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Effect of fluoride toothpaste with nano-sized trimetaphosphate on enamel demineralization: An *in vitro* study

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ABSTRACT

Objective: This study evaluated the effect of toothpastes containing 1100 ppm F associated or not with micrometric or nano-sized sodium trimetaphosphate (TMP) on enamel demineralization *in vitro*, using a pH cycling model.
Design: Bovine enamel blocks (4 mm × 4 mm, $n = 96$) were randomly allocated into eight groups ($n = 12$), according to the test toothpastes: Placebo (without fluoride or TMP); 1100 ppm F (1100F); 1100F plus micrometric TMP at concentrations of 1%, 3% or 6%; and 1100F plus nanosized TMP at 1%, 3% or 6%. Blocks were treated 2×/day with slurries of toothpastes and submitted to a pH cycling regimen for five days. Next, final surface hardness (SHf), integrated hardness loss (IHL), differential profile of integrated hardness loss (ΔIHL) and enamel fluoride (F) concentrations were determined. Data were analyzed by ANOVA and Student-Newman-Keuls' test ($p < 0.05$).
Results: The use of 1100F/3%TMPnano led to SHf 30% higher ($p < 0.001$) and IHL ~80% lower ($p < 0.001$) when compared to 1100F. This toothpaste also resulted in ~64% reduction of mineral loss (ΔIHL) when compared to 1100F. Moreover, the addition of nano-sized TMP promoted increases in enamel F uptake of 90%, 160% and 100%, respectively for the concentrations of 1%, 3% and 6%, when compared to 1100F ($p < 0.001$).
Conclusion: The addition of nano-sized TMP at 3% to a conventional toothpaste significantly decreased enamel demineralization when compared to its counterparts without TMP or supplemented with micrometric TMP.

1. Introduction

In recent years, several studies have assessed the effects of nano-sized calcium phosphates on enamel mineral loss, which have been shown to have unique remineralizing properties (Comar, Souza, Gracindo, Buzalaf, & Magalhães, 2013; Hanning & Hanning, 2010; Li et al., 2008; Roveri et al., 2008). Similarly, attempts to enhance the effects of fluoride (F) products against dental caries have included the use of inorganic phosphates in conventional (micrometric) particles, which have been proven to produce a synergistic effect on enamel de- and remineralization (Danelon, Takeshita, Sassaki, & Delbem, 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014; Manarelli, Delbem, Lima, Castilho, & Pessan, 2014; Takeshita, Castro, Sassaki, & Delbem, 2009; Takeshita, Exterkate, Delbem, & ten Cate, 2011). This has later prompted to studies assessing the impact of nano-sized calcium-phosphates added to F toothpastes on the process of enamel remineralization (Huang, Gao & Yu, 2009; Karlinsey, Fontana, González-Cabezas, Haider & Stookey, 2007).

Sodium trimetaphosphate (TMP) is a cyclic polyphosphate which adsorbs on the enamel surface, thereby reducing enamel demineralization (Barbour, Shellis, Parker, Allen & Addy, 2005; Gonzalez, 1971; Gonzalez, Jeansonne, & Feagin, 1973), enhancing enamel remineralization (Manarelli, Delbem, Binhardi & Pessan, 2015) decreasing hydroxyapatite solubility and mineral exchange (McGaughey & Stowell, 1977), and changing the affinity between the enamel surface and salivary proteins (Nordbö & Rölla, 1972; Pruitt, Jamieson, & Caldwell, 1970). The addition of TMP in micrometric particles to fluoridated products has been shown to significantly decrease enamel demineralization when compared to counterparts without TMP (Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014; Moretto, Magalhães, Sassaki, Delbem, & Martinhon,

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2010; Takeshita, Castro, Sassaki, & Delbem, 2009; Takeshita, Exterkate, Delbem, & ten Cate, 2011). Given the scarcity of studies assessing the effects of TMP added to conventional (1100 ppm F) toothpastes on the dynamics of dental caries and considering the promising effects of nanoparticles, it would be interesting to assess whether the addition of nano-sized TMP to a conventional F toothpaste would further enhance the protective effects of micrometric TMP.

