

LILIANA CAROLINA BÁEZ QUINTERO

**Efeito de vernizes fluoretados suplementados com nanopartículas de
Trimetafosfato de Sódio sobre a remineralização de lesões de cárie e erosão
de esmalte dental *in vitro***

ARAÇATUBA

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Orientador: Prof. Dr. Juliano Pelim Pessan

Coorientadores: Prof. Tit. Alberto Carlos Botazzo Delbem

Dra. Marcelle Danelon

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Liliana Carolina Báez Quintero

Nascimento	22.12.1979- Soatá-Boyaca-Colômbia
Filiação	Ana Helda Quintero Parra Luis Carlos Báez Suescun
1997/2002	Curso de Graduação em Odontologia pela Faculdade de Odontologia da Universidade Nacional de Colômbia
2004-2008	Desenvolvimento do projeto de Mestrado em Saúde Pública, Faculdade de Medicina, Universidade Nacional de Colômbia.
2014-2017	Desenvolvimento de Projeto de Doutorado, com auxílio do Programa de Apoio a Estudantes de Doutorado do Exterior PADEX/AUIP/PROPG, na Faculdade de Odontologia de Araçatuba-UNESP.
2015-2016	Especialização em Odontopediatria pela Faculdade de Odontologia de Araçatuba- UNESP.
Associações	CROSP – