Mayra Frasson Paiva

Efeito de vernizes fluoretados suplementados com nanopartículas de Trimetafosfato de Sódio sobre o desgaste dental erosivo *in vitro* e *in situ*

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Orientador: Prof. Dr. Juliano Pelim Pessan
Coorientador: Prof. Dr. Alberto Carlos Botazzo Delbem
Coorientadora: Prof. Dr. Annette Wiegand

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Dedicatória

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"Na realidade, todas as coisas,
todos os acontecimentos, para quem os sabe ler com
profundidade, encerram uma mensagem que, em
definitivo, remete para Deus."

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Resumo

PAIVA, M.F. **Efeito de vernizes fluoretados suplementados com nanopartículas de Trimetafosfato de Sódio sobre o desgaste dental erosivo *in vitro* e *in situ*.** 2020 99 f. Tese (Doutorado em Ciência Odontológica, Área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

RESUMO

O presente estudo avaliou o efeito de vernizes fluoretados suplementados com nanopartículas de Trimetafosfato de Sódio (TMP) sobre o desgaste erosivo do esmalte dental bovino, em protocolos *in vitro* e *in situ*. Para a 1ª fase, blocos de esmalte dental bovino (*n*=100) foram selecionados por meio de dureza de superfície (DS) e aleatoriamente divididos em 5 grupos experimentais (*n*=20/grupo), de acordo com os vernizes testados: (a) Placebo (Pla - sem F ou TMP), (b) 5% NaF, (c) 5% NaF + 5% TMP microparticulado (5% Micro), (d) 5% NaF + 2,5% TMP nanoparticulado (2,5% Nano), (e) 5% NaF + 5% TMP nanoparticulado (5% Nano). Os blocos receberam uma única aplicação dos vernizes e foram imersos em saliva artificial por 6 h. Em seguida, os vernizes foram removidos e todos os blocos, submetidos a 4 desafios erosivos diários durante 5 dias (ERO, imersão em ácido cítrico 0,05 M, pH 3,2, 90 s/ciclo, sob agitação). Após ERO, metade dos blocos foi submetida a abrasão por escovação (15 s/ciclo) com dentifrício placebo (ERO+ABR). Os blocos foram analisados por perfilometria, dureza de superfície (DS) e dureza em secção longitudinal (ΔKHN). Os dados foram submetidos a ANOVA a dois critérios e Teste de Fisher LSD ($p<0,05$). O desgaste do esmalte foi significativamente menor para ERO comparado a ERO+ABR para todos os vernizes testados ($p<0,001$), seguindo o padrão 5% Nano < 5% Micro < 5% NaF < 2,5% Nano < Pla (ERO e ERO+ABR). A maior perda de DS foi observada para o Pla e a menor para 5% NaF (ERO) e 2,5% Nano (ERO+ABR), sem diferenças significativas entre 2,5% Nano, 5% NaF e 5% Micro. Os maiores valores de ΔKHN foram observados para 5% Micro e 5% Nano a 5-30 µm, com diferenças menos acentuadas entre os grupos a 30-70 µm (ERO e ERO+ABR). Para a 2ª fase, blocos de esmalte bovino (*n*=224) foram selecionados por DS e

distribuídos aleatoriamente entre os grupos: (a) Placebo (Pla - sem F ou TMP), (b) 5% NaF, (c) 5% NaF + 5% TMP microparticulado (5% Micro), e (d) 5% NaF + 5% TMP nanoparticulado (5% Nano). Os blocos foram inseridos em dispositivos acrílicos palatinos (*n*=4/dispositivo), e tratados com os vernizes uma única vez, permanecendo na cavidade bucal dos voluntários (*n*=14) por 6 h. Em seguida, os vernizes foram removidos e os blocos, submetidos à ERO (imersão *ex vivo* em ácido cítrico 0,05 M, pH 3,2, 90 s, 4x/dia), enquanto dois blocos foram adicionalmente submetidos a abrasão por escovação com dentifrício fluoretado (ERO+ABR), totalizando 5 dias em cada etapa experimental, seguindo um protocolo duplo-cego e cruzado. As análises dos blocos e dos dados foram idênticas às da 1ª fase. Os valores do desgaste seguiram um padrão similar em ambas as condições experimentais (ERO ou ERO+ABR), com 5% Nano < 5% Micro < 5% NaF < Pla. Um padrão similar foi observado para dureza em secção longitudinal (ΔKHN), apesar de não serem verificadas diferenças significativas entre 5% Micro×5% Nano (5-30 µm). Quanto à perda de DS, o maior valor foi observado para Pla e o menor para 5% Nano (ERO ou ERO+ABR), sem diferenças significativas entre Pla×5% NaF (ERO), 5% NaF×5% Micro (ERO+ABR), e 5% Micro×5% Nano (ERO+ABR). Diante dos resultados, conclui-se que a adição de TMP a vernizes fluoretados melhorou significativamente a proteção contra o desgaste erosivo do esmalte *in vitro* e *in situ*. O uso de 5% de TMP em escala nanométrica aumentou ainda mais esses efeitos.

Palavras–chave: Esmalte dentário, Fluoretos, Fosfatos, Erosão dentária, Verniz.

Abstract

PAIVA, M.F. **Effect of fluoride varnishes supplemented with nanosized Sodium Trimetaphosphate on enamel erosive wear *in vitro* and *in situ.*** 2020 99 f. Tese (Doutorado em Ciência Odontológica, Área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

ABSTRACT

The present study evaluated the effect of fluoride (F) varnishes supplemented with sodium trimetaphosphate (TMP) nanoparticles on erosive tooth wear, using *in vitro* and *in situ* protocols. For the first phase, bovine enamel blocks (*n*=100) were selected by surface hardness (SH) and randomly divided into 5 experimental groups (*n*=20/group), according to the varnishes tested: (a) Placebo (Pla - without F or TMP), (b) 5% NaF, (c) 5% NaF + 5% micrometric TMP (5% Micro), (d) 5% NaF + 2.5% nano-sized TMP (2.5% Nano), (e) 5% NaF + 5% nano-sized TMP (5% Nano). Blocks received a single varnish application, and were immersed in artificial saliva for 6 h. Varnishes were then removed and all blocks, subjected to 4 daily erosive challenges during for 5 days (ERO, immersion in 0.05 M citric acid, pH 3.2, 90 s/cycle, under agitation). After ERO, half of the blocks were subjected to abrasion by brushing (15 s/cycle) with placebo dentifrice (ERO+ABR). Blocks were analyzed by profilometry, surface hardness (SH) and cross-sectional hardness (ΔKHN). The data were submitted to 2-way ANOVA and Fisher's LSD test ($p<0.05$). Enamel wear was significantly lower for ERO compared to ERO+ABR for all varnishes tested ($p<0.001$), following the pattern 5% Nano < 5% Micro < 5% NaF < 2.5% Nano < Pla (ERO and ERO+ABR). The highest SH loss was observed for Pla, and the lowest for 5% NaF (ERO) and 2.5% Nano (ERO+ABR), without significant differences between 2.5% Nano, 5% NaF and 5% Micro. The highest values of ΔKHN were observed for 5% Micro and 5% Nano at 5-30 µm, with less marked differences between the groups at 30-70 µm (ERO and ERO+ABR). In the second phase, bovine enamel blocks (*n*=224) were selected by SH and randomly distributed among the groups: (a) Placebo (Pla - without F or TMP), (b) 5% NaF, (c) 5% NaF + 5% micrometric TMP (5% Micro), and (d) 5% NaF + 5% nano-sized TMP

(5% Nano). The blocks were inserted in acrylic palatal devices (*n*=4/device), and treated with the varnishes only once, remaining in the oral cavity of the volunteers (*n*=14) for 6 h. Then, the varnishes were removed and the blocks, subjected to ERO (*ex vivo* immersion in 0.05 M citric acid, pH 3.2, 90 s, 4x/ day), while two blocks were additionally subjected to abrasion by brushing with fluoride dentifrice (ERO+ABR), totaling 5 days in each experimental stage, following a double-blind, crossover protocol. The blocks and the data were analyzed as described for the first phase. The wear values followed a similar pattern under both experimental conditions (ERO or ERO+ABR), with 5% Nano < 5% Micro < 5% NaF < Pla. A similar pattern was observed for hardness in depth (ΔKHN), although no significant differences were found between 5% Micro×5% Nano (5-30 µm). As for SH loss, the highest value was observed for Pla, and the lowest for 5% Nano (ERO or ERO+ABR), without significant differences between Pla×5% NaF (ERO), 5% NaF×5% Micro (ERO+ABR), and 5% Micro×5% Nano (ERO + ABR). In view of the results, it was concluded that the addition of TMP to fluoride varnishes significantly improved protection against erosive enamel wear *in vitro* and *in situ*. The use of 5% nano-sized TMP further increased these effects.

Keywords: Dental enamel, Fluorides, Phosphates, Dental erosion, Varnish.

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CAPÍTULO 1

CAPÍTULO 2

Lista de abreviaturas e símbolos

LISTA DE ABREVIATURAS E SÍMBOLOS

ANOVA	Análise de Variância
α	Alfa
β	Beta
CAAE	Certificado de Apresentação para Apreciação Ética
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CNPq	Conselho Nacional de Desenvolvimento Científico
CaF_2	Fluoreto de cálcio
Ca^{2+}	Íon cálcio
DDE	Desgaste dentário erosivo
DS	Dureza de superfície
ERO	Erosão
ERO+ABR	Erosão+Abrasão
ETW	Erosive tooth wear/Desgaste dentário erosivo
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
F	Fluoreto
g	Grama
h	Hora (s)
i.e.	id est/isto é
KHN	Knoop Hardness Number/Número de Dureza Knoop
Kgf/mm^2	Kilograma força por milímetro quadrado
L	Litro
LSD	Least Significant Difference/Diferença Menos Significativa
mg	Miligrama
mL	Mililitro
mm	Milímetro
mmol	Milimol
mV	Milivoltagem/Milivolt
min	Minuto
M	Molar
µ	Micro
µg	Micrograma

µm	Micrômetro
nm	Nanômetro
NaF	Fluoreto de sódio
pH	Potencial Hidrogeniônico
ppm	Partes por milhão
q.s.p.	Quantidade suficiente para
s	Segundo (s)
SH	Surface Hardness/Dureza de superfície
TMP	Trimetafosfato de sódio
UFSCar	Universidade Federal de São Carlos
UNESP	Universidade Estadual Paulista
XRD	X-ray diffraction/Difração de raio-x
ΔKHN	Integrated hardness loss in depth/Perda integrada da dureza em profundidade
~	Approximately/Aproximadamente

Sumário

SUMÁRIO

Introdução Geral

INTRODUÇÃO GERAL

A erosão dentária é considerada uma lesão não cariosa, definida como a dissolução química da estrutura mineralizada dos dentes, causada pela exposição a ácidos não provenientes de bactérias (Schlueter et al., 2019). Sua etiologia envolve fatores intrínsecos ao indivíduo (como distúrbios de refluxo e vômito) e, principalmente, fatores extrínsecos, relacionados ao consumo frequente de alimentos e bebidas ácidas (Lussi & Carvalho, 2014). O contato entre o ácido e a superfície dentária leva ao amolecimento da mesma, tornando-a mais suscetível a impactos mecânicos, como a abrasão por escovação e atrito com os alimentos. A interação entre os fatores químicos e físicos pode resultar na perda cumulativa da superfície mineralizada, a qual é denominada desgaste dentário erosivo (DDE) (Schlueter et al., 2019).