Therefore, the purpose of the present study was to evaluate the effect of conventional toothpastes containing 1100 ppm F associated or not with different concentrations of micrometric or nano-sized TMP on enamel demineralization, using a pH cycling model. The null hypothesis was that fluoride toothpastes associated to nano-sized TMP would present a similar ability to reduce enamel demineralization when compared to their counterparts without TMP or supplemented with micrometric TMP.

2. Materials and methods

2.1. Experimental design

Enamel blocks (4 mm × 4 mm, $n = 96$) were obtained from bovine incisors; enamel surfaces were polished (outer enamel removed ~120 μm), and the blocks were selected by initial surface hardness test (SHi; 320.0 to 380.0 KHN). Blocks were then randomly distributed into 8 groups ($n = 12$). The experimental toothpastes contained either micrometric TMP (TMP) or nano-sized TMP (TMPnano) at concentrations of 1, 3, and 6%. NaF (Merck, CAS 7681-49-4, Germany) was also added at 1100 ppm F. In addition, toothpastes without F/TMP (Placebo), as well as with 1100 ppm F without TMP (1100F) were prepared. Blocks were subjected to pH cycling and treatment with toothpaste slurries. Next, final surface hardness (SHf), integrated hardness loss (IHL), differential profile of integrated hardness loss (ΔIHL) and enamel fluoride (F) concentrations were determined.

2.2. Synthesis and characterization of nano-sized TMP particles

To prepare nano-sized TMP, 70 g of pure (micrometric) sodium trimetaphosphate ($Na_3O_9P_3$, Aldrich, purity ≥ 95% CAS 7785-84-4) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol. After 48 h, the powder was separated from the alcoholic media and ground in a mortar. The powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC difractometer in the 2θ range from 10 to 80° with a scanning rate of 2°/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation:

$$L = \frac{K\lambda}{B\cos\theta_B}$$

where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9. The scanning electron microscopy (SEM) images were collected using a Philips XL-30 FEG.

2.3. Toothpaste formulation and fluoride and pH assessment

The toothpastes were produced with the following components: titanium dioxide, carboxymethyl cellulose, methyl *p*-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate and deionized water. Toothpastes containing micrometric or nano-sized TMP were prepared (Aldrich Chemistry, CAS 7785-84-4, China) at concentrations of 1, 3, and 6%. To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpastes without F/TMP (Placebo), as well as with 1100 ppm F without TMP (1100F) were prepared. F concentrations (Delbem et al., 2009) and pH of the all toothpastes were checked (Moretto, Magalhães, Sassaki, Delbem, & Martinhon, 2010) prior to the beginning of the study.

2.4. Treatment with toothpastes and pH cycling

The blocks were subjected to five pH cycles during 7 days, at constant temperature of 37 °C (Vieira, Delbem, Sassaki, Rodrigues, Cury, & Cunha, 2005). Enamel blocks were kept in a demineralizing solution (DE) (6 h; 2.0 mmol/L calcium and phosphate in 75 mmol/L acetate buffer, pH 4.7; 0.04 μg F/mL, 2.2 mL/mm^2) followed by their immersion in a remineralizing solution (RE) (18 h; 1.5 mmol/L calcium; 0.9 mmol/L phosphate; 150 mmol/L KCl in 0.02 mol/L cacodylic buffer, pH 7.0; 0.05 μg F/mL, 1.1 mL/mm^2). The treatment consisted of a 1-min soak under agitation in 2 mL/block of toothpaste:deionized water slurry (1:3 w/w), prior to immersion in the DE and RE solutions (twice a day) (Vieira, Delbem, Sassaki, Rodrigues, Cury, & Cunha, 2005). Deionized water rinses were performed between each step. The blocks were kept in a fresh remineralizing solution during the last 2 days.

2.5. Hardness measurements

Surface hardness was determined before (SHi) and after (SHf) pH cycling using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the Buehler OmniMet software (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations spaced 100 μm apart were produced in the center of enamel blocks (SHi). After pH cycling, 5 indentations spaced 100 μm from the baseline indentations were produced for the determination of SHf.

