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ORIGINAL ARTICLE

Fluoride toothpastes containing micrometric or nano-sized sodium trimetaphosphate reduce enamel erosion *in vitro*

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ABSTRACT

Objective: To evaluate the effect of fluoride toothpastes supplemented with micrometric or nano-sized sodium trimetaphosphate (TMP or TMPnano, respectively) on enamel erosion *in vitro*, as well as the influence of salivary acquired pellicle and saliva.

Material and methods: Bovine enamel blocks ($n = 120$) were randomly assigned into the following experimental toothpastes: no F/TMP/TMPnano (Placebo); 1100 ppm F (1100 ppm F); 1100 ppm F plus 3% TMP or 3% TMPnano (1100 TMP or 1100 TMPnano, respectively) and 5000 ppm F (5000 ppm F). Erosive challenge was performed by immersion of the blocks in citric acid for 5 min, followed by 2 h immersion in human or artificial saliva, 4×/day, during 5 days. After each erosive challenge, blocks were exposed to slurries of the toothpastes. Enamel erosion (μm), surface hardness (SHf) and cross-sectional hardness (ΔKHN) were analyzed as response variables and the data were submitted to two-way ANOVA, followed by the Student–Newman–Keuls test ($p < .05$).

Results: 1100 TMPnano significantly reduced enamel loss when compared to 1100 TMP ($p = .002$), reaching values similar to those promoted by 5000 ppm F ($p = .96$). 1100 ppm F presented significantly lower enamel loss than Placebo ($p < .001$), and higher than 1100 TMP ($p < .001$). Significantly higher SHf and lower ΔKHN was observed for 1100 TMPnano and 5000 ppm F when compared with the other groups ($p < .001$). The type of saliva did not influence enamel erosion, SHf and ΔKHN for the groups treated with TMP-containing toothpastes.

Conclusion: The addition of 3% TMPnano to 1100 ppm F toothpastes significantly increases the protective effect against enamel erosion *in vitro* when compared with its counterparts with micrometric TMP or without TMP. This effect was not influenced by the presence of acquired enamel pellicle and saliva.

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Introduction

Dental erosion is a multifactorial condition resulting from cumulative chemical and mechanical damage of the hard dental tissue [1,2]. The interaction of factors related to the patient and/or to the dietary habits may lead to either progression of the lesion or to the protection of the surface [3].

Topical fluoride application has been used as preventive strategy to reduce tooth erosion [4]. Nonetheless, moderate or low fluoride (F) concentrations have been shown to be unable to provide significant preventive effects against erosion [5]. Despite F may have a protective effect under conditions in which the erosive factors are not excessive [6], some studies have shown a limited beneficial effect of 1000 ppm F toothpaste compared to F-free toothpastes on the abrasion of eroded dentin and enamel [7,8].

Several studies have been conducted attempting to enhance the effects of topically applied F products against erosion, among which the use of inorganic phosphates has been shown to produce additional protective effects when added to toothpastes [9,10], mouthrinses [11], gels and varnishes [12,13]. The addition of sodium trimetaphosphate (TMP) in micrometric particles to F toothpastes was shown to be significantly more effective against enamel erosion when compared with their counterparts without TMP [9,10]. This has later prompted to studies aiming to assess the impact of nano-sized TMP added to F toothpastes on the process of enamel rehardening in a pH-cycling caries model, showing increased enamel rehardening compared to conventional toothpaste (1100 ppm F) [14]. The above-mentioned benefits of using nano-sized TMP have not been studied on enamel erosion.

In addition to therapeutic agents, human saliva has also been shown to protect enamel against erosive challenges through its clearing and buffering effects, and also due to the formation of the acquired pellicle on the enamel surface [15,16]. It is an organic film formed by the continuous selective adsorption of salivary proteins and glycoproteins onto the enamel surface which, in turn, also possesses buffering capacity and reduces the dissolution of hydroxyapatite [17]. Moreover, constant exchange of the proteins occurs during the formation of the layer [18].

