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Short communication

Remineralizing effect of a fluoridated gel containing sodium hexametaphosphate: An *in vitro* study

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ABSTRACT

Objectives: To evaluate *in vitro* the effect of neutral pH topical gels with reduced fluoride concentration (F), supplemented or not with sodium hexametaphosphate (HMP) on the remineralization of dental enamel, using a pH-cycling model. Materials and methods Bovine enamel blocks with caries-like lesions were randomly treated with five gels (n = 24/group): without F/HMP (Placebo); 4500 ppm F (4500F), 4500F plus 9% HMP (4500F + HMP); 9000 ppm F (9000F); and 12,300 ppm F (Acid gel). After pH-cycling, the percentage of surface hardness recovery (%SHR), integrated loss of subsurface hardness (ΔKHN), and concentrations of loosely- (CaF_2) and firmly-bound (FA) fluoride formed and retained in/on enamel were determined. The results were analyzed by ANOVA followed by the Student-Newman-Keuls test ($p < 0.001$).
Results: The 4500F + HMP gel promoted the highest %SHR among all groups; the lowest ΔKHN was achieved by 4500F + HMP and Acid gel, without significant differences between these. The Acid gel group presented the highest CaF_2 and FA formed and retained on/in enamel ($p < 0.001$).
Conclusion: Based on the present results, the addition of 9% sodium hexametaphosphate to a gel with reduced fluoride concentration (4500F) was able to significantly enhance the remineralization of artificial carious lesions *in vitro* when compared to 4500F, reaching protective levels similar to an acidic formulation with ~3-fold higher fluoride concentration.

1. Introduction

Fluoride gels have been widely used as a caries-preventive measure in several countries, both for professional application or self-applied. Even though its clinical efficacy has been demonstrated for both primary and permanent dentitions, these products are not typically recommended for children under 6 years of age due to concerns related to acute toxicity resulting from product ingestion during application (Marinho, Worthington, Walsh, & Chong, 2015). To minimize the possibility of side-effects without compromising the therapeutic effect of gels, strategies to enhance the preventive and therapeutic effects of fluoride by the association with inorganic phosphates have been intensively studied over the last decade.

The addition of sodium hexametaphosphate (HMP) to toothpastes and gels with reduced fluoride concentration was shown to promote a synergistic protective effect against enamel demineralization *in vitro* (Danelon et al., 2012; da Camara, Miyasaki, Danelon, Sassaki, & Delbem, 2014). Sodium hexametaphosphate interferes with the enamel de-remineralization processes due to its ability to bind to the enamel surface and reduce its solubility. This phosphate also has antimicrobial activity, due to its ability to increase the permeability of the bacterial outer membrane (Vaara & Jaakkola, 1989), as well as inhibitory activity against biofilm formation (Shibata & Morioka, 2001). Considering that gels are also used as therapeutic vehicles for the reversal of non-cavitated caries lesions, the study of the remineralizing effect of sodium hexametaphosphate containing gels with reduced fluoride concentration could provide additional information on the real benefits of such formulations. Thus, this study assessed the remineralizing effect of a low-fluoride gel containing HMP *in vitro*, using a pH-cycling model. The null hypothesis was that the test gel would present a similar remineralizing effect when compared with its counterpart without HMP.

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2. Material and methods

2.1. Experimental design

Enamel blocks (4 mm × 4 mm, n = 120) were obtained from bovine incisors kept in formaldehyde (2%, pH 7.0) for 30 days prior to the experimental procedures (Danelon, Takeshita, Sassaki, & Delbem, 2013). The enamel surface of the blocks was serially polished and selected by surface hardness (SH, 369.0 to 377.0 KHN). The blocks were then demineralized (induction of subsurface lesions), analyzed by surface hardness after demineralization (SH1), and randomly assigned into five treatments (n = 24/group): without F/HMP (Placebo); 4500 ppm F (4500F); 4500F plus 9% HMP (4500F + HMP); 9000 ppm F (9000F); and 12,300 ppm F (Acid gel). The gels were applied only once (1 min). Half of the blocks (n = 12/group) were used for determination of loosely- (CaF_2) and firmly-bound (FA) fluoride formed on/in enamel. The other blocks (n = 12/group) were subjected to six pH cycles. The percentage of hardness surface recovery (%SHR) and cross-sectional hardness (ΔKHN) were assessed, and the concentrations of CaF_2 and FA retained on/in enamel were determined.