For cross-sectional hardness measurements, blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed. They were then gradually polished until the enamel was totally exposed. One sequence of 14 indentations was created at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 μm) from the surface of the enamel in the central region using a Micromet 5114 hardness tester (Buehler Lake Bluff, IL, USA) with a Knoop diamond indenter under a 5-g load for 10 s. The averages were calculated for each distance. The integrated area (IH; KHN × μm) of the lesions was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the IH for sound enamel to obtain the integrated area of the subsurface region in the enamel, which was termed integrated hardness loss (IHL) (Danelon, Takeshita, Sassaki, & Delbem, 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014).

To analyze the patterns of demineralization, differential hardness profiles for F and F + TMP (i.e., value of 1100-TMP groups minus 1100F group), for each of the micrometric TMP concentrations, were determined. Also, the differential hardness profiles were calculated for the F + nano-sized TMP and F + micrometric TMP at each TMP concentrations. These differential profiles were then integrated over three depth zones in the lesion (zone A, 5–15 μm; zone B, 15–50 μm; zone C, 50–130 μm) and underlying sound enamel to yield ΔIHL values (Danelon, Takeshita, Sassaki, & Delbem, 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014).

2.6. Analysis of the F concentration in enamel

Blocks measuring 2 mm × 2 mm ($n = 96$) were obtained from half of the longitudinally sectioned blocks, and were fixed to a mandrel. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50.0 ± 0.05 μm was removed from each enamel block (Alves et al., 2007; Weatherell, Robinson, Strong, & Nakagaki, 1985). A total of 0.5 mL of 0.5 mol/L HCl was added to the enamel powder retained on the polishing disc fixed to the polystyrene crystal tube. This mixture was agitated for 1 h, and 0.5 mL of 0.5 mol/L NaOH solution was added (Alves et al., 2007; Takeshita, Castro, Sassaki, & Delbem, 2009). For F analysis, samples were buffered with TISAB II and analyzed with an ion-specific electrode (Orion 9609) connected to an ion analyzer (Orion 720$^+$). A 1:1 ratio (TISAB:sample) was used. The electrode was previously calibrated with standards containing from 0.125 to 2.00 mg F/mL under the same conditions of the samples. The results were expressed as μg/mm^3.

2.7. Statistical analysis

For statistical analysis, SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) was used, and the significance limit was set at 5%. The data presented normal (Shapiro-Wilk test) and homogenous (Cochran test) distribution. Data from SHf, IHL and F were submitted to one-way ANOVA followed by the Student–Newman–Keuls test. The results of ΔIHL (considering% of TMP and zone) were submitted to two-way ANOVA followed by the Student–Newman–Keuls test.

3. Results

The milling processing reduced the particle size of TMP without affecting its crystalline structure. The X-ray diffraction (XRD) pattern of the nano-sized TMP 48 h after milling shows broader peaks due to the smaller crystallites (Fig. 1), which could be used to estimate an average particle size of 22.7 nm. Fig. 2 shows SEM images of the TMP powder (a) before milling and (b) 48 h after milling, in which the agglomerated particles before milling can be observed.

Total (TF) and ionic (IF) fluoride concentrations in the placebo toothpaste were 9.5 (1.1) and 9.7 (0.4) ppm F, respectively. For the 1100F toothpastes, mean (SD) values were 1,162.0 (44.1) and 1,157.2 (16.8), respectively for TF and IF, ranging from 1,111.0 to 1,183.9 ppm F. Mean pH of toothpastes was 7.3 (0.3) ranging from 6.8 to 7.7. Mean (SD) SHi considering all blocks was 372.8 (0.2) KHN, with no significant differences among the groups after the random allocation ($p = 0.610$).