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Based on the information above, the aim of this study was to evaluate the effect of F toothpastes supplemented with micrometric or nano-sized sodium trimetaphosphate (TMPnano) on enamel erosive, and to assess the influence of acquired pellicle and saliva on the effects of TMP/TMPnano. It was hypothesized that the toothpaste supplemented with nano-sized TMP would lead to additional protective effect against enamel erosive when compared to counterparts with micrometric TMP or without TMP, and that this effect would be decreased by the presence of acquired pellicle and saliva.

Materials and methods

Experimental design

Bovine enamel blocks (4 mm × 4 mm, $n = 120$) were selected by surface hardness and randomly assigned to the following experimental toothpastes: no F/TMP/TMPnano (Placebo); 1100 ppm F (1100 ppm F); 1100 ppm F plus 3% TMP or 3% TMPnano (1100 TMP or 1100 TMPnano, respectively) and 5000 ppm F (5000 ppm F). The sample size of 12 enamel blocks was calculated based on previous study [9] considering as primary enamel erosion (μm) data, adopting the mean difference between the groups and standard deviation of 0.8 and 0.5, respectively, α-error of 5% and a β-error of 20%. Blocks were further divided into two conditions of erosion: in the presence of human saliva (ERO-HS, $n = 60$) or artificial saliva (ERO-AS, $n = 60$). The erosive challenge was produced by immersion in citric acid four times per day. The factors studied were toothpastes and type of saliva. Enamel erosion, surface hardness and cross-sectional hardness were analyzed as response variables.

Synthesis and characterization of nano-sized (TMP) particles

The synthesis and characterization of nano-sized TMP was based on Danelon et al. [14] study. X-ray diffraction (XRD) was used to identify the crystalline structure and estimating crystallographic coherency domain TMP and milled for 48 h (TMPnano).

Toothpaste formulation and fluoride and pH assessment

The toothpastes were prepared in the laboratory of Pediatric Dentistry (São Paulo State University (Unesp), School of Dentistry, Araçatuba) with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulphate and deionized water. TMP (Aldrich Chemistry, CAS 7785-84-4, China) was added at concentration of 3%, for both micrometric and nano-sized particles. To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpastes without TMP, TMPnano and F (Placebo), as well as with 1100 and 5000 ppm F (without TMP/TMPnano) were also prepared. A single batch of each toothpaste was prepared. The total (TI) and ionic (IF) concentrations of fluoride and the pH of the toothpastes were determined in triplicate prior to the beginning of the study [9,19].

Enamel sample preparation

Enamel blocks (4 mm × 4 mm, $n = 120$) were prepared from extracted bovine incisors, which were stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature [20]. The enamel surface of the blocks was ground flat with water-cooled Carborundum disks (400, 600, 800 and 1200 grades of Al_2O_3 papers; Buehler, Lake Bluff, IL) for the removal of ~200 μm of enamel surface. After polishing, enamel surface knoop hardness (SHi) was determined using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, IL) with a Knoop diamond indenter under a 25 g load for 10 s. Blocks ($n = 120$) with initial hardness surface (SHi) numbers ranging from 372.0 up to 377.0 KHN ($p = .610$) were selected, and randomly allocated into five groups of 12 blocks each. In order to maintain reference surfaces for analysis of enamel loss by profilometry, two layers of acid-resistant nail varnish (Risqué®-Brazil) were applied in the middle of each block.

Saliva collection

Saliva collection was performed in accordance with the protocol approved by the Human Ethics Committee of São Paulo State University (Unesp), School of Dentistry, Araçatuba, Brazil (Protocol: 15018213.8.0000.5420). Paraffin-stimulated saliva samples from healthy donors of both genders (aged 22–35 years, $n = 10$) were collected into ice-chilled vials and pooled. Immediately after collection, whole saliva samples were centrifuged for 20 min at 4 °C and 2000*g*. The supernatants were divided into 13 mL aliquots and stored at −80 °C [21].