O DDE tem sido observado com frequência na prática clínica atual. Sua prevalência mundial é estimada entre 30 a 50% para dentes decíduos e 20 a 45%, para os permanentes (Schlueter & Luka, 2018). No Brasil, a prevalência entre crianças e adolescentes varia entre 20% a 78% (Rios et al., 2007; Gurgel et al., 2011, Salas et al., 2017), sendo esta ampla variação possivelmente devida a diferentes critérios de diagnóstico, idade dos pacientes, dentre outros fatores. Devido à dificuldade na realização do diagnóstico em estágios precoces, lesões erosivas são frequentemente diagnosticadas em estágios moderado ou avançado, quando já ocorreu perda de estrutura dentária. Considerando o caráter cumulativo e irreversível do DDE, estratégias têm sido propostas com a finalidade de prevenir ou minimizar seus efeitos sobre a estrutura dentária. Estas incluem a redução na ingestão de alimentos e bebidas ácidas, e a aplicação de fluoretos (F) tópicos (Ganss, 2008; Salas et al., 2015).

Apesar da eficácia clínica comprovada no controle da cárie dentária (Featherstone, 1999; Marinho, 2009; O'Mullane et al., 2016), os produtos fluoretados tópicos apresentam efeito limitado sobre a erosão (Ganss et al., 2011), de forma que alternativas para potencializar o efeito do F sem aumentar seus efeitos adversos têm sido intensivamente estudadas. A adição de sais de fosfato, como o trimetafosfato de sódio (TMP), a dentifrícios, géis e enxaguatórios fluoretados, demonstrou aumentar significativamente seus efeitos protetores e terapêuticos contra a cárie dentária (Danelon et al., 2014;

Favretto et al., 2013; Takeshita et al., 2009, 2015), bem como sobre a erosão e DDE (Cruz et al., 2015; Manarelli et al., 2011; Moretto et al., 2010; Pancote et al., 2014). Esta associação também foi avaliada para vernizes fluoretados, demonstrando haver um efeito sinérgico sobre a redução do DDE, em protocolos *in vitro* e *in situ* (Manarelli et al., 2013; Moretto et al., 2013).

A fim de se aumentar ainda mais os efeitos preventivos e terapêuticos de veículos fluoretados contendo TMP, nanopartículas deste fosfato (TMPnano) foram adicionadas a um dentifrício de 1100 ppm F, o que reduziu significativamente a desmineralização do esmalte *in vitro* (Danelon et al., 2017) e aumentou seu efeito remineralizador *in situ* (Danelon et al., 2015). Esta formulação também se mostrou eficaz em aumentar os efeitos protetores sobre a erosão quando comparada a formulações de mesma concentração de F, sem suplementação de TMP (Danelon et al., 2018).

Considerando as vantagens anteriormente citadas com relação à suplementação de dentifrícios fluoretados com TMPnano, aventou-se a possibilidade de que a adição de TMPnano a vernizes promovesse um aumento do potencial preventivo e terapêutico destas formulações. Esta hipótese, testada por Báez-Quintero (2017), demonstrou que a adição de TMPnano a vernizes fluoretados convencionais (contendo 5% NaF) levou a um aumento no potencial remineralizador destes produtos em comparação a vernizes suplementados com TMPmicro, em um modelo *in vitro* utilizando lesões de cárie artificial em esmalte dental bovino. Tais efeitos foram também observados sobre a erosão dental, demonstrando que o verniz contendo TMPnano reduziu significativamente a progressão de lesões erosivas iniciais do esmalte, com um efeito adicional do TMPnano sobre o TMPmicro. Ressalta-se, entretanto, que estes resultados foram obtidos por meio da análise de dureza de superfície, que é considerada como o método mais adequado para o estudo de lesões erosivas iniciais (Schlueter et al., 2016), onde ainda não ocorreu perda da estrutura dentária (ou seja, DDE, aferido por perfilometria).

Os resultados promissores obtidos no estudo supracitado (lesões erosivas iniciais) motivaram a investigação do efeito dos mesmos vernizes considerando outras variáveis relevantes, a fim de se aumentar a capacidade de extrapolação dos resultados para a prática clínica. Estas incluem (1) o efeito adicional de forças mecânicas sobre a perda de estrutura de esmalte (DDE);

(2) a formação da película adquirida do esmalte, a qual poderia afetar a interação entre os princípios ativos dos vernizes (F e TMP) e a superfície do esmalte; (3) análise da perda estrutural do esmalte (perfilometria) somada à avaliação do conteúdo mineral (dureza); e (4) exposição simultânea ao F a partir do dentifrício.

Com base no exposto, propusemos investigar os efeitos de vernizes fluoretados suplementados com TMPnano, em comparação a um verniz contendo TMPmicro e um verniz contendo apenas NaF, avaliando tanto o processo de erosão dental (efeito químico) como o DDE (efeito químico + desgaste mecânico), contemplando as variáveis listadas acima. Este estudo poderia trazer informações importantes sobre os reais efeitos das formulações testadas em condições mais próximas das observadas clinicamente, bem como sobre os mecanismos de ação envolvidos (Anexo A).

Para abordar o tema proposto, o estudo será apresentado em dois capítulos, conforme descrito abaixo:

- Capítulo 1: **"Effect of fluoride varnishes supplemented with nano-sized sodium trimetaphosphate on enamel erosive wear *in vitro*"***
- Capítulo 2: **"Fluoride varnish containing nano-sized sodium trimetaphosphate reduces enamel erosive wear *in situ*"***

*artigos formatados de acordo com as normas do periódico Journal of Dentistry (Anexo B)

Effect of fluoride varnishes supplemented with nano-sized sodium trimetaphosphate on enamel erosive wear *in vitro*

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Short tittle: F varnishes containing nanosized TMP reduce enamel erosive wear

Key words: Dental enamel, Fluoride, Polyphosphates, Prevention, Tooth erosion.

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Declaration of interest

The authors declare no conflict of interest that may affect the manuscript judgment.

Effect of fluoride varnishes supplemented with nano-sized sodium trimetaphosphate on enamel erosive wear *in vitro*

Abstract

Objective: To evaluate the effect of fluoride (F) varnishes with Sodium Trimetaphosphate (TMP) on erosive tooth wear (ETW) *in vitro*. *Methods:* Enamel blocks (*n*=100) were divided into 5 experimental groups (*n*=20/group): Placebo (Pla - without F/TMP); 5% NaF; 5% NaF + 5% micrometric TMP (5% Micro); 5% NaF + 2.5% nano-sized TMP (2.5% Nano), and 5% NaF + 5% nano-sized TMP (5% Nano). Blocks received a single varnish application (6 h contact), and were submitted to 4 daily erosive challenges (ERO, 0.05 M citric acid, pH 3.2, 90 s, under agitation), during 5 days. After ERO, half of the blocks (*n*=10/group) was subjected to brushing abrasion (ERO+ABR). Profilometry, surface hardness (SH) and cross-sectional hardness (ΔKHN) were determined. The data were submitted to 2-way ANOVA and Fisher LSD test ($p<0.05$). *Results:* Enamel wear was significantly lower for ERO compared with ERO+ABR for all varnishes tested ($p<0.001$), following the pattern 5% Nano < 5% Micro < 5% NaF < 2.5% Nano < Pla (both for ERO and ERO+ABR). The highest SH loss was observed for Pla and the lowest for 5% NaF (ERO) and 2.5% Nano (ERO+ABR), without significant differences among 2.5% Nano, 5% NaF and 5% Micro. The highest ΔKHN values were observed for 5% Micro and 5% Nano at 5-30 µm, with less marked differences among the groups at 30-70 µm (ERO and ERO+ABR). *Conclusion:* The addition of TMP to F varnishes significantly improves protection against ETW *in vitro*. The use of 5% nano-sized TMP further enhances such effects. *Clinical significance:* F varnishes containing TMP can reduce enamel loss caused by ERO or ERO+ABR.

Introduction

With the reduction of dental caries prevalence observed in recent years worldwide, erosive tooth wear (ETW) has becoming an increasingly frequent clinical condition due to changes in lifestyle and eating habits [1,2]. Early diagnosis of erosive lesions is difficult to perform, as it is a painless process involving discrete alterations at the onset which, thus, may go unnoticed. Despite ETW can be considered, to a certain degree, as a physiological condition, it can turn into a pathological lesion over time, and can cause damages from tooth sensitivity to pulp exposure, thus requiring repeated expensive restorative treatment [3,4].

Some alternatives have been proposed to prevent the onset and progression of dental erosion, such as changing eating habits (reducing consumption of acidic foods and beverages) and the use of topical fluorides (F), such as dentifrices, gels, mouthrinses and varnishes [5-9]. F products promote the formation of calcium fluoride (CaF_2) on dental surfaces, which is slowly released under acidic challenges, favoring the process of dental remineralization [10]. Despite their proven clinical efficacy against the development of dental caries (characterized by initial subsurface lesions), F vehicles have limited effect on erosion (characterized by irreversible mineral loss at the surface), so that their effects can be essentially related to the protection of the surface against mineral loss rather than its remineralization [11].

This scenario has prompted to investigations of alternatives to enhance the anti-erosive effects of F vehicles, with promising results reported for products supplemented with calcium salts and/or organic or inorganic phosphates [12]. Sodium trimetaphosphate (TMP) is an inorganic phosphate with high affinity to mineralized tissues, acting as a stable barrier against acid diffusion, which reduces the dissolution of hydroxyapatite [8]. This polyphosphate has been shown to be effective in reducing erosion and ETW when combined with F in dentifrices, mouthrinses, gels and varnishes [8,9,13,14]. Considering the advantages of F varnishes related to ease of application, safety and efficacy [15,16], a series of studies has assessed the effects of the addition of TMP to these products, demonstrating a benefit on

enamel de- and re-mineralization, as well as on ETW, using *in vitro* and *in situ* protocols [8,16-19].