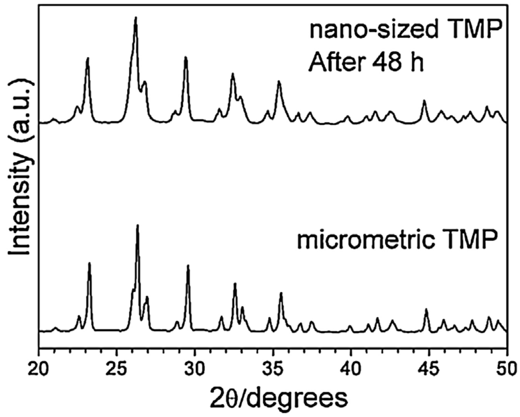

Fig. 1. X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

The use of 1100F (Table 1) led to a 3-fold higher SHf when compared with the placebo ($p < 0.001$), and the addition of TMP to 1100F further reduced enamel softening when compared to 1100F without TMP ($p < 0.001$). Blocks treated with 1100F/3%TMPnano had SHf 30% higher than those treated with 1100F ($p < 0.001$), while the corresponding value seen for 1100F/3% micrometric TMP was around 15% higher when compared with 1100F ($p < 0.001$). TMP concentrations over 3% did not promote significant reductions in enamel surface softening regardless of the particle size.

The integrated hardness loss (IHL) was 2-fold lower in the presence of F (placebo × 1100F), and the association F/TMP further reduced IHL when compared with 1100F without TMP ($p < 0.001$). The use of 1100F/3%TMPnano lead to the lowest mineral loss, which was ~80% lower when compared to 1100F ($p < 0.001$). Treatment with 1100F/3%TMP resulted in ~64% reduction of mineral loss when compared to the 1100F.

The addition of micrometric TMP at 1 and 3% increased enamel F concentration in 20% and 50%, respectively, when compared to 1100F. TMPnano led to increases in enamel fluoride uptake of 90%, 160% and 100% when compared to 1100F, respectively for the concentrations of 1%, 3% and 6%. Significant correlations were observed between enamel F concentration and SHf (Pearson's $r = 0.689$; $p < 0.001$) as well as between enamel F concentration and IHL (Pearson's $r = -0.658$; $p < 0.001$).

Differential hardness profiles (Fig. 3 and Table 2) show distinct subsurface lesion patterns. The addition of TMP to 1100F lead to higher values of mineral content in the middle part of the lesion (15–50 μm) which was improved for TMP concentrations up to 3% ($p < 0.001$). For TMP at 6% (Fig. 3a), the mineral content was lower in zones A (5–15 μm) and B (15–50 μm) when compared to TMP at 3% ($p < 0.001$). The differential hardness profile from Fig. 3b show the additional effect of nano-sized TMP when compared to micrometric TMP. For nano-sized TMP at 1%, hardness was only higher ($p < 0.001$) in the inner part of the lesion (zone C, 50–130 μm) when compared with the other TMPnano concentrations. The addition of 3%TMPnano lead to the highest hardness among the concentrations tested ($p < 0.001$), with a more marked effect at zone B ($p < 0.001$). Nano-sized TMP at 6% promoted a lower mineral loss in zones A and B only when compared to 1% ($p < 0.001$).

4. Discussion

The addition of micrometric TMP to topically applied fluoride products, especially toothpastes, has been shown to significantly improve their effect against enamel demineralization (Takeshita, Castro, Sassaki, & Delbem, 2009; Takeshita, Exterkate, Delbem, & ten Cate, 2011). For low-fluoride toothpastes (500 ppm F), TMP concentrations up to 3% increased their capacity to reduce enamel demineralization and was positively and significantly correlated to enamel F concentrations. For TMP at concentrations higher than 3%, however, enamel fluoride uptake and enamel hardness were significantly reduced. As the effect of TMP is related to its ability to adsorb on the enamel, the above-mentioned studies clearly demonstrate that the TMP:F molar ratio is paramount to achieve a maximum protective effect against enamel demineralization (Favretto, Danelon, Castilho, Vieira, & Delbem, 2013; Manarelli, Vieira, Matheus, Sassaki, & Delbem, 2011; Takeshita, Castro, Sassaki, & Delbem, 2009).