2.2. Gel formulation and determination of fluoride and pH in products

The experimental gels were prepared in the laboratory of Pediatric Dentistry, School of Dentistry, Araçatuba (UNESP, Brazil) using the following ingredients: 8.0 g carboxymethyl cellulose (Synth, Diadema, São Paulo, Brazil), 0.1 g sodium saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), 28.0 g glycerol (Sigma-Aldrich Co., St. Louis, MO, USA), 0.5 g peppermint oil (Synth, Diadema, São Paulo, Brazil), and adjusted with deionized water to 100 g. Fluoride (NaF, Merck, Darmstadt, Germany) was added to the gel at concentrations of 4500 (1.0 g of NaF), or 9000 ppm F (2.0 g of NaF), besides a fluoride-free gel (Placebo, negative control). An HMP-containing gel was also produced by the addition of HMP (Sigma-Aldrich Co., St. Louis, MO, USA) at a concentration of 9% to the 4500 ppm F gel. A commercial acidic gel was used as positive control (12,300 ppm F, Acid gel, pH = 4.5; DFL Indústria e Comércio S.A., Rio de Janeiro, RJ, Brazil). The ionic fluoride (IF) concentration in the gels and the pH were determined as previously described (Danelon et al., 2013; Danelon, Takeshita, Peixoto, Sassaki, & Delbem, 2014). Mean (SD, n = 2) IF concentrations were 23.7 (4.1); 4,519.2 (41.0); 4,514.6 (22.7); 9,487.5 (18.2); and 12,889.0 (15.4), respectively for Placebo, 4500F, 4500F + HMP, 9000F, and Acid gel. The mean (SD) pH of the neutral gels was 6.4 (0.2), ranging from 6.0 to 6.7. The pH of the Acid gel was 4.0 (0.6).

2.3. Induction of subsurface lesions

All surfaces of each block, except for the enamel surface, were coated with acid-resistant varnish (Risqué®-Brazil), and subsurface enamel demineralization was produced by immersing each enamel disc in 32 mL of a solution with 1.3 mmol/L Calcium, 0.78 mmol/L Phosphorus in 0.05 mol/L acetate buffer, pH 5.0; 0.03 ppm F; for 16 h at 37 °C (Queiroz, Hara, Leme, & Cury, 2008). Thereafter, surface hardness after demineralization (SH1) was determined and the mean and standard deviation (SD) for all enamel blocks was 58.9 (14.7) KHN, the lowest mean (SD) value of the groups was 57.1 (22.6) and the highest was 65.5 (11.2), without significant differences between groups (p = 0.631).

2.4. Treatment with the gels and pH-cycling

The gels were applied for 1 min to each block (3 g/block) only once (Danelon et al., 2013), removed with gauze and washed with deionized water for thirty seconds. After that, twelve blocks from each group were immersed (8 a.m.) in the remineralizing solution (1.5 mmol/L Calcium, 0.9 mmol/L Phosphorus, 0.15 mol/L Potassium Chloride in 0.02 mol/L cacodylate buffer, 0.04 ppm F, pH 7.0; 4 mL/block), for 4 h. Following (12 p.m.), the blocks were washed with deionized water, gently dried and immersed in the demineralizing solution (2.0 mmol/L Calcium and Phosphorus in 0.075 mol/L acetate buffer, 0.03 ppm F, pH 4.7; 12 mL/block) for 2 h (12 p.m. to 2 p.m.). At 2 p.m., the blocks were washed and immersed in the same remineralizing solution previously used. At 4 p.m., the blocks were washed and immersed in a fresh remineralizing solution until 8 a.m. on the following day. The blocks were subjected to pH-cycling in individual vials for 6 days at 37 °C (Vieira et al., 2005). Fresh de- and remineralizing solutions were used every day.

2.5. Analysis of enamel hardness

Surface hardness (SH) was determined with Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) and Buehler Omni Met software (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations, 100 μm apart, were made in the center of each block to analyze the SH. SH was measured again after the induction of artificially demineralized lesions (SH1) and after pH-cycling (SH2), at 100 mm from the initial indentations (SH) (Vieira et al., 2005). The percentage of SH recovery was then calculated (% SHR = [{SH2 − SH1}/{SH − SH1}] × 100). For cross-sectional hardness measurements, the blocks were sectioned at the center and one of the halves was embedded in acrylic resin and gradually polished. A sequence of 14 indentations was created at 5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 μm from the enamel surface, in the central region of the blocks, using a Micromet 5114 hardness tester (Buehler Lake Bluff, IL, USA) with a Knoop diamond indenter under a 5 g load, for 10 s. Integrated hardness (KHN × μm) for the lesion into sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN × μm) (Danelon et al., 2013).