The synergistic effects of F and TMP have recently been shown to be potentiated with the use of nanoparticles of this phosphate. For F varnishes, a recent study reported a superior effect of nano-sized TMP on initial erosive enamel lesions compared with micrometric particles [20]. Despite the promising results, this study only assessed early erosive lesions and used a single analytical tool (surface hardness). Although this method is suitable for the study of initial erosive lesions [21], the results on mineral loss obtained cannot be directly extrapolated to structural loss resulting from the cumulative effect of successive erosive challenges, associated or not with abrasion.

Based on the above, the aim of this study was to evaluate the effect of F varnishes supplemented with TMPnano on enamel erosive wear *in vitro*, using complementary analytical tools for a better understanding of the effects of these products both on enamel loss and on the mineral content of the remaining enamel. The null hypothesis was that enamel erosive wear would be similar among groups treated with varnishes supplemented with TMP at micrometric or nano-sized particles.

Materials and Methods

Experimental design

Bovine enamel blocks were selected by surface hardness (SH), and randomly divided into 5 experimental groups (*n*=20/group) according to the varnishes to be tested: Placebo (Pla - without F/TMP); 5% NaF; 5% NaF + 5% micrometric TMP (5% Micro); 5% NaF + 2.5% nano-sized TMP (2.5% Nano), and 5% NaF + 5% nano-sized TMP (5% Nano), following a blind protocol. All blocks received a single application of the varnishes and were immersed in artificial saliva for 6 h. Following, varnishes were removed and all blocks were submitted to 4 daily erosive challenges for 5 days (ERO, 0.05 M citric acid, pH 3.2, 90 s/cycle, under agitation). Half of the blocks were subsequently immersed in placebo dentifrice slurry during 15 seconds (ERO), and the other half was subjected to abrasion by brushing (5 strokes/s) with a placebo dentifrice (ERO+ABR) [9]. Blocks were analyzed by profilometry and by surface and cross-sectional hardness.

Enamel blocks preparation

Enamel blocks (*n*=100) measuring 4 × 4 × 2 mm were obtained from bovine incisors previously stored in 2% neutral formaldehyde solution (pH 7.0) for 1 month. They were obtained from the flattest portion of the crowns and fixed in acrylic resin discs using sticky wax. Firstly, the dentine was polished (320 grit) and subsequently the enamel (600, 800, 1200 grit), followed by polishing with felt paper and 1µ diamond suspension [22,23] (Anexo E). Sample size was calculated based on a pilot study, according to which 9 blocks would be required to detect significant differences in mean enamel wear of blocks treated with a 5% NaF varnish (positive control) and a 5% NaF varnish supplemented with 5% nano-sized TMP (mean difference = 2.0, standard deviation = 1.1), considering a power of 80% (α=0.05). Due to the possibility of losses during the processing of the specimens, 10 blocks were included in each group.

Formulation and dosage of fluoride of experimental varnishes

The varnishes were manufactured by SS White Produtos Odontológicos (Rio de Janeiro, RJ, Brazil), containing the following components: film-forming polymer (artificial resin), solvent (ethanol), essence (saccharin) and demineralised water (q.s.p.) (Anexo F). The synthesis and characterization of nanosized TMP was performed at the Federal University of São Carlos (UFSCar). The resulting powder was analyzed by X-ray diffraction (XRD), as reported by Danelon et al. (2017) [24]. The X-ray diffraction analysis showed an average particle size estimated at 22.7 nm, without alteration of its crystalline structure. The varnishes contained 5% NaF with or without the addition of 5% micrometric TMP or 2.5% or 5% nano-sized TMP. A placebo varnish (without the addition of NaF or TMP, negative control) was also produced. F concentrations was determined using an ion-specific electrode (9609 BN - Orion) and ion analyzer (Orion 720 A+), previously calibrated with standards at 2.0, 4.0, 8.0, 16.0 and 32.0 µg F/mL, according to the protocol used by Manarelli et al. (2014) [16]. The data obtained in mV were converted to µg F/mL.

Treatment of the blocks and erosive challenges

Half of the surface of each block was protected with an acid-resistant nail varnish (Risqué®, Brazil) (control area), so that the other half (test area) was subjected to treatment with the varnishes and to the ERO or ERO+ABR challenges. The varnishes were applied in a thin layer and the blocks were immersed in artificial saliva (1.5 mmol.l^{-1} $Ca(NO_3)_2.4H_2O$; 0.9 mmol.l^{-1} $NaH_2PO_4.2H_2O$; 150 mmol.l^{-1} KCl; 0.1 mol.l^{-1} Tris buffer) [13] for 6 h. Then, the varnishes were removed with spatula and acetone (Sigma-Aldrich CO., St. Louis, MO, USA), the blocks were washed with deionized water and dried with absorbent paper. All blocks were submitted to 4 daily acid treatments (ERO, immersion in 0.05 M citric acid (Synth®, Brazil), pH 3.2, 90 s/cycle, under agitation frequency 35 rpm/min; Shaker SK-300, São Paulo, Brazil) over 5 days. Subsequently, half of the blocks were immersed in placebo dentifrice slurry for 15 seconds (under agitation frequency 35/min) [9]. ERO+ABR were produced on half of the blocks of each group, by brushing using a brushing machine (250 g axial load, 5 strokes/s, 15 s; Elquip Maq Escovação, São Carlos, Brazil) and a slurry of a placebo (F-free) dentifrice. Brushing was performed immediately after the immersion in citric acid [21]. All blocks were stored in artificial saliva [8] for 2 h between the challenges (Anexos G e H).

Analysis of enamel wear - Profilometry

At the end of the treatment, the nail varnish was carefully removed from the control areas of each block with spatula and acetone, starting from the edges to avoid damages to the surface (Sigma-Aldrich CO., St. Louis, MO, USA) for the analysis. Enamel loss was performed by profilometry (Surftest SJ 401 - Mitutoyo American Corporation), and the Surftest software (Anexo I). The surface of each block was scanned from the reference (control) surfaces through the exposed surfaces, with measuring length of 0.8 mm. The average value of 5 readings was calculated for each block [8].

Analysis of surface and cross-sectional hardness

The surface microhardness was determined before (SH_1) (Anexo J) and after (SH_2) (Anexo K) the experiments using the Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and Knoop indenter (KHN), under 25 g for 10 s. Five indentations spaced 100 μm

from each other were performed at the center of the block (SH_1), and five equidistant impressions were performed in relation to the initial impressions (SH_2). After, enamel blocks were cross-sectioned at the center, and half of each block were embedded in acrylic resin and subsequently polished. A sequence of 9 impressions at distances of 5, 10, 15, 20, 25, 30, 40, 50, 70 µm from the external enamel surface was performed in the center of blocks, for both the control and the test areas (Anexo L). The integrated area of hardness (KHN × µm) of the lesion up to the intact enamel was calculated using the Trapezoidal rule (ΔS*(Y1+Y2)/2; GraphPad Prism, version 3.02). The difference of each depth of the healthy enamel and the value of the integrated area loss was calculated (ΔKHN = loss of hardness in depth in the enamel - area under the curve) [25].

Statistical analysis

The data were analyzed using the SigmaPlot version 12.0. Data of Enamel loss (profilometry) and ΔKHN presented normal distribution (Shapiro-Wilk), while %SH loss did not. Enamel loss and %SH loss were submitted to 2-way ANOVA, followed by Fisher's LSD test. For ΔKHN, data of ERO and ERO+ABR (both normally distributed) were analyzed separately by 2-way ANOVA, followed by Fisher's LSD test, considering the varnishes and depths (5 to 30 µm and 30 to 70 µm) as independent variables. A significance level of 5% was adopted.

Results

Enamel wear was higher for ERO+ABR than ERO for all groups, with significant differences for all pairwise comparisons except for the 5% Micro varnish. The lowest values were observed for 5% Nano, followed by 5% Micro, 5% NaF, 2.5% Nano and Pla, with significant differences among all groups both for ERO and ERO+ABR. The only exception was the comparison between 5% NaF and 2.5% Nano under ERO+ABR (Figure 1).

The highest SH loss was observed for Pla, and the lowest for 5% NaF (ERO) or 2.5% Nano (ERO+ABR), without significantly differences among 2.5% Nano, 5% NaF and 5% Micro (Figure 2). As for ΔKHN (area under the curve), differences among the groups were more pronounced at 5-30 µm compared

with 30-70 µm from the enamel surface (Figure 3). For both for ERO and ERO+ABR, the highest ΔKHN values were observed for 5% Micro and 5% Nano, followed by 5% NaF, 2.5% Nano, and Pla, with no significant differences between 5% Micro and 5% Nano, and between 5% NaF and 2.5% Nano.

Discussion

The present study demonstrated that the addition of TMP to a conventional F varnish significantly increased the protective effect against enamel erosion and erosive wear, and that the use of nanoparticles of this polyphosphate led to an additional protective effect. Consequently, the null hypothesis has to be rejected.

The highest protective effect was observed for nano-sized TMP at 5% compared to 2.5%, emphasizing the need for establishing an appropriate F:TMP molar ratio in order to achieve maximum benefits. The results corroborate with a previous study assessing the same varnishes as in the present study on initial enamel erosion [20]. These authors showed that the benefit of TMP micro was around 43% higher compared with 5% NaF, and such effects further increased to 60-67% when TMP nano was added to the varnishes, clearly demonstrating the influence of particle size. The above mentioned results were attributed to the higher contact surface of nanoparticles, as well as higher percentage of atoms on surface compared to micrometric ones, thus enhancing their reactivity [27]. The lower particle size of TMP nano might also be associated with a higher interaction with the varnish matrix, which may result in a lower mobility to the oral environment. Therefore, TMP nano at 5% promoted a more pronounced effect than at 2.5%, possibly due to a higher release of TMP from the varnish [20]. Furthermore, a recent study with F gels supplemented with TMP nano also reported higher protective effects of TMP nano over TMP micro on enamel erosive wear *in vitro* [28], corroborating the present findings. Despite these *in vitro* observations cannot be directly extrapolated to *in vivo* conditions, they clearly show that such formulations may be a good alternative against enamel erosive wear.

It is important to note that, under erosive conditions, F essentially has a protective effect against mineral loss, and does not act on the remineralization of hydroxyapatite crystals as occurs in caries lesions. This is due to the

permanent loss of crystals of the surface layer after ERO, leaving the outer layer softened. For ERO+ABR, the softened enamel was immediately subjected to abrasion, leading to loss of the acid-softened layer. This event explains the higher percentage of erosive enamel wear after ERO+ABR than after ERO only.