Erosive challenge and treatment with toothpastes

Before the first erosive challenge and treatment with the toothpastes, enamel blocks were kept immersed in 3 mL of human (ERO-HS; $n = 60$) or artificial saliva (ERO-AS; $n = 60$) in individual containers, at 37 °C, unstirred, during 24 h [22]. Following, blocks were removed from HS/AS and subjected to erosive challenges (0.05 mol/L citric acid, pH = 3.2 [22]; Labsynth Diadema, SP, Brazil) during 5 days, 4×/day (under gentle agitation on an orbital shaker, 5 min each, 2 mL/block) [9]. Immediately following each erosive challenge, the enamel blocks were exposed to slurries (1 g of toothpaste: 3 mL of water) of the toothpastes for 1 min (under gentle agitation on an orbital shaker, 6 mL/block), at room temperature, resulting in four cycles of erosive/treatment. The enamel blocks were stored in HS/AS (3 mL) after the last erosive/treatment process, at 37 °C, for a period of 2 h [23,24]. The composition of the artificial saliva was: 1.5 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$, 0.9 mmol/L $NaH_2PO_4 \cdot 2H_2O$, 150 mmol/L KCl, 0.1 mol/L Tris buffer, 0.03 ppm F, pH 7.0 [9–11].

Table 1. Mean (SD) enamel erosion (μm), final surface hardness (SHf), integrated subsurface hardness (ΔKHN) after erosive challenge, according to the toothpastes used and type of saliva ($n = 12$).

	SHf (KHN)		Wear (μm)		ΔKHN (KHN × μm)	
Groups	Artificial	Human	Artificial	Human	Artificial	Human
Placebo	41.7^{a,A} (5.1)	39.7^{a,A} (4.4)	4.42^{a,A} (0.09)	4.25^{a,A} (0.30)	3,563.7^{a,A} (694.3)	2,858.9^{a,B} (427,1)
1100 ppm F	77.7^{b,A} (8.0)	76.9^{b,A} (8.3)	1.23^{b,A} (0.25)	1.19^{b,A} (0.30)	2,357.0^{b,A} (337.2)	2,337.8^{b,A} (259.4)
1100 TMP	142.4^{c,A} (3.4)	141.0^{c,A} (7.4)	0.65^{c,A} (0.10)	0.67^{c,A} (0.20)	1,466.1^{c,A} (387.9)	1,247.1^{c,A} (396.5)
1100 TMPnano	214.8^{d,A} (6.8)	213.9^{d,A} (4.1)	0.46^{d,A} (0.10)	0.48^{d,A} (0.10)	1,063.4^{d,A} (127.4)	1,075.5^{d,A} (218.4)
5000 ppm F	214.0^{d,A} (3.6)	210.3^{d,A} (6.5)	0.48^{d,A} (0.12)	0.47^{d,A} (0.10)	978.0^{d,A} (170.2)	836.0^{d,A} (166.2)

Distinct lowercase letters show significant difference among the toothpastes in each column. Distinct capital letters show significant difference between the two conditions of saliva (ANOVA, Student–Newman–Keuls, $p < .05$).

Profilometric analysis

The nail varnish on the reference surfaces was carefully removed with acetone-soaked cotton wool [9,25]. Enamel loss was determined in relation to the reference surfaces by profilometry (Hommel Tester T1000, Hommelwerke, VS-Schwenningen, Germany). Profilometric traces were taken from the reference surfaces (baseline) across the exposed surfaces (length = 2 mm). Five readings were performed on each block, and the average erosion depth was calculated. The final hardness surface (SHf) was determined as described previously and the mean of five other indentations was calculated.