In the present study, the use of nano-sized TMP significantly reduced IHL in 20% compared to micrometric TMP, which suggests an increased capacity of adsorption of nano-sized TMP to enamel; therefore the null hypothesis could be rejected. TMP has been shown to enhance incorporation of Ca (Takeshita, Castro, Sassaki, & Delbem, 2009; Takeshita, Exterkate, Delbem, & ten Cate, 2011; Souza, Amaral, Moraes, Sassaki, & Delbem, 2013) and F ions into

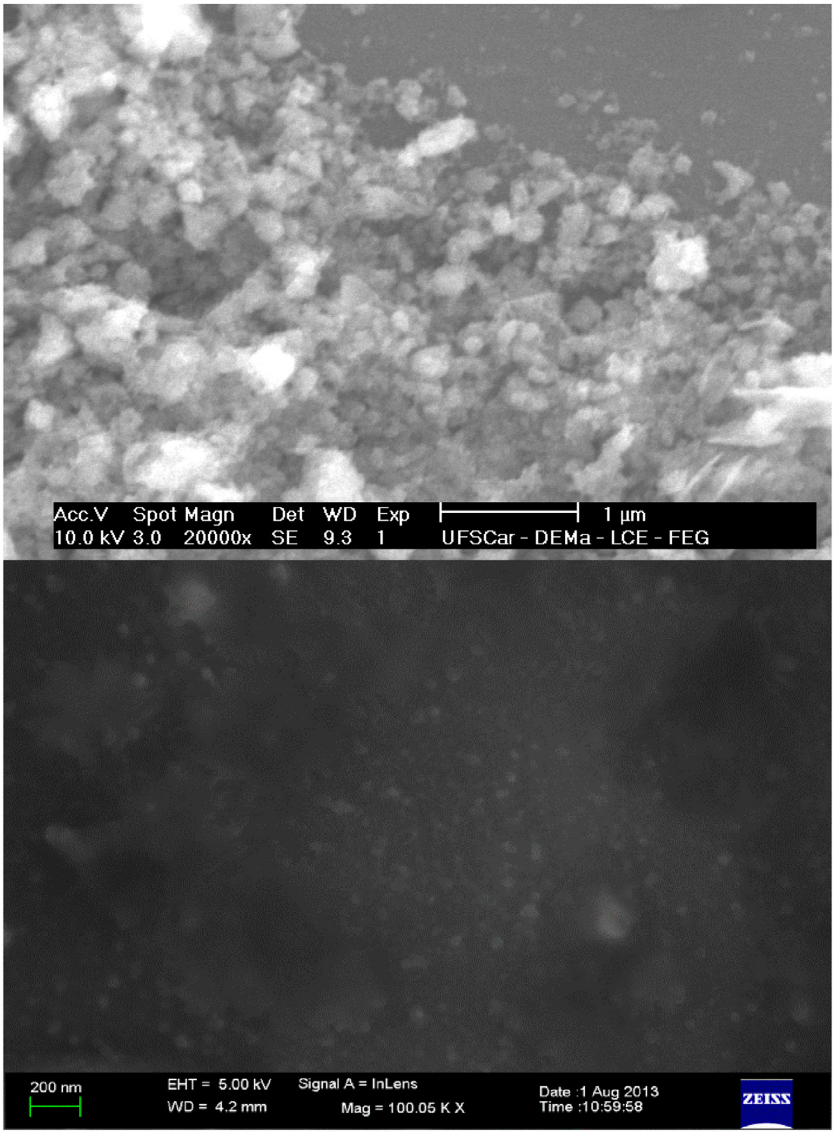

Fig. 2. SEM images of the TMP powders (a) before milling and (b) after milling for 48 h.

Table 1
Mean values (SD) of surface hardness (SHf), integrated hardness loss (IHL) and fluoride in enamel (F) according to groups ($n = 12$).

Groups	SHf (KHN)	IHL, KHN × μm	F (μg/mm^3)
Placebo	73.7^{a}	$9,871.9^{a}$	0.4^{a}
	(8.0)	(694.4)	(0.1)
1100 ppm F	241.0^{b}	$5,775.9^{b}$	1.0^{b}
	(8.7)	(920.0)	(0.2)
1100 1%TMP	254.9^{c}	$4,419.0^{c}$	$1.2^{b,d}$
	(7.1)	(548.8)	(0.2)
1100 1%TMPnano	280.3^{d}	$4,335.0^{c}$	1.9^{c}
	(5.5)	(315.1)	(0.6)
1100 3%TMP	276.6^{d}	$2,236.3^{d}$	1.5^{d}
	(4.4)	(693.5)	(0.3)
1100 3%TMPnano	311.5^{e}	$1,260.3^{e}$	2.6^{e}
	(4.4)	(211.9)	(0.7)
1100 6%TMP	223.2^{f}	$4,104.8^{c}$	1.0^{b}
	(4.5)	(680.6)	(0.4)
1100 6%TMPnano	261.4^{g}	$2,951.3^{f}$	2.0^{c}
	(9.9)	(298.1)	(0.4)