As for SH loss, on the other hand, higher values were observed for ERO compared with ERO+ABR, and the addition of TMP did not affect the outcomes, regardless of the particle size tested. Such trend seems to be in line with data of enamel wear, given that SH data on enamel subjected to ERO is related to a more softened surface in comparison with ERO+ABR [13]. Thus, these results emphasize the concept that SH analysis provides limited data in studies assessing advanced erosive lesions.

Data on mineral loss in depth (ΔKHN), in turn, provide more robust information on the mineral changes on the remaining enamel resulting from erosive challenges, followed or not by abrasion [15]. Considering that the effects of erosive challenges on enamel are more restricted to its outermost layers, ΔKHN data in the present study were analyzed at two depths (*i.e.,* 5-30 or 30-70 µm from the enamel surface). At 5-30 µm, the results showed that TMP-containing varnishes led to significantly lower mineral loss compared to the conventional varnish (without TMP). Given that the formation of CaF_2 is significantly reduced for these varnishes [16], their superior protective effect cannot be explained by the formation of such deposits, but instead by the high affinity of TMP to enamel surfaces, by replacing phosphate groups or binding to calcium (Ca^{2+}) on hydroxyapatite. TMP adsorption thus forms an acid diffusion barrier, leading to the formation of a more stable mineral, affecting the de- and remineralization processes [15,16]. Interestingly, the results show that the effects of TMP were sustained even after the superficial enamel layers were removed due to the initial ERO/ERO+ABR challenges. This suggests that, differently CaF_2, which is dissolved during the acid challenge, TMP stay attached to the remaining enamel, when there is saturation of Ca, P and TMP in the microenvironment. This is very important from a clinical standpoint, since F products are known to be most effective at the outermost enamel layers (*i.e.*, promoting its hypermineralization) with less pronounced effects in depth [10]. Within the context of preventive measures for enamel erosion, the formation of a barrier resistant to acid attacks is more desirable than promoting effects on

enamel rehardening, given that successive erosive challenges may hinder mineral precipitation [29,30].

The addition of TMP (at both particle sizes) to a conventional fluoride varnish led to the lowest wear and highest values of integrated hardness in depth (ΔKHN). A similar trend was reported by Manarelli et al. (2013) [8]. It is noteworthy, however, that the overall wear values of the present study were 5-, 3- and 4-fold higher to those observed in the above-mentioned study [8] for Pla, 5% NaF and 5% Micro. Differences in the type of acid and pH, mode of exposure to the acid (static or dynamic) and duration of exposure seem to be the reasons for the above-mentioned differences. In the study by Manarelli et al. (2013) [8], the erosive challenge was performed by immersing the samples in a soft drink (Sprite, pH = 2.8) during 5 minutes, without agitation. It is known that static challenges imply in initial calcium and phosphate release, followed by saturation of the micro environment closer to the enamel surface, thus reducing mineral loss. The dynamic model used in the present study [21] bypasses the issue of saturation, and more closely resembles acid exposure occurring in the oral cavity. Dynamic conditions at the enamel-acid interface are determinant for the extent of tissue loss [31,32] because new hydrogen ions are supplied, so that dissolved minerals are removed faster than in slow motion or static conditions, where local saturation phenomenon may occur [33]. Another important issue to be considered is the acid type and the titratable acidity. The effects of citric acid on enamel dissolution have been intensively studied [34-37]. It dissociates in three steps, therefore can act as a buffer and can resist pH changes. Other components associated with acid may also influence its erosive power. The use of citric acid (1%) associated with agitation in the present study may also explain the higher wear compared to other studies that used static methodology during the challenge [33].

Based on the aforementioned considerations, we can conclude that TMP significantly improves the protective effects of F varnishes against enamel erosive wear *in vitro,* and the use of nano-sized TMP at 5% further enhances such effects. Furthermore, as TMP can reduce enamel softening after erosive challenges, it can be considered as a promising alternative for preventing ETW.

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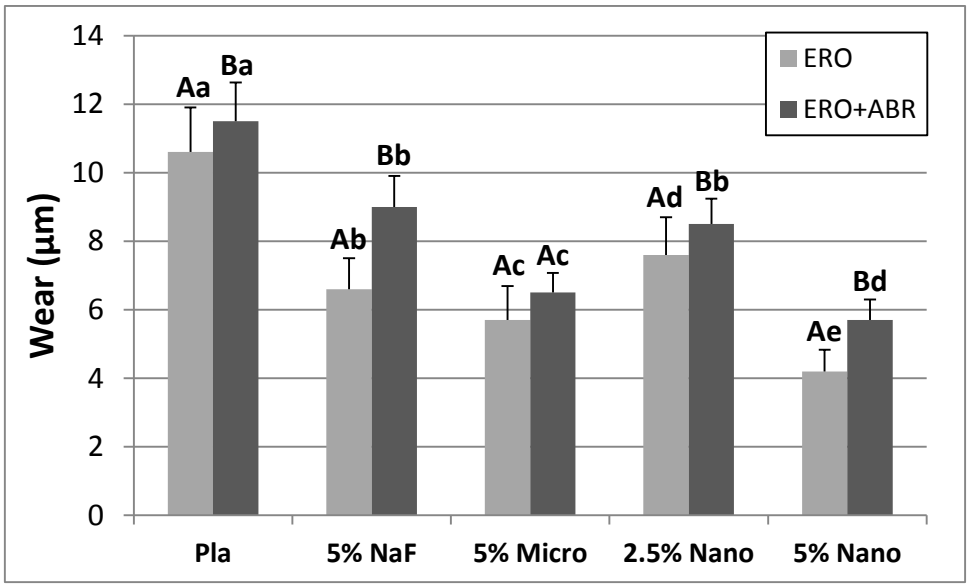


Figure 1. Enamel wear after erosive (ERO) or erosive+abrasive (ERO+ABR) challenges. Capital letters indicate significant differences between the challenges within each varnish/treatment. Lower case letters indicate differences among varnishes within each challenge (Two-way ANOVA and Fisher's LSD test, $p<0.05$, $n=10$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "2.5% Nano" = 5% NaF + 2.5% nano-sized TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.

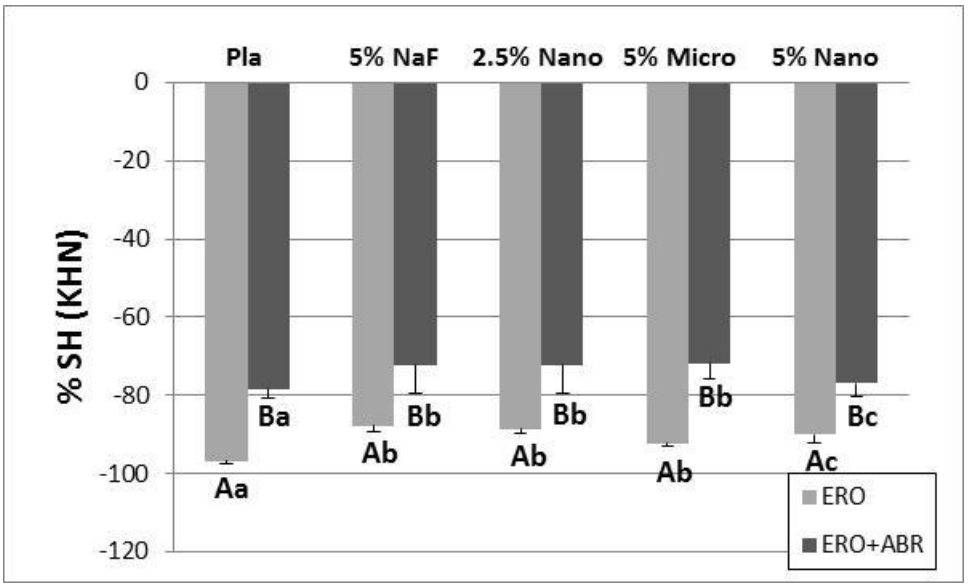
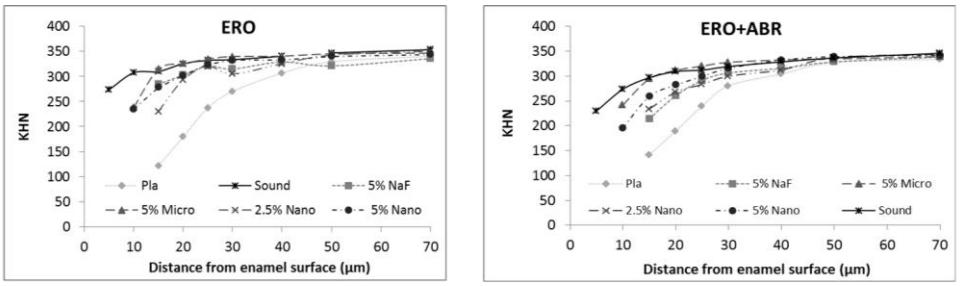


Figure 2. Percentage of surface hardness loss (%SH) of enamel after erosive (ERO) or erosive+abrasive (ERO+ABR) challenges. Capital and lower case letters indicate significant differences among the varnishes for ERO and ERO+ABR, respectively. Values observed for ERO and ERO+ABR were significantly different for all pairwise comparisons within each varnish (Two-way ANOVA and Fisher's LSD test, $p<0.05$, $n=10$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "2.5% Nano" = 5% NaF + 2.5% nano-sized TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.



Groups	Distance from enamel surface (µm)	
	5-30 µm	30-70 µm
Pla	3304.7 (±606.7) ^{A}	12754.3 (±734.0) ^{A}
5% NaF	5324.3 (±472.6) ^{B}	13028.0 (±526.0) ^{AB}
5% Micro	6910.6 (±283.4) ^{c}	13741.6 (±345.1) ^{C}
2.5% Nano	4977.3 (±430.2) ^{B}	13416.8 (±454.4) ^{BC}
5% Nano	6518.5 (±538.2) ^{C}	13530.5 (±402.4) ^{BC}

Groups	Distance from enamel surface (µm)	
	5-30 µm	30-70 µm
Pla	3561.2 (±723.5) ^{A}	12699.3 (±767.6) ^{A}
5% NaF	4599.1 (±453.5) ^{B}	13001.8 (±355.0) ^{AB}
5% Micro	6661.5 (±270.0) ^{c}	13374. (±387.0) ^{B}
2.5% Nano	4676.6 (±513.3) ^{B}	13123.1 (±629.4) ^{AB}
5% Nano	5969.8 (±635.2) ^{C}	13404.0 (±527.4) ^{B}

Figure 3. Differential hardness profiles and the values of the integrated area loss (ΔKHN) for blocks submitted to erosive challenges (ERO, left) or erosive+abrasive challenges (ERO+ABR, right). Capital letters indicate significant differences between the depths. Lower case letters indicate significant differences among the groups within each distance (Two-way ANOVA and Fisher's LSD test, $p<0.05$, $n=10$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "2.5% Nano" = 5% NaF + 2.5% nano-sized TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.

