect of NaF-based toothpastes on the prevention of erosion.<sup>9</sup> NaF is a saliva-dependent compound, as it demonstrates similar efficacy to fluoride-free toothpastes in low salivary flow conditions.<sup>29</sup>

In general, Sn-based toothpastes showed the best results. The combination of fluoride with Sn and Chitosan is more efficient than NaF.<sup>12,16,29</sup> Their mode of action is mostly based on the reaction between Sn ions and hard dental tissues to form  $\text{Sn}_2\text{OHPO}_4$ ,  $\text{Sn}_3\text{F}_3\text{PO}_4$ , and  $\text{Ca}(\text{SnF}_3)_2$  salts.<sup>30</sup>  $\text{SnF}_2$  can interact with and be incorporated in the eroded enamel<sup>21</sup>, forming salts with Ca and  $\text{PO}_4^{3-}$ ,<sup>30</sup> which are more acid-resistant<sup>31</sup>. Additionally, several studies have confirmed the effectiveness of  $\text{SnF}_2$  in the presence of an acquired pellicle<sup>11,29</sup>. Sn ions were suggested to have a cross-linking action with proteins of a previously formed

acquired pellicle,<sup>32</sup> including mucins and albumins.<sup>11</sup> In the 3D images (Figure 2), demineralization had a similar honeycomb appearance with the use of distilled water and  $\text{SnF}_2$ , but with less depth, which was probably a reason for the low Ra values.

The combination of chitosan with Sn and fluoride compounds showed the same effect as SnF on tissue loss and Ra ( $p < 0.05$ ) (Table 3). They had the best effect against erosion. Furthermore, chitosan showed lower %SMH2 and higher SMH3 than Sn ( $p > 0.05$ ) (Table 2). This result shows that after erosive challenge, F/Sn/Chitosan hardens the enamel more than SnF does, which may benefit in facing subsequent erosive challenges. Chitosan is a natural polymer derived from chitin de-acetylation. This polymer binds to salivary proteins,<sup>33</sup> fluoride,<sup>7</sup> and other ions on the enamel surface,<sup>34</sup> forming layers that are acid-resistant and providing better protection against demineralization.<sup>34</sup> Moreover, this layer can increase Sn retention,<sup>16</sup> thereby increasing its protective effect. In this toothpaste, Sn is combined with AmF. The Sn ion needs to be stabilized in an aqueous solution, particularly in neutral formulations.<sup>12</sup> Sn is better stabilized when combined with AmF.<sup>30</sup> In addition, this toothpaste has  $\text{SnCl}_2$ , thus increasing the Sn amount in the toothpaste. The effects of chitosan and Sn were evident in the 3D images of the enamel surface (Figure 2), as a reduced pattern of demineralization and an aprismatic layer, allowing the identification of crystals precipitated from the remineralizing agents. However, %SMH2 showed that the effect of chitosan-based toothpastes is limited after enamel is exposed to smoke. Probably, contaminants prevent the formation of chitosan layers under the eroded enamel. In contrast, the protection of  $\text{SnF}_2$  was higher in the enamel exposed to cigarettes. Being a metal, Sn can deposit itself and form stable salts with hydroxyapatite crystals, as shown above.<sup>30</sup>

Another toothpaste evaluated in this study had F/ $\text{CaSiO}_3$ / $\text{Na}_3\text{PO}_4$ . Studies show that  $\text{CaSiO}_3$  can deposit on the enamel surface and release Ca ions into oral fluids under acidic conditions, increasing the degree of hydroxyapatite saturation, and inhibit the dissolution of tooth enamel;<sup>35</sup> however, in this study, this deposit did not resist the acidic effect. F/ $\text{CaSiO}_3$ / $\text{Na}_3\text{PO}_4$  showed a great loss of tissue, better

only than distilled water (Table 3). Furthermore,  $\text{CaSiO}_3$  can nucleate hydroxyapatite crystals,<sup>36</sup> as seen in the topographic 3D images (Figure 2). This may have favored the results of SMH and %SMH because this toothpaste presented results similar to F/Sn/chitosan ( $p < 0.05$ ). Similarly, F/bioactive glass promoted the precipitation of Na, Ca and  $\text{PO}_4^{3-}$  ions, which also interact with oral fluids to form a layer of crystallized hydroxy-carbonate apatites, similar to the enamel<sup>37</sup>, and minimize any changes in SMH. However, these beneficial effects may be verified in the remaining enamel that is underneath the lost enamel after the erosive challenge. The surface profilometry results (Table 3) showed that F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$  and F/bioactive glass were not enough to prevent mineral loss, as they did not differ statistically from NaF.

F/ $\text{CaSiO}_3/\text{Na}_3\text{PO}_4$  and F/bioactive glass have MFP as a fluoride agent. In cases of in vitro studies on MFP-based toothpastes, the type of diluent used in the slurry should be considered. The bioavailability of fluoride can be affected not only by the composition<sup>38</sup> but also by interactions with human saliva. MFP hydrolysis is catalyzed by salivary enzymes that decrease the efficacy of MFP-containing toothpastes in in vitro studies.<sup>39</sup> Therefore, there are few published studies testing the effectiveness of MFP against erosive challenges. These factors may have contributed to the low efficacy of these toothpastes.

## Conclusion

Cigarette smoking may be considered a modulator of dental erosion by causing superficial changes in the enamel. The increase in SMH exposed to cigarette smoke was not enough to inhibit demineralization of the tooth enamel in the erosion cycle. However, the exposure to cigarette smoke resulted in a greater loss of tissue for all groups, except F/ $\text{CaCO}_3/\text{Na}_3\text{PO}_4$ , as seen in surface profilometry analysis. The NaF-containing toothpaste showed a limited efficacy against dental erosion. Sn and its combinations with chitosan and fluoride would yield promising compounds, as it had the best performance and anti-erosive action among all evaluated toothpastes.

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