Analysis of cross-sectional hardness

Blocks were cross-sectioned at the centre, and half of each block was embedded in acrylic resin and subsequently polished. Cross-sectional hardness was determined using a hardness tester (Micromet 5114, Lake Bluff, IL, USA) and the Buehler OminiMet software (Lake Bluff, IL, USA), with a Knoop diamond indenter under a 5 g-load for 10 s [26]. A sequence of eight indentations at distances of 10, 15, 20, 25, 30, 40, 50 and 70 μm from the external surface of the enamel was performed in the centre of blocks, for both the control and the test areas. The integrated area of hardness (KHN × μm) of the demineralized and sound enamel was calculated using the trapezoidal rule (GraphPad Prism, version 3.02; GraphPad Software Inc, La Jolla, CA, USA) and subtracted from the integrated area of the hardness of sound enamel loss resulting integrated hardness (ΔKHN) [27].

Statistical analysis

For statistical analysis, SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA) was used, and the significance limit was set at 5%. The values of erosion (μm), SHf and ΔKHN were considered as outcome measures and the toothpastes and saliva, as the variation factor. The data presented normal (Shapiro–Wilk test) and homogenous (Cochran test) distribution and were submitted to two-way ANOVA followed by the Student–Newman–Keuls test.

Results

The total and ionic F concentrations (TF and IF) (mean [SD]; ppm F; $n = 3$) in the toothpastes were, respectively 9.5 [1.1] and 9.7 [0.4] for the Placebo; 1106.5 [8.2] and 1102.9 [4.5] for 1100 ppm F; 1112.6 [13.2] and 1110.3 [17.6] for 1100 TMP; 1103.8 [5.1] and 1135.3 [4.9] for 1100 TMPnano; and 5387.0 [26.3] and 5028.8 [24.8] for 5000 ppm F toothpaste. The pH (mean [SD]; ppm F; $n = 3$) of the toothpastes were 7.5 [0.1] for the Placebo; 7.7 [0.1] for 1100 ppm F; 7.4 [0.1] for 1100 TMP; 7.1 [0.3] for the 1100 TMPnano and 7.5 [0.2] for 5000 ppm F toothpastes.

The addition of TMP to the 1100 ppm F toothpaste reduced enamel erosion ~48% when compared to 1100 ppm F without TMP ($p < .001$). Nano-sized TMP further increased the effect against erosion in ~30% when compared to 1100 TMP ($p = .002$). Moreover, 1100 TMPnano and 5000 ppm F toothpastes presented similar rates of enamel loss ($p = .955$) (Table 1). The type of saliva (HS/AS) did not influence enamel erosion ($p = .332$).

After the erosive challenges, the remaining enamel was significantly harder (SHf) for groups treated with fluoride toothpastes in comparison with the Placebo group ($p < .001$). The addition of micrometric TMP to 1100 ppm F toothpaste reduced enamel softening at 84% when compared to the counterpart without TMP ($p < .001$). The 1100 TMPnano improved this effect in 50% in comparison with micrometric TMP ($p < .001$), reaching levels similar to those obtained after the use of the 5000 ppm F ($p = .202$). As for enamel erosion, the type of saliva (HS/AS) did not influence the enamel surface hardness ($p = .113$).

The demineralization area (ΔKHN) resulting from the fluoridated toothpastes was significantly lower than the Placebo, reaching reduction levels around 27%, 58% and 67%, respectively for 1100 ppm F, 1100 TMP and 1100 TMPnano ($p < .001$); similar values were observed for 1100 TMPnano and 5000 ppm F toothpastes ($p = .212$). In addition, blocks treated with Placebo and kept in AS had ΔKHN significantly higher than those kept in HS ($p < .001$). The profile analysis showed that enamel mineral loss was not restricted to the outermost part of the enamel (Figure 1). The hardness at 10 μm was significantly lower when enamel blocks treated with Placebo and 1100 ppm F were exposed to human saliva ($p < .028$). Also, blocks treated with the Placebo toothpaste showed lower hardness values at 15, 20 and 30 μm when stored in artificial saliva ($p < .001$). The results of the other groups and depth in the enamel were not influenced by the storage medium ($p > .150$).