Fluoride varnish containing nano-sized sodium trimetaphosphate reduces enamel erosive wear *in situ*

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Short tittle: F varnishes containing nanosized TMP on enamel erosive wear

Key words: Dental enamel, Fluoride, Polyphosphates, Prevention, Tooth erosion.

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Declaration of interest

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Abstract

Objective: To assess the effects of fluoride (F) varnishes containing Sodium Trimetaphosphate (TMP) on erosive tooth wear (ETW) *in situ*. *Methods:* Bovine enamel blocks (*n*=224) were divided into 4 groups: Placebo (Pla - without F/TMP); 5% NaF; 5% NaF + 5% micrometric TMP (5% Micro) and 5% NaF + 5% nano-sized TMP (5% Nano). The blocks were inserted in palatal devices (*n*=4/device), treated with the varnishes and remained in the oral cavity of the subjects (*n*=14) for 6 h. The varnishes were removed and all blocks were submitted to 4 daily erosive challenges during 5 days (ERO, 0.05 M citric acid, pH 3.2, 90 s) *ex vivo*, while two blocks were additionally subjected to abrasion by brushing (ERO+ABR), following a double-blind, crossover protocol. Profilometry, surface hardness (SH) and cross-sectional hardness (ΔKHN) were determined. The data were submitted to 2-way ANOVA and Fisher's LSD test ($p<0.05$). *Results:* Wear values followed a similar trend ERO and ERO+ABR, with 5% Nano < 5% Micro < 5% NaF < Pla. A similar pattern was observed for hardness in depth (ΔKHN), despite no significant differences were verified between 5% Micro×5% Nano (at 5-30 µm from the enamel surface). As for SH loss, the highest value was observed for Pla and the lowest for 5% Nano (ERO or ERO+ABR), without significant differences between Pla×5% NaF (ERO), 5% NaF×5% Micro (ERO+ABR), and 5% Micro×5% Nano (ERO+ABR). *Conclusion*: F varnish containing TMP significantly reduced the ETW *in situ*, and the use of nanoparticles provided an additional protective effect. *Clinical significance:* the addition of TMP to fluoride varnishes, especially as nanoparticles, reduces enamel erosive wear *in situ*, being a promising alternative for the prevention of ETW.

Introduction

Erosive tooth wear (ETW) has been increasingly observed in the clinical practice, with an estimated prevalence of 30-50% and 20-45%, respectively for primary and permanent teeth [1]. Changes in contemporary eating habits, with higher consumption of industrialized juices and soft drinks, have been identified as one of the most important factors causing ETW [2].

Alternatives to prevent or minimize the progression of ETW have been intensively investigated, including fluoride (F) therapy. Although topical F products are very effective against dental caries [3-5], their effect against ETW is less prominent, as the pH of the acids involved on the erosion process is lower than the critical values for dissolution of hydroxyapatite and fluorapatite [6]. Consequently, F concentration and/or the frequency of application have to be increased to reduce or prevent erosion [7]. In order to overcome such limitations, strategies such as the addition of sodium trimetaphosphate (TMP) to F products have been shown to be effective in reducing ETW.

TMP is a condensed inorganic phosphate which has the ability to adsorb on enamel surface and increase the incorporation of calcium, phosphate and F [8]. In this way, it acts as a barrier to acid diffusion, leading to the formation of a more stable mineral [9,10]. Concerning vehicles for professional applications, studies have shown that F varnishes containing TMP significantly reduced ETW under *in vitro* and *in situ* conditions, both for enamel [11,12] and dentine [13].

These favorable results have prompted to further studies assessing the effects of TMP as nanoparticles. The addition of nano-sized TMP to a F varnish was shown to promote an additional protective effect both on initial erosive lesions [14] and on ETW [15] using different *in vitro* models. Noteworthy, the effects of this formulation were observed not only on the enamel surface (assessed by surface hardness and profilometry), but also in depth (assessed by cross-sectional hardness). This aspect would be relevant from a clinical standpoint, given that the enamel treated with this formulation would be more resistant to further erosive challenges.

Due to the limitations inherent to *in vitro* protocols, it would be interesting to evaluate the effects of this varnish under conditions that better resemble clinical situations, concerning interactions with the enamel pellicle, and the influence salivary properties (composition, flow and clearance), in individuals

who regularly use a F dentifrice. Based on this, the objective of the present study was to evaluate the effect of F varnishes supplemented with nano-sized TMP on ETW *in situ*. The null hypothesis was that particle size (*i.e.*, micrometric or nano-sized) would not influence ETW.

Materials and Methods

Ethical aspects, sample size calculation and inclusion criteria

This study was approved by the IRB of School of Dentistry, Araçatuba - UNESP (CAAE 94240218.1.0000.5420) (Anexo C). Participants signed an informed consent form and received written and verbal instructions on the research protocol prior to the beginning of the study (Anexo D). Fourteen individuals (20–30 years old), living in the city of Araçatuba-SP, Brazil (0.6-0.8 mg F/L in the public water supply) [16], were enrolled. Sample size calculated based on a pilot study conducted with 4 subjects, according to which 12 subjects would be required to detect significant differences in mean enamel wear of blocks treated with a 5% NaF varnish (positive control) and a 5% NaF varnish supplemented with 5% nano-sized TMP (mean difference = 2.0, standard deviation = 1.1), assuming α-error of 5% and β-error of 20% [18]. Considering a dropout rate of 20%, 14 subjects were enrolled. The inclusion criteria involved participants in good general and oral health, assessed by the application of a questionnaire. Individuals who used medicines that could interfere with the salivary flow or microbiological composition, smokers, those who received F applications 2 weeks before the experiment, were using orthodontic appliances or had systemic diseases were excluded from the study.

Experimental design

Bovine enamel blocks were sequentially polished and selected by surface microhardness (SH) [17]. Fourteen volunteers were randomly divided into 4 experimental groups according to the varnishes to be tested: Placebo (Pla - without F/TMP); 5% NaF; 5% NaF + 5% micrometric TMP (5% Micro) and 5% NaF + 5% nano-sized TMP (5% Nano), following a double-blind, crossover protocol. Half of the surface of each block was protected with acid-resistant nail varnish (Risqué®, Brazil) (control area), so that only the test area was subjected to treatment with the varnishes and to erosive challenges (ERO, 0.05 M citric

acid, pH 3.2, 90 s), followed or not by abrasion by brushing with a fluoridated dentifrice (ERO+ABR) 4 times a day, during 5 days. Blocks were analyzed by profilometry and by surface and cross-sectional hardness.

Enamel blocks preparation and appliances preparation

Enamel blocks (*n*=224) measuring 4 × 4 × 2 mm were obtained from bovine incisors previously stored in 2% neutral formaldehyde solution (pH 7.0) for 1 month. They were obtained from the flattest portion of the crowns and fixed in acrylic resin discs using sticky wax. Firstly, the dentine was flattened (320 grit), and subsequently the enamel was polished (600, 800, 1200 grit), followed by polishing with felt paper and 1µ diamond suspension [19] (Anexo E). Four enamel blocks were fixed in 2-mm deep spaces of custom-made acrylic palatal devices at the level of the acrylic surface.

Experimental protocol

The night before applying the varnishes, the volunteers slept with the palatal appliances inside their mouth, allowing the formation of acquired enamel pellicle on the blocks. In the next morning, the devices were gently rinsed with deionized water and a standard layer of varnish was applied on the blocks. The palatal appliances were then reinserted in the mouth, and the varnishes remained on the blocks for 6 h. Following, the varnish was removed with a blade and acetone (Sigma-Aldrich CO., St. Louis, MO, USA).

All blocks were submitted to 4 daily erosive challenges *ex vivo*, for five days, by immersion of the palatal appliances in 20 mL of citric acid (Synth®, Brazil) (ERO, 0.05 M, pH 3.2, 90 s). Subsequently, the device was gently rinsed in tap water and placed in the oral cavity, and the subjects brushed their own teeth with a F dentifrice (1450 ppm F, Colgate Total 12, NaF) for 15 s, allowing the formation of a natural dentifrice:saliva slurry. Following, the devices were removed from the oral cavity, and two blocks (highlighted on the device) were brushed, totaling 15 back and forth strokes (ERO+ABR). Before starting the experiment, the volunteers were individually trained on the brushing procedure and received written and cell phone guidance. All volunteers used a placebo dentifrice (F-free) 1 week prior each experimental phase, to eliminate possible

residual effects from the treatments. Between each erosive challenge, the devices remained for at least 2 h in oral cavity (Anexo M).

Formulation and dosage of fluoride of experimental varnishes

The varnishes were manufactured by SS White Dental Products (Rio de Janeiro, RJ, Brazil), containing the following components: film-forming polymer (artificial resin), solvent (ethanol), essence (saccharin) and demineralised water (q.s.p.) (Anexo F). The synthesis and characterization of nano-sized TMP was performed as reported by Danelon et al. (2017) [20]. The X-ray diffraction analysis showed an average particle size estimated at 22.7 nm, without alteration of its crystalline structure. The varnishes contained 5% NaF without or with the addition of 5% micrometric or 5% nano-sized TMP. A placebo varnish (without the addition of NaF or TMP, negative control) was also produced. F concentrations were determined using an ion-specific electrode (9609 BN - Orion) and ion analyzer (Orion 720 A+), previously calibrated with standards at 2.0, 4.0, 8.0, 16.0 and 32.0 µg F/mL, according to the protocol used by Manarelli et al. (2014) [21]. The data obtained in mV were converted to µg F/mL.

Analysis of enamel wear - Profilometry

At the end of each experimental phase, the nail varnish was carefully removed from the control areas of each block with spatula and acetone, starting from the edges to avoid damages to the surface (Sigma-Aldrich CO., St. Louis, MO, USA). Enamel wear was performed by profilometry (Surftest SJ 401 - Mitutoyo American Corporation), and the Surftest software (Anexo I). The surface of each block was scanned from the reference (control) surfaces through the exposed surfaces with measuring length of 0.8 mm. The average value of 5 readings was calculated for each block [11].