Different superscript lowercase letters indicate statistical significance in each row (1-way ANOVA, Student-Newman Keuls test, $p < 0.001$).

enamel (Takeshita, Castro, Sassaki, & Delbem, 2009; Takeshita, Exterkate, Delbem, & ten Cate, 2011; Favretto, Danelon, Castilho, Vieira, & Delbem, 2013; Souza, Amaral, Moraes, Sassaki, & Delbem, 2013). These results have led to the hypothesis that the effect of TMP is probably related to the retention of charged ions of CaF^{+} and Ca^{2+} by replacing Na^{+} from cyclic structure (Manarelli, Delbem, Lima, Castilho, & Pessan, 2013). At acidic pH, these linkages would be broken, releasing Ca^{++} and CaF^{+}, which could further take part in a series of events that ultimately would lead to the formation of neutrally charged species (CaHPO$_4^0$ and HF^0) that have a higher diffusion coefficient into the enamel (Cochrane, Saranathan, Cai, Cross, & Reynolds, 2008). This mechanism is strengthened when considering the two different particle sizes used in the present study: due to the higher ratio of surface area per volume of nanoparticles, as well as to their higher percentage of atoms on the surface compared to larger (micrometric) particles, nanoparticles can be regarded as more reactive than microparticles. The mechanism above also seems to explain why 1100F supplemented with 3% of nano-sized TMP reduced the mineral loss in $\sim$44% compared to its micrometric counterpart, mainly in the depth of 15 – 50 μm (Fig. 3 and Table 1). The impact of this effect can also be

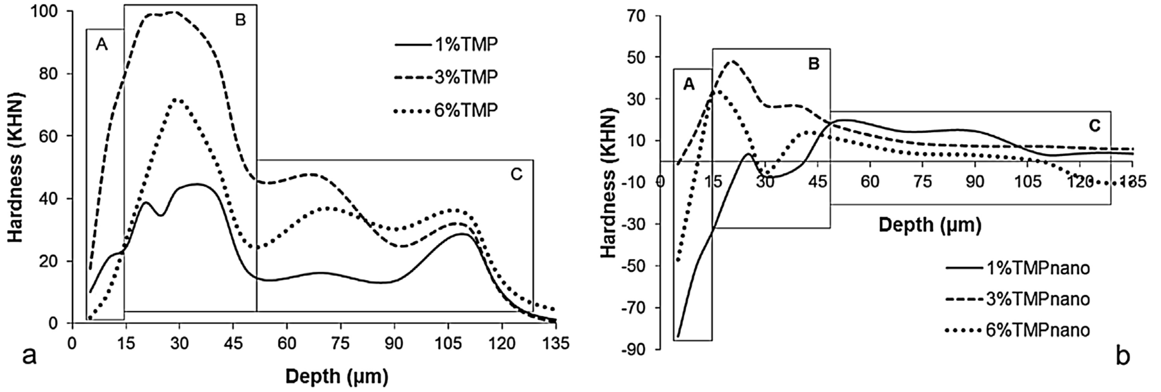

Fig. 3. Differential profile hardness as a function of depth according to the groups: Zone A (5–15 µm), Zone B (15–50 µm) e Zone C (50–130 µm) ($n = 12$). a: Differential hardness profiles (hardness vs depth) for F and F + TMP (i.e. value of 1100 TMPs groups minus 1100 group), at each of the micrometric TMP concentrations. b: Differential hardness profiles were calculated for the F + nano-sized TMP and F + micrometric TMP at each TMP concentrations.

Table 2
Mean (SD) of differential profile of integrated mineral loss (ΔIHL) calculated for three zones in the enamel lesions according to groups ($n = 12$).