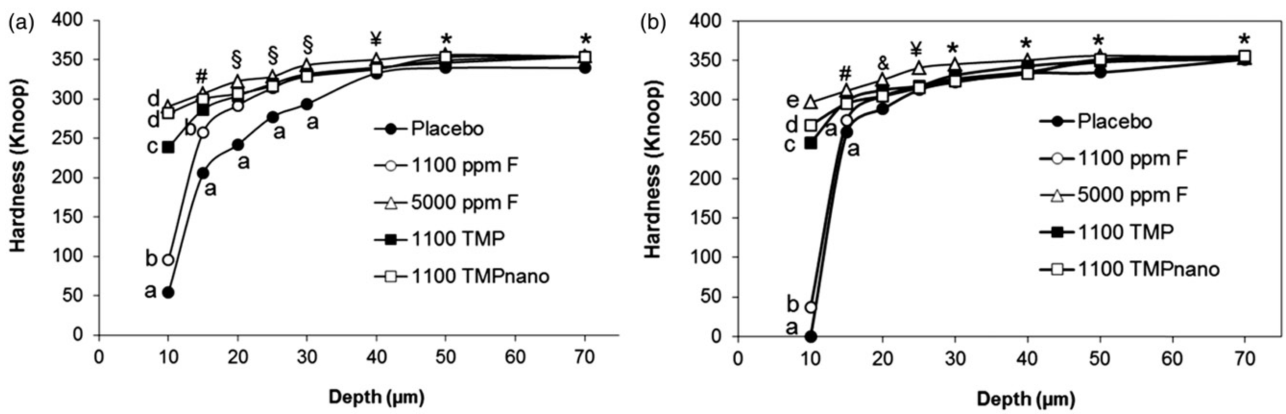

Figure 1. Cross-sectional hardness profile according to the groups exposed to artificial (a) or human (b) saliva ($n = 12$). Distinct letters show significant difference between groups (Student–Newman–Keuls, $p < .05$). (#): indicates no significant difference between 5000 ppm F, 1100 TMP and 1100 TMPnano. (§): indicates no significant difference between 5000 ppm F, 1100 ppm F, 1100 TMP and 1100 TMPnano. (&): indicates significant difference between 5000 ppm F and Placebo and 1100 TMPnano. (¥): indicates significant difference between 5000 ppm F and Placebo. (*): indicates no significant difference among the groups. Placebo and 1100 ppm F at 10 µm of depth presented different results between artificial and human saliva ($p < .028$). Placebo at 15, 20, 25 and 30 µm of depth presented different results between artificial and human saliva ($p < .001$).

Discussion

Recently, the association of F and phosphate salts has been shown to be a promising alternative to reduce enamel loss in an *in vitro* erosion models [9,10,13]. The present results showed that the addition of TMPnano significantly increased the protective effect against enamel erosion, reaching levels similar to those promoted by the 5000 ppm F toothpaste, and superior to other treatments, thus confirming the study's hypothesis. As in the study of Cruz et al. [10], formulations containing only TMP (without F) have not been tested because the effects of this salt alone is lower than that observed for F.

Although several studies showed a limited effect of 1100 ppm F toothpaste against enamel erosion [8,28,29], our results demonstrated this toothpaste promoted a significantly higher protective effect when compared with the Placebo, and that the association of 1100 ppm F and TMP further improved such effects [9,10]. It is known that F in toothpastes leads to the formation of CaF_2 on the eroded enamel surface, partially reducing enamel erosion after erosive cycles, as demonstrated in the study of Magalhães et al. [8], and in line with the present results. The remaining, less softened enamel (SHf and ΔKHN) observed for the 5000 ppm F group can be related to the greater CaF_2 formation on the blocks, which minimizes mineral loss in a subsequent erosive challenge [6,13,30,31]. The CaF_2 globules act as a physical barrier inhibiting the contact of acid with the enamel and also act in the rehardening processes [25,32]. As the enamel blocks were stored in HS/AS saliva after the last erosive/treatment process, it would be more correct to state that the hardness testing (superficial and cross-sectional) analyzed the rehardening ability of the formulations on the remaining enamel. A greater formation of CaF_2 occurs when F is associated to TMP [33] and can also explain the lower erosion values when compared to 1100 ppm F toothpaste. In addition, the adsorption of TMP on enamel is able to block acid diffusion [9–11,13] minimizing the enamel softening in a depth of 10 µm (Figure 1) [12,13].