Analysis of surface and cross-sectional hardness

The surface microhardness was determined before (SH_1) (Anexo J) and after (SH_2) (Anexo K) the experiments using the Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and Knoop indenter (KHN) under 25 g for 10 s. Five indentations spaced 100 µm

from each other were performed at the center of the block (SH_1), and five equidistant impressions were performed in relation to the initial impressions (SH_2). After that, the enamel blocks were cross-sectioned at the center, and half of each block was embedded in acrylic resin and subsequently polished. A sequence of 9 impressions at distances of 5, 10, 15, 20, 25, 30, 40, 50, 70 µm from the external enamel surface was performed in the center of blocks, for both the control and the test areas (Anexo L). The integrated area of hardness (KHN × µm) of the lesion up to the intact enamel was calculated using the Trapezoidal rule (ΔS*(Y1+Y2)/2; GraphPad Prism, version 3.02). The difference of each depth of the sound enamel and the value of the integrated area loss was calculated (ΔKHN = loss of hardness in depth in the enamel – area under the curve) [22].

Statistical analysis

SigmaPlot version 12.0 was used to conduct the statistical analysis. Enamel wear (profilometry) and SH data were submitted to 2-way, repeated measures ANOVA, followed by Fisher's LSD test. For ΔKHN, data of ERO and ERO+ABR were analyzed separately by 2-way ANOVA, followed by Fisher's LSD test, considering the varnishes and depths (5 to 30 µm or 30 to 70 µm) as independent variables. All data were normally distributed (Shapiro-Wilk's test). A significance level of 5% was adopted.

Results

Enamel wear was significantly higher for ERO+ABR than ERO for all groups, with significant differences for all pairwise comparisons. The lowest values were observed to 5% Nano, followed by 5% Micro, 5% NaF and Pla, with significant differences among all groups, under both experimental conditions (Figure 1).

For ΔKHN (area under the curve), differences among the groups were more pronounced at 5-30 µm compared with 30-70 µm from the enamel surface. For both for ERO and ERO+ABR, the highest ΔKHN values were observed for 5% Nano and 5% Micro, followed by 5% NaF and Pla. No significant differences were observed between 5% Micro and 5% Nano (5-30 µm) and among 5% NaF, 5% Micro and 5% Nano (30-70 µm) (Figure 2). As for

SH loss, the highest values were observed for Pla, and the lowest, for 5% Nano (ERO or ERO+ABR), without significantly differences between Pla and 5% NaF (ERO), 5% NaF and 5% Micro (ERO+ABR), and 5% Micro and 5% Nano (ERO+ABR) (Figure 3).

Discussion

The results of the present study demonstrated that the addition of 5% micrometric TMP to a conventional F varnish significantly reduced the ETW under *in situ* conditions, in individuals using a F toothpaste. As the addition of nano-sized TMP led to a significantly greater reduction of ETW in comparison with micrometric TMP, the study's null hypothesis was rejected.

In vitro studies with different F vehicles, such as toothpastes, mouthrinses, gels and varnishes, have shown that the addition of conventional (*i.e.*, micrometric) TMP is effective in reducing erosion/ETW [11, 23-25]. Under *in situ* conditions, the present data demonstrated a 20% additional protective effect of 5% Micro over 5% NaF; for the 5% Nano, the protective effect was even greater, leading to a reduction of 40% compared with 5% NaF. This better effect results from the greater contact surface of nanoparticles in comparison with micrometric ones [20]. In varnish formulations, the use of nanoparticles can also favor higher nanoparticles interaction with the varnish matrix, which can result in higher release to the oral environment, consequently promoting greater reactivity with the dental surfaces.

It was noteworthy that the mean wear values were ~4-fold lower in the present *in situ* study compared to previous *in vitro* data [15]. Several factors might explain the above-mentioned differences, among which the presence of natural saliva (*in situ*), the employment of dynamic exposure to the erosive challenge (*in vitro*) and the use of F dentifrice are likely to have played a major role. As for the first, studies have shown that human saliva can provide higher stability to the CaF_2 layer formed on the enamel compared to samples exposed only to artificial saliva *in vitro* [26,27]. Also, salivary proteins (including those forming the enamel pellicle) and phosphates can stabilize F precipitation on the enamel [28]. Regarding the mode of exposure to the erosive challenge, static protocols are known to promote the saturation of the microenvironment around the exposed tooth surfaces due to the initial release of calcium and phosphate

[29]. In this way, the reaction reaches a plateau, leading to a reduction in the rate of mineral loss from enamel surface [29]. For dynamic protocols, on the other hand, such saturation does not occur, so that a fairly constant rate of mineral loss is achieved during the entire exposure time [30]. Concerning the use of F dentifrice, the frequent exposure to F from this source promotes the formation of CaF_2 deposits on the tooth surfaces, as well as F uptake by intraoral retention sites (*e.g.*, dental biofilm, soft and hard tissues) [31]. These reservoirs promote a favorable environment for mineral deposition, which may have greatly influenced the results.

The analysis of enamel hardness in depth allowed the observation of the effects of varnishes on enamel mineral content, thus providing additional information to the main study variable (*i.e.*, enamel wear). Also, the ΔKHN data (area under the curve) were obtained at two depths, which allowed to verify the effects closer to the enamel surface (5-30 µm) and in deeper regions (30-70 µm). The results showed that varnishes containing TMP led to significantly lower mineral loss compared with 5% NaF at 5-30 µm, but such differences were not significant for the 30-70 µm depth. Such a trend is in line with the mechanism of action of TMP, which is related to its adsorption to the enamel surface, forming a protective layer that prevents acid diffusion [10,32]. This explains the favorable data obtained for the TMP-containing varnishes both for enamel wear and hardness in depth. Furthermore, the marked effects of TMP in depth observed in Figure 2 are paramount from a clinical point of view, since F products at high concentrations are known to have a more pronounced effect at the outermost enamel layers [31]. The analysis of both variables together (wear and hardness in depth) indicate that TMP-containing varnishes are effective in reducing enamel wear during the 5-day experimental protocol, and that the remaining enamel is more resistant to further ERO or ERO+ABR challenges.

Regarding SH loss, values observed under ERO were higher than those under ERO+ABR, demonstrating that the brushing procedure (for ERO+ABR) is able to remove the softened enamel resulted from acid dissolution, thus exposing deeper enamel layers with higher hardness values [12,23]. Despite data on SH loss must be interpreted with caution in studies assessing ETW, the simultaneous analysis of SH loss and the other variables provided interesting insights on the benefits of TMP, as well as the additional advantages on the use

of nanoparticles. For ERO, although the conventional varnish (5% NaF) promoted a significant protective effect on enamel wear (Figure 1) and on enamel hardness in depth (Figure 2), its effects on enamel surface were not significantly different compared with Pla. On the other hand, the addition of micrometric TMP significantly increased the effects of the conventional varnish both on surface hardness and in depth. It has previously been shown that the non-dissociated citric acid diffuses on enamel porosities, dissociating in the layer close to the surface called “near-surface” [33], acting as a source of H^+ that maintains the near-surface dissolution. In this sense, the data from the 5% Micro varnish suggest that TMP prevents the effect of acid directly on the surface (Figure 3) and near-surface (Figure 2). However, the effect on the surface was removed by the mechanical effect (*i.e.*, brushing), which may explain the lack of significant difference between 5% NaF and TMPmicro on enamel SH loss (Figure 3, ERO+ABR). Interestingly, the superior effects of TMPnano (compared with 5% NaF) were maintained even after brushing (ERO+ABR), which seems to be related to its improved ability in rehardening the eroded enamel after the erosive challenges compared with TMPmicro (Figure 3, ERO data).

Based on the results above, we can conclude that TMP enhances the protective effect of conventional F varnishes against ETW *in situ*. The additional effect of 5% Nano over 5% Micro under both study conditions justifies the use of nanoparticles of this polyphosphate in varnish formulations.

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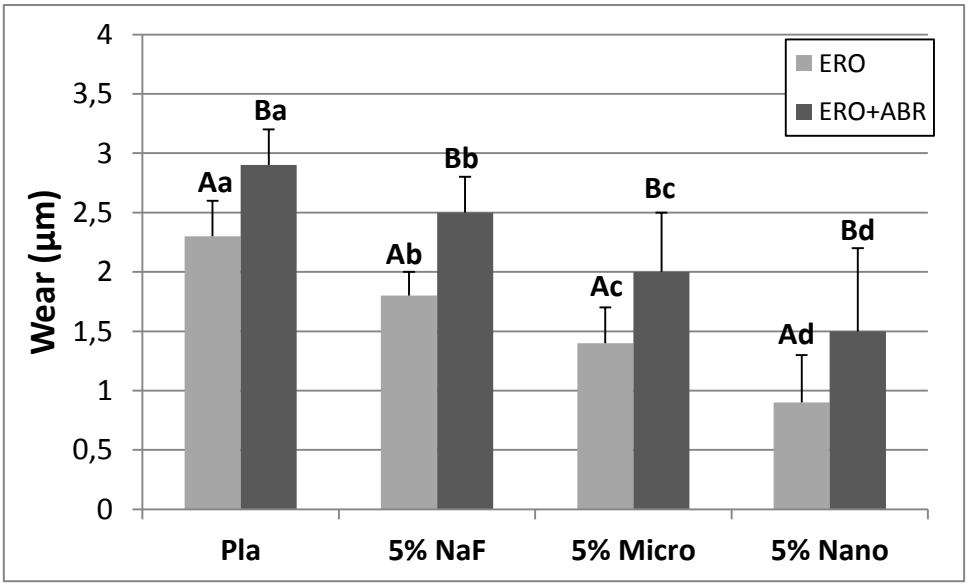
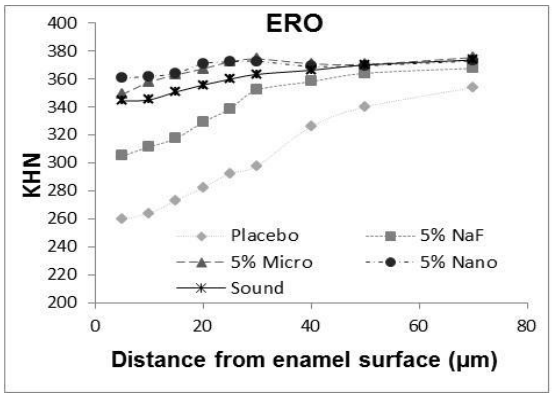
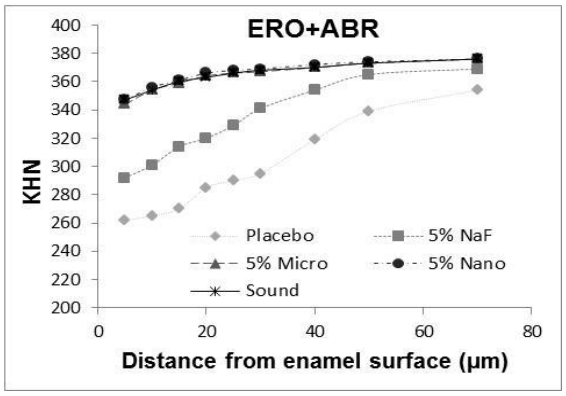


Figure 1. Enamel wear after erosive (ERO) or erosive+abrasive (ERO+ABR) challenges. Capital letters indicate significant differences between the challenges within each varnish. Lower case letters indicate differences among varnishes within each challenge (Two-way repeated measures ANOVA and Fisher's LSD test, $p<0.05$, $n=14$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.