Groups	ΔIHL[a], KHN × µm		
	Zone A (5–15 µm)	Zone B (15–50 µm)	Zone C (50–130 µm)
1%TMP	$189.8^{a,A}$ (58.6)	$890.0^{a,B}$ (355.8)	$360.3^{a,A}$ (35.0)
3%TMP	$547.5^{b,A}$ (136.4)	$2{,}226.0^{b,B}$ (458.6)	$635.0^{b,A}$ (122.6)
6%TMP	$120.4^{a,A}$ (46.2)	$1{,}278.3^{a,B}$ (355.7)	$578.0^{b,C}$ (114.4)
1%TMPnano	$-548.5^{a,A}$ (48.2)	$-120.6^{a,B}$ (42.9)	$221.5^{a,C}$ (79.3)
3%TMPnano	$152.2^{b,A}$ (48.5)	$833.2^{b,B}$ (192.4)	$209.2^{a,A}$ (46.0)
6%TMPnano	$-65.2^{c,A}$ (39.6)	$349.2^{c,B}$ (77.4)	$19.0^{b,C}$ (19.6)

Distinct superscript capital letters indicate the differences between zones A, B and C in each line (Student–Newman–Keuls test, $p < 0.001$). Values denote means with SD in parentheses. Distinct superscript lowercase letters in the first three rows indicate statistical significance in each column considering 1%TMP, 3%TMP and 6%TMP groups (Student–Newman–Keuls test, $p < 0.001$). Distinct superscript lowercase letters in the next three rows indicate differences between groups in each columns considering 1%TMPnano, 3%TMPnano and 6%TMPnano groups (Student–Newman–Keuls test, $p < 0.001$).

[a] ΔIHL: positive values denote higher integrated hardness and vice versa.

observed in the amount of F present in the enamel when nano-sized TMP is used, since an increase of ~75% on enamel F uptake was observed when compared to micrometric TMP. An important aspect that promotes such effects is the ability of TMP to remain bound to enamel for longer periods than other polyphosphates (McGaughey and Stowell, 1977).

The current study shows that TMP significantly affects enamel demineralization, especially in the deeper regions of the enamel lesion (mainly at 15–50 µm), while in the outer part of the lesion (5 µm) only a small additional effect was produced by the presence of TMP in the toothpastes, what is consistent with previous findings (Danelon, Takeshita, Sassaki, & Delbem, 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014; Takeshita, Exterkate, Delbem, & ten Cate, 2011). Recent studies have also shown that TMP has little additional effect on the remineralization of the outer enamel layers, and reduces the precipitation of CaF_2 and firmly bound fluoride (Danelon, Takeshita, Sassaki, & Delbem, 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014; Manarelli, Delbem, Lima, Castilho, & Pessan, 2013; Souza, Amaral, Moraes, Sassaki, & Delbem, 2013).

Since the adsorption of polyphosphates to enamel occurs rapidly after exposure (Anbar, Farley, Denson, & Maloney, 1979) and is followed by the adsorption of F, an appropriate molar proportion between TMP and F must be used for achieving optimum results. For conventional F toothpastes (1100 ppm F) the maximum inhibitory effect on enamel demineralization was shown to be achieved with micrometric TMP at 3% (TMP/F: 1.7), while at higher TMP concentrations (TMP/F: 2.5, 3.4 and 5.1), the bonding strength of hydroxyapatite to TMP reduces the link sites of F on enamel, what decreases the synergistic anticaries effect between NaF and TMP (Takeshita, Exterkate, Delbem, & ten Cate, 2011). When the fluoride concentration is raised to 3000 ppm F, the bond strength of the F to enamel is greater than TMP at 3%, with no further improvement in anticaries effect (Takeshita, Exterkate, Delbem, & ten Cate, 2011).

On the basis of the findings of this *in vitro* study, it was concluded that the addition of TMP to a 1100 ppm F toothpaste produced a synergistic effect on the inhibition of enamel demineralization. This effect was further enhanced by the use of nano-sized particles.

Conflict of interest

The authors Marcelle Danelon, Alberto Carlos Botazzo Delbem, Juliano Pelim Pessan and Emerson Rodrigues de Camargo hold a patent request for a product used in the study, by the National Institute of Industrial Property – INPI/SP, on 04/29/2008 under number 018080026091, PI0801811-1, and published on January 11, 2011. All authors approved the publishing of the manuscript.

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