The synergistic effect of F and TMP was more pronounced when TMP was added as nano-sized particles, which reduced enamel loss (erosion) in ∼30% when compared to 1100 TMP. These results can be explained due to the properties of nano-sized materials, such as their high ratio of surface area to volume, as well as a high percentage of atoms on the surface compared to larger particles, which makes them more reactive when compared micrometric particles [34]. As previously reported, the F/TMP or F/TMPnano molar ratios has a strong influence on enamel demineralization/remineralization [9,14,35,36]. It is possible that higher F concentrations would reduce the adsorption of TMP on enamel owing to the higher strength of chemical bonding of F, resulting in an antagonistic effect between the agents (i.e. F × TMP; F × TMPnano). This would further reduce Ca^{2+} and CaF^{+} retention [12,13,31], which in turn would minimize the formation of more reactive species under acidic conditions [37], and decrease the protective effect against demineralization during erosive challenges [12,14,31]. Thus, the size of the trimetaphosphate particles used (22.7 nm) [14] support the hypothesis that the smaller particle size promotes greater reactivity with the enamel surface and, therefore, reduces enamel erosion.

Human saliva was used in this study, aiming to better simulate a clinical condition, since it has been suggested that the presence of the acquired salivary pellicle can reduce the loss and softening of enamel, due to its diluent action, buffering capacity, and supersaturation with respect to tooth mineral content [18,25,38]. This was confirmed for the Placebo group when analyzed by cross-sectional hardness (Table 1 and Figure 1), for which the mineral content differed under the two conditions (HS/AS). Nonetheless, no influence of human saliva was observed in the presence of F/TMP or F/TMPnano during the *in vitro* experiment, showing that F enhances rehardening and significantly reduces the demineralization in acidic environments [39]. The differences observed for F when comparing *in vitro* and *in situ* protocols could be related to salivary clearance in the oral environment [39], since the dilution of the toothpaste was the same for all groups. It is noteworthy, however, that the use of human saliva in *in vitro* studies has some limitations. The collection of natural saliva is time consuming and demands its use in compliance with cross-infection procedures, due to its fast decomposition [21]. Furthermore, human saliva is considered as a biological material, thus requiring legal consent for its use [40].

Although it is known that the erosion *in vitro* models are appropriate for analysis of new preventive agents, they also have some limitations related to the faster periods of erosive challenge and rehardening and the lower reactivity of F when compared with *in vivo* conditions. Also, this study did not allow the full validation of the efficacy of the toothpastes tested due to the lack of abrasion by toothbrushing, which may result in incorrect conclusions on the anti-erosion potential of the toothpastes. This is of concern because the effects of the toothpastes seen in the present study could potentially be counteracted by abrasive challenges or other ingredients in the formulation. Thus, although some studies assessing the effects of toothpastes on erosion provided a very similar trend of enamel erosion after erosive challenges alone or followed by abrasion by toothbrushing, differing only in the magnitude of the erosion [11–13,28], other investigation do not support such assumption [29]. Therefore, the results should be evaluated with caution, and, *in situ* study models of dental erosion, erosion/abrasion and clinical studies, are recommended.

Conclusion

On the basis of the findings of this *in vitro* study, the addition of 3% TMPnano to a 1100 ppm F toothpaste promoted a significantly higher protective effect against enamel erosion when compared with its counterparts with micrometric TMP or without TMP, reaching protective levels similar to those seen for the 5000 ppm F toothpaste. This effect was not influenced by the presence of acquired enamel pellicle.

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Disclosure statement

The authors Marcelle Danelon, Juliano Pelim Pessan, Emerson Rodrigues de Camargo and Alberto Carlos Botazzo Delbem hold a patent request for a product used in the study, by the National Institute of Industrial Property – INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3. All authors approved the publishing of the manuscript.

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