Groups	ΔKHN (KHN × µm)	
	Distance from enamel surface	
	5-30 µm [A]	30-70 µm [B]
Pla	6953.3 (±287.4) [a]	13390.5 (±817.2) [a]
5% NaF	8127.5 (±200.2) [b]	14484.3 (±191.8) [b]
5% Micro	9110.8 (±90.2) [c]	14359.6 (±318.4) [b]
5% Nano	9181.0 (±145.8) [c]	14830.2 (±316.9) [b]

Groups	ΔKHN (KHN × µm)	
	Distance from enamel surface	
	5-30 µm [A]	30-70 µm [B]
Pla	6943.3 (300.9) [a]	13160.5 (993.0) [a]
5% NaF	7900.8 (194.9) [b]	14409.1 (254.7) [b]
5% Micro	8990.4 (177.6) [c]	14895.9 (166.1) [b]
5% Nano	9040.3 (119.6) [c]	14669.8 (970.8) [b]

Figure 2. Differential hardness profiles and the values of the integrated area loss (ΔKHN) for blocks submitted to erosive challenge (ERO, left) or erosive+abrasive challenges (ERO+ABR, right) as a function of depth. Capital letters indicate significant differences between the depths. Lower case letters indicate significant differences among the groups within each distance (Two-way repeated measures ANOVA and Fisher's LSD test, $p<0.05$, $n=14$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.

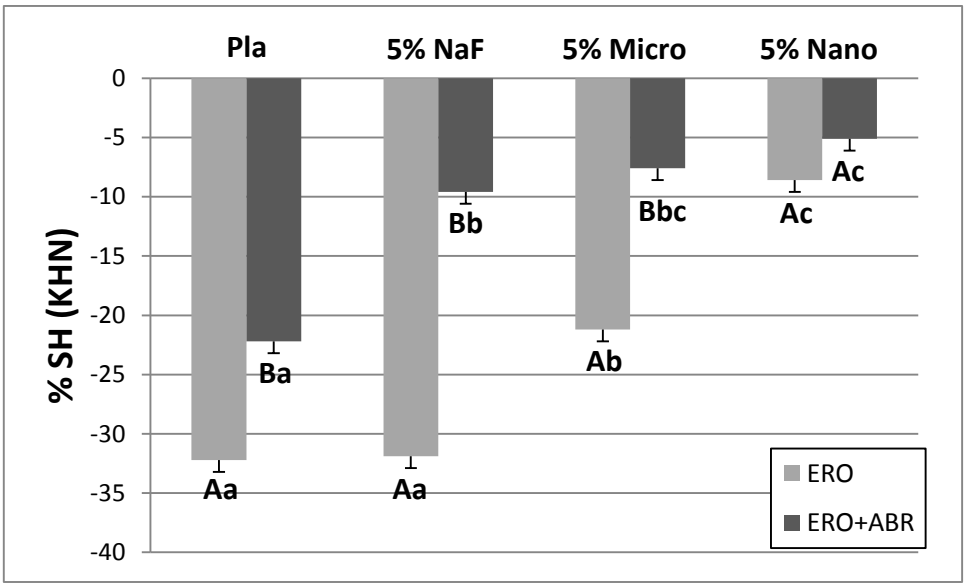


Figure 3. Percentage of surface hardness loss (%SH) of enamel after erosive (ERO) or erosive+abrasive (ERO+ABR) challenges. Capital and lower case letters indicate significant differences between the challenges within each varnish/treatment. Lower case letters indicate differences among varnishes within each challenge (Two-way repeated measures ANOVA and Fisher's LSD test, $p<0.05$, $n=14$/group).
Legends: "Pla" = Placebo (without NaF or TMP); "5% Micro" = 5% NaF + 5% micrometric TMP; "5% Nano" = 5% NaF + 5% nano-sized TMP.

Anexos

ANEXOS

ANEXO A

Referências Introdução Geral

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ANEXO B

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[5] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/
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Título da Pesquisa: Efeito de vernizes fluoretados suplementados com Trimetafosfato de Sódio nanoparticulado sobre o desgaste erosivo do esmalte dental in vitro e in situ

Pesquisador: Mayra Frasson Paiva

Área Temática:

Versão: 2

CAAE: 94240218.1.0000.5420

Instituição Proponente: Universidade Estadual Paulista Júlio de Mesquita Filho

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Número do Parecer: 2.924.682

Apresentação do Projeto:

A pesquisadora pretende realizar dois subprojetos. O primeiro será in vitro e não envolve a participação de humanos. A saliva utilizada será artificial e os dentes serão bovinos. Indico que a mesma deva submeter este projeto também ao Comitê de Ética em Pesquisa. O 2os subprojeto também envolve dentes bovinos, mas a pesquisadora pretende inseri-los em dispositivos acrílicos palatinos (n=4/dispositivo), sendo tratados com os vernizes uma única vez, permanecendo na cavidade bucal por 6 h. Os vernizes serão removidos e os blocos, submetidos a ERO (imersão ex vivo em ácido cítrico 0,05 M, pH 3,2, 5 min, 4x/dia), enquanto dois blocos serão adicionalmente submetidos a abrasão com dentifrício fluoretado (ERO+ABR), totalizando 5 dias em cada etapa experimental. Assim, esse 2o subprojeto envolve humanos que utilizaram aparelhos acrílicos por 5 dias, com descanso de 7 dias e então voltarão a usar novamente até que todos os grupos de estudos estejam concluídos.

Objetivo da Pesquisa:

O objetivo do presente estudo será avaliar o efeito de vernizes fluoretados suplementados com nanopartículas de Trimetafosfato de Sódio (TMP)sobre o desgaste erosivo do esmalte dental bovino, em protocolos in vitro e in situ.

Avaliação dos Riscos e Benefícios:

Riscos: mínimos. Somente desconforto.

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Benefícios: não há benefícios diretos, mas o paciente receberá sessões de profilaxia dental e acompanhamento clínico. Diante de problemas dentários, haverá encaminhamento à FOA para o tratamento necessário.

Comentários e Considerações sobre a Pesquisa:

A pesquisadora adequou o projeto conforme recomendação do CEP.

Considerações sobre os Termos de apresentação obrigatória:

TCLE foi apresentado nas normas do CEP.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

O projeto está adequado e recomenda-se a sua aprovação.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/03/2019.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1169984.pdf	24/08/2018 17:28:09		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.doc	24/08/2018 17:26:37	Mayra Frasson Paiva	Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	24/08/2018 17:22:33	Mayra Frasson Paiva	Aceito
Folha de Rosto	FR.pdf	05/07/2018 15:42:38	Mayra Frasson Paiva	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

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Continuação do Parecer: 2.924.682

Não

ARACATUBA, 28 de Setembro de 2018

Assinado por:
Aldiéris Alves Pesqueira
(Coordenador(a))

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ANEXO D

Instruções aos Voluntários

- Antes do início de cada fase experimental, o voluntário deverá fazer uso do dentifrício placebo (sem F) por 7 dias;
- Para cada fase experimental, o voluntário receberá uma escova dental específica. A primeira escova (somente utilizada com o dentifrício placebo) deverá ser guardada para posterior uso entre uma fase e outra (washout 7 dias);
- Durante cada fase experimental e entre elas, não será permitido o uso de produtos fuoretados;
- Durante o período de descanso (washout – 7 dias) entre uma fase e outra, utilizar somente o dentifrício placebo fornecido pelos pesquisadores.

Protocolo do estudo:

- Na tarde que antecede cada fase, o voluntário receberá o kit a ser utilizado na pesquisa, contendo: 1 escova dental, 1 dentifrício fluoretado (1450 ppm F), 1 copo de vidro para a realização do desafio erosivo, 1 dispositivo palatino contendo 4 blocos de esmalte dental bovino, 1 frasco de solução de ácido cítrico (pH=3,2) e gaze;
- Neste mesmo dia, o voluntário deverá instalar o dispositivo na cavidade bucal antes de dormir;
- Na manhã seguinte, o voluntário deve remover o aparelho da boca, enxaguar com água corrente, escovar seus dentes normalmente com o dentifrício fornecido e colocar o aparelho novamente na boca, até entregar para o pesquisador. Após a aplicação do verniz fluoretado, o dispositivo permanecerá por 6 h na cavidade bucal do voluntário. Após esse período, o verniz será removido e os desafios erosivos serão iniciados.

Protocolo para o desafio erosivo:

- Os desafios erosivos serão realizados fazendo a imersão do dispositivo em ácido cítrico (pH=3,2) 4 vezes ao dia, com intervalo mínimo de 2 horas entre cada desafio (Exemplos de horários: 8:00, 10:00, 12:00 e 14:00). É muito importante respeitar o intervalo mínimo de 2 h entre cada desafio e, se possível, realizar os desafios todos os dias nos mesmos horários.

Como realizar o protocolo:
1º Colocar o ácido cítrico no copo de vidro fornecido pelo pesquisador;
2º Cronometrar 1 minuto e 30 segundos, remover o dispositivo da boca e mergulhar no ácido cítrico;
3º Remover o dispositivo do ácido, enxaguar com água corrente;
4º Colocar o dispositivo na boca, escovar os seus dentes com o dentifrício (quantidade de um grão de ervilha) fornecido pelo pesquisador por 15 segundos para formação do “slurry”;

5º Remover o dispositivo da boca e escovar o lado direito (destacado) fazendo 15 movimentos de vai-e-vem;
6º Terminar normalmente a escovação dos dentes sem o dispositivo na boca, enxaguar os dentes, enxaguar o dispositivo com água corrente;
7º Reinserir o dispositivo na cavidade bucal;

- Realizar este protocolo por 5 dias. Após o último desafio do 5º dia, o voluntário deve permanecer com o aparelho por mais 2 horas na cavidade bucal. Por exemplo: O último desafio foi realizado as 14 h, então o voluntário deve permanecer com o aparelho na boca até as 16 h e depois guardá-lo na caixinha e colocar na geladeira até entregar para o pesquisador.