2.6. Analysis of loosely-bound fluoride (CaF_2) on enamel

The concentration of loosely-bound fluoride (CaF_2) on enamel was analyzed after application of gels (CaF_2 formed) and after pH cycling (CaF_2 retained). A digital caliper (Mitutoyo CD-15B, Mitutoyo Corporation, Japan) was used to measure the surface area of the enamel blocks (n = 120) (Akabane et al., 2018). Assessment of loosely bound fluoride (alkali-soluble fluoride – CaF_2 formed and retained) was performed following the methodology of Caslavska, Moreno, and Brudevold (1975). The surface of each specimen, except for the treated surface, was coated with wax. Then, blocks were immersed in 0.5 mL of Potassium hydroxide 1.0 mol/L solution for 24 h under constant agitation. The solution was neutralized and buffered with 0.5 mL of TISAB II modified Hydrochloric acid. Fluoride content was determined using an ion-specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A$^+$, Thermo Scientific, Beverly, MA, USA) previously calibrated with standards 4.00–64.00 ppm F (100 ppm F, Orion 940907) that were used for the readings. The data obtained in mV were converted to μg F/cm^2 using Microsoft Excel.

2.7. Analysis of firmly-bound fluoride (FA) in enamel

Blocks measuring 2 mm × 2 mm (n = 120) were obtained from half of the longitudinally sectioned blocks and fixed to a mandrel coupled to a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, Athol, MA, USA) to measure enamel wear. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of a polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50.0 ± 0.03 μm each was removed from the enamel blocks by grinding the enamel surface against the polishing discs, in circular movements. The vials with the enamel

powder on the polishing discs, after the addition of 0.5 mL Hydrochloric acid (1.0 mol/L), were kept under constant stirring for 1 h (Weatherell, Robinson, Strong, & Nakagaki, 1985; Akabane et al., 2018). For firmly-bound fluoride analysis (FA formed and retained), an ion-specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and micro-electrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A$^+$, Thermo Scientific, Beverly, MA, USA) were used. The electrodes were calibrated with standards ranging from 0.25 to 4.00 ppm F (100 ppm F, Orion 940907) under the same conditions as the samples. The readings were conducted in 0.25 mL of the biopsy solution with the same volume of TISAB II modified Sodium hydroxide (Akabane et al., 2018). The results were expressed in μg/mm^3.

2.8. Statistical analysis

SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) was used, at a significance limit of 5%. Data presented normal (Shapiro-Wilk test) and homogenous (Cochran test) distributions and thus were analyzed by 1-way ANOVA (%SHR, and ΔKHN, on the natural outcomes) and 2-way ANOVA (CaF_2 and FA, log transformed data). Student-Newman-Keuls test was used for individual comparisons.

3. Results

Table 1 shows%SHR, ΔKHN, CaF_2 and FA formed and retained on/in enamel according to the groups. The%SHR of the group treated with 4500F + HMP was 18% and 22% higher than 9000F and Acid gel, respectively ($p < 0.05$), without significant differences between 9000F and Acid Gel ($p = 0.97$). As for ΔKHN, the 4500F + HMP gel promoted an approximate reduction of 16% compared to 9000F ($p < 0.001$). No significant differences were observed between 4500F + HMP and Acid gel ($p = 0.739$).

The highest CaF_2 concentrations formed on enamel were observed for the group treated with the Acid gel ($p < 0.001$), followed by 9000F > 4500F > 4500F + HMP > Placebo ($p < 0.001$). After pH cycling, CaF_2 concentrations were significantly reduced for all groups ($p < 0.001$). Regarding FA concentrations formed in enamel, a dose-response relationship was observed according to the fluoride concentration in the gels. After pH cycling, the highest concentrations were achieved by the Acid gel group, which promoted values 2.4 and 4.8 times higher compared with 9000F and 4500F + HMP, respectively ($p < 0.001$). Similar FA values retained in enamel were observed for the 4500F and 9000F groups ($p = 0.489$). The results observed for the Placebo group showed similarity between the concentrations of FA formed and retained ($p = 0.180$).