Informações importantes:

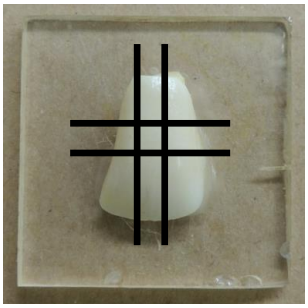
- O voluntário deve remover o aparelho da boca SOMENTE para comer ou beber. Não deve ingerir nenhum líquido (nem água) ou sólido enquanto o aparelho estiver na boca;
- Quando remover o aparelho da boca, guarde-o em sua caixinha, a qual deve estar sempre com uma gaze úmida em seu interior;
- O ácido fornecido pelo pesquisador deve permanecer na geladeira, se possível, ou em locais frescos. Nunca deixar em locais quentes
- O voluntário não deve fazer uso de nenhum produto fluoretado, além do dentifrício fornecido pelo pesquisador;
- Não ingerir chá mate, verde ou preto e tereré durante a pesquisa;
- O voluntário não deve estar fazendo uso de medicamentos que interfiram no fluxo salivar, nem antibiótico. Caso precisar, informe imediatamente o pesquisador.

Qualquer dúvida, entrar em contato com a pesquisadora responsável (Mayra) através do telefone: (44) 99868-3041.

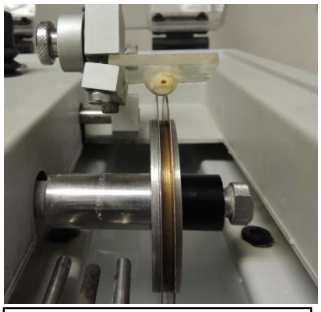
ANEXO E

Preparo dos Blocos de Esmalte

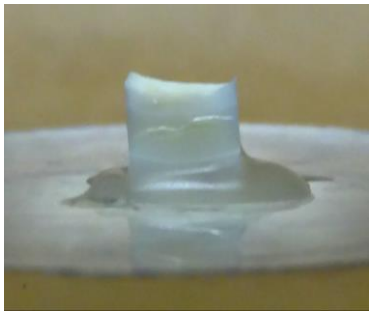
1. Separação coroa/raiz



2. Região da superfície vestibular (V) a ser seccionada

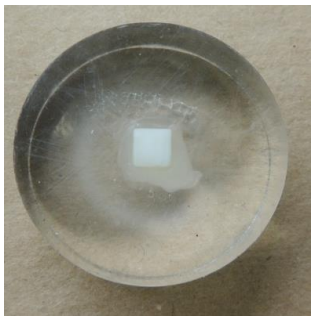


3. Secção da superfície V em cortadeira



4. Superfície dentinária antes da planificação

5. Planificação da dentina e polimento do esmalte em politriz



6. Bloco de esmalte após polimento

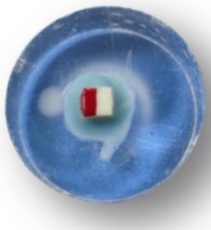
ANEXO F

Vernizes Experimentais

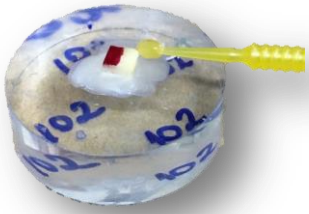
Vernizes experimentais: Placebo, 5% NaF, 5% NaF + 2,5% TMPnano, 5% NaF + 5% TMPnano, 5% NaF + 5% TMPmicro
(SS White Produtos Odontológicos Rio de Janeiro, RJ, Brasil)

ANEXO G

Tratamento dos Blocos de Esmalte



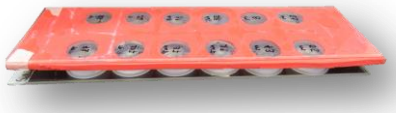
1. Esmalte ácido-resistente



2. Aplicação do verniz F

3. Blocos posicionados em placa de acrílico

4. Saliva artificial 4 mL



5. Blocos em contato com a saliva

6. Agitação 95 rpm/6 h

ANEXO H

Desafios Erosivos

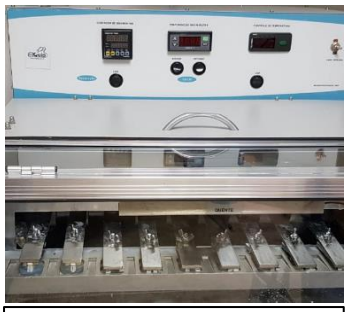
1. Remoção dos vernizes com espátula e acetona

2. Agitação em ácido cítrico (pH=3,2) 90 s/35 rpm

ERO (n=50)

3. Agitação em dentifrício placebo 15 s

ERO+ABR (n=50)



4. Abrasão em Máquina de Escovação 250 g/15 s

ANEXO I

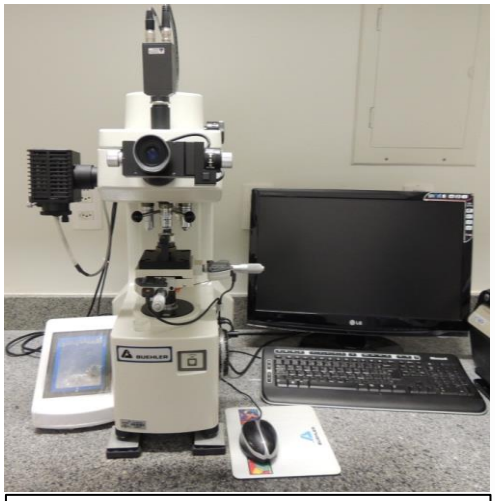
Perfilometria

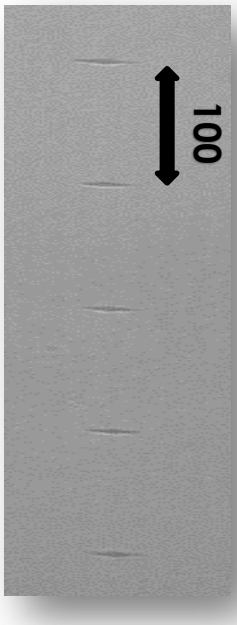
Rugosímetro adaptado para Perfilometria (Programa Surfpak – Surftest Mitutoyo)

Cinco varreduras são feitas da área hígida do esmalte para área erodida

ANEXO J

Dureza de Superfície final (DS_1)

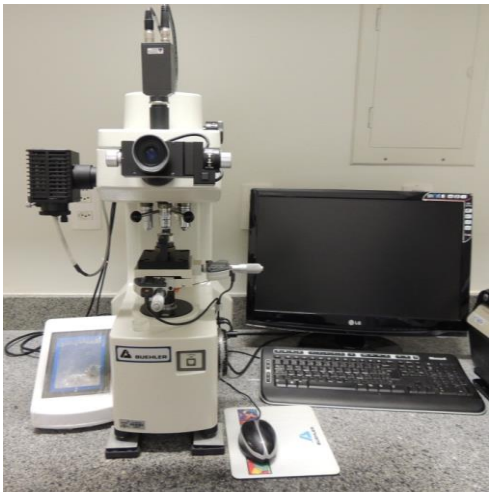


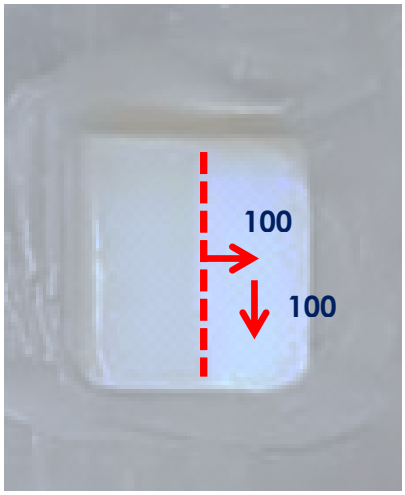


DS_1: 25 g/10 s
5 impressões a 100 µm entre si

ANEXO K

Dureza de Superfície final (DS_2)

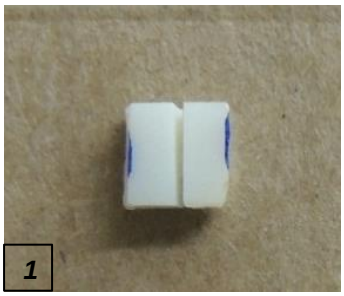


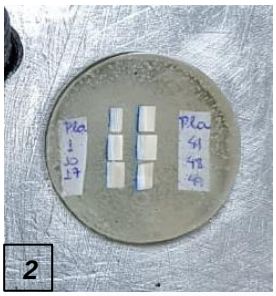


DS_2: 25 g/10 s
5 impressões a 100 µm do início da área erodida e eqüidistantes

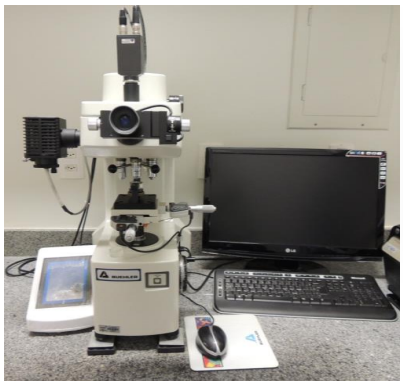
ANEXO L

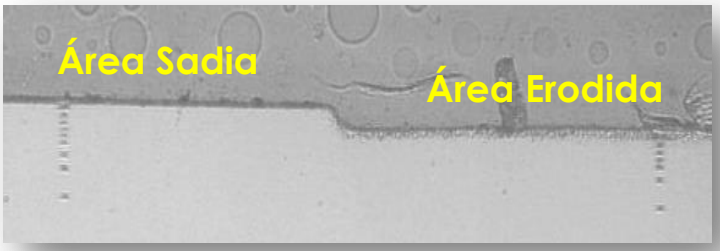
Dureza em secção longitudinal


1


2

1. Secção dos blocos no sentido longitudinal
2. Blocos embutidos em resina acrílica com a superfície interna voltada para cima





Carga 5g/10 s
9 impressões a partir da superfície externa do bloco
5, 10, 15, 20, 25, 30, 40, 50, 70 µm

ANEXO M

Fase experimental *in situ*

1


2

1. Kit recebido pelos voluntários contendo: dispositivo palatino, escova e dentifrício fluoretado, copo de vidro, ácido cítrico (pH=3,2) e gaze
2. Dispositivo palatino contendo 4 blocos de esmalte dental bovino

3

3. ERO: imersão em 20 mL de ácido cítrico (pH=3,2) 90 s
4. ABR: escovação com dentifrício fluoretado (15 s)