4. Discussion

The sodium hexametaphosphate concentration used was based on a previous *in vitro* study showing that the 4500F + HMP gel achieved a similar protective effect against enamel demineralization when compared with 9000F and Acid gel (surface hardness), and a superior effect on the subsurface when compared with 9000F (Danelon et al., 2012). Similar results were observed in the present study with the%SHR, showing a superior remineralizing effect of artificial carious lesions when compared with the 9000F and Acid gel groups. In addition, the sodium hexametaphosphate containing gel reduced the lesion area (integrated loss of subsurface hardness) by 30% when compared with 4500F. It is noteworthy that the test gel promoted a similar remineralizing effect as the Acid gel, despite the ~3-fold difference in fluoride concentration and the more reactive pH of the Acid gel. It is known that the application of an Acid gel is an effective method to reduce the incidence of dental caries (Marinho et al., 2015; Winter, Jablonski-Momeni, Ladda, & Pieper, 2017), and some *in vivo* and *in situ* studies have shown that it also enhances the remineralization of initial caries lesions and provides resistance to further enamel demineralization (Jardim, Pagot, & Maltz, 2008; Winter et al., 2017). It is also important to say that the cyclic phosphate used in the present study is different from the linear polymer of pyrophosphate used in stannous fluoride toothpaste, which also, confusingly, bears the name hexametaphosphate (Cochrane, Saranathan, Cai, Cross, & Reynolds, 2008).

One of the main advantages of applying topical fluorides at high concentrations is to promote the formation of loosely-bound fluoride on the enamel surface, which act as a slow-release, pH-dependent fluoride reservoir (Buzalaf, Pessan, Honório, & ten Cate, 2011; Carey, 2014). In our study, loosely-bound fluoride concentration measured immediately after gel application (formed) was shown to be dependent on the fluoride concentration (gels without sodium hexametaphosphate) and intensified by the acidic pH (Acid gel) which, along with other parameters assessed, validated the *in vitro* model used. The remineralizing effect of topical products with high fluoride concentration is related to loosely-bound fluoride deposition on enamel and its dissolution during the experiment *in vitro* (Danelon et al., 2012; Danelon et al., 2013; Danelon et al., 2014; Nagata et al., 2017). The pH of the vehicle also had a large influence on fluoride deposition and the degree of mineral loss. The remineralizing effect of the Acid gel (Table 1) was related to the high deposition of loosely-bound fluoride, seven times greater than that of the 9000F group (Table 1). Nonetheless, the light load used (25 g) and the greater loosely-bound fluoride clusters deposited by Acid gel group can partially explain the absence of difference between 9000F and Acid gel, since the loosely-bound fluoride clusters are softer than enamel and can affect the surface hardness measurements. The partial adherence to this hypothesis is because the hardness analyses were performed after the pH-cycling regimen, which consumed most of the loosely-bound fluoride reservoirs, as shown by the results. In addition to that point, the acidic gel also left the outer enamel more porous, which allowed the diffusion of calcium phosphate ions into deeper regions of the subsurface (as shows by cross-sectional hardness data), while the remineralization promoted by the 9000F gel was more restricted to the outer enamel regions. In the presence of sodium hexametaphosphate, a greater calcium flux inside the lesions in the deeper

Table 1
Mean (SD) values of percentage of surface hardness recovery (%SHR), integrated loss of subsurface hardness (ΔKHN), and concentrations of loosely-bound fluoride (CaF_2) and firmly-bound fluoride (FA) formed on/in enamel (6 h after application) and retained (after pH-cycling).

Groups	%SHR (KHN)	ΔKHN (KHN × μm)	CaF_2 (μg/cm^2)		FA (μg/mm^3)	
			Formed	Retained	Formed	Retained
Placebo	18.5^{a} (2.3)	8,305.0^{a} (902.5)	0.62^{a,A} (0.20)	0.47^{a,B} (0.06)	0.27^{a,A} (0.10)	0.34^{a,A} (0.10)
4500F	26.8^{b} (3.1)	6,559.2^{b} (466.8)	19.07^{b,A} (2.19)	0.72^{b,B} (0.16)	0.46^{b,A} (0.31)	1.06^{b,B} (0.31)
4500F + HMP	41.0^{c} (1.8)	4,578.3^{c} (354.2)	13.56^{c,A} (2.49)	0.68^{b,B} (0.29)	0.38^{b,A} (0.09)	0.54^{c,B} (0.13)
9000F	34.7^{d} (3.2)	5,847.3^{d} (354.2)	24.05^{d,A} (2.41)	1.20^{c,B} (0.4)	0.64^{c,A} (0.19)	1.00^{b,B} (0.58)
Acid gel	33.4^{d} (3.7)	4,684.3^{c} (686.1)	173.21^{e,A} (9.56)	2.10^{d,B} (0.52)	0.84^{d,A} (0.29)	2.37^{d,B} (0.87)

Lowercase letters indicate differences between groups in each column. Capital letters indicate the differences between CaF_2 formed and retained, and between FA formed and retained (Student-Newman-Keuls test, $p < 0.001$, $n = 12$).

layers can block the interprismatic pores at the lesion front, thus reducing acid diffusion to the underlying sound enamel (da Camara et al., 2015; da Camara et al., 2016). The study of da Camara et al. (2015) showed that sodium hexametaphosphate produces higher concentration of calcium enamel, probably due to formation of sodium hexametaphosphate – ion calcium layer on enamel, which reduces acid diffusion into enamel (van Dijk, Borggreven, & Driessens, 1980; da Camara et al., 2015). Sodium hexametaphosphate seems to facilitate the diffusion of ions during ionic exchanges (van Dijk et al., 1980), which is a consequence of its mechanism of action. Nevertheless, sodium hexametaphosphate cannot be considered as a source of phosphate that reacts spontaneously with the dental enamel, since it is not a hydrolysable compound (Choi, Wen, & Smith, 1993; Castellini, Lusvardi, Malavasi, & Menabue, 2005). Instead, it forms strong complexes with metal ions (Andreola, Castellini, Manfredini, & Romagnoli, 2004; Cochrane et al., 2008) in the oral environment, which are adsorbed by the enamel surface and the charged ions calcium fluoride and ion calcium are retained, replacing ion sodium in the cyclic structure, leading to a reticular formation (van Wazer & Campanella, 1950), by binding of ion calcium to one or more HMP molecules. As a result of these multiple connections, sodium hexametaphosphate molecules form a layer of condensed phosphates, changing the selective permeability of enamel (Cochrane et al., 2008) and, in this case, increased the cations selectivity. These data agree with the findings of da Camara et al. (2015) showing that the ionic activity of species such as ions calcium fluoride and calcium, as well as neutral species of neutral hydrogen fluoride and Phosphate hydrogenated calcium neutral in the dental biofilm formed *in situ*, were significantly higher when compared with the 1100 ppm F toothpaste.

Similarly, the amount of firmly-bound fluoride retained in enamel after pH-cycling is another relevant parameter of effect, which was also shown to be dose-dependent in the present study. Nonetheless, the addition of sodium hexametaphosphate to the 4500F significantly reduced both the amount of loosely-bound fluoride deposition on enamel immediately after application, and the amount of firmly-bound fluoride retained in enamel after pH-cycling. The results of enamel fluoride uptake (loosely- and firmly-bound) apart from those of the surface and cross-sectional hardness could suggest that the addition of sodium hexametaphosphate would not be a good strategy to enhance the remineralizing effect of the 4500F gel. However, taking all variables together, the results reinforce the concept that sodium hexametaphosphate enhances the therapeutic effect of fluoride both at the surface and subsurface following a different mechanism than that described for fluoride alone. Regarding the application time, this study adopted 1 min for topical gel treatments based on Delbem and Cury (2002). In that study, the results of enamel firmly-bound F forming after Acid gel and neutral gel application showed that 74% and 94%, respectively, had been deposited on enamel during the first minute, producing a similar resistance to enamel demineralization when compared with the 4-min application.

Although this hypothesis cannot be fully established on the basis of the study protocol used, the above effect may be related to an increase in the formation of neutral species (Phosphate hydrogenated calcium neutral and Hydrogen fluoride neutral) during the demineralization and remineralization cycles, which have a higher coefficient of diffusion to enamel, as compared with charged species (Cochrane et al., 2008). Similar results were also found in the same study performed by da Camara et al. (2015), demonstrating that the sodium hexametaphosphate/fluoride association led to an increase in phosphate hydrogenated calcium neutral and hydrogen fluoride neutral, producing a superior effect on reducing enamel demineralization when compared with a conventional toothpaste. This formation becomes even greater when the phosphate is in a molar ratio after adjusting for fluoride (hexametaphosphate/fluoride), thereby increasing the remineralizing effect, because both will act synergistically. In our study, the results for integrated loss of subsurface hardness summarize significant information, confirming the results obtained by Danelon et al. (2012) showing that the gel supplemented with sodium hexametaphosphate presents a similar effect when compared with the Acid gel.

Despite the promising data presented, results from *in vitro* studies cannot be directly extrapolated to clinical situations. It is, therefore, crucial to mention that cariogenic bacteria and different salivary compounds will influence the outcome during application of a particular fluoride product. In addition, the present study was limited to a 6-day period, while the de/remineralization processes are long-term processes. However, *in vitro* studies can estimate the role of new anticaries compounds in screening tests.

Based on the present results, it can be concluded that the addition of 9% sodium hexametaphosphate concentration to a gel with reduced fluoride concentration (4500F) was able to promote the remineralization of artificial carious lesions in this in *vitro study,* reaching protective levels similar to an acidic formulation with ~3-fold higher fluoride concentration.

Declaration of interest

The second author has a patent for a product used in the study, by the National Institute of Industrial Property – INPI/SP, on April 11, 2017 under number C1 0801811-1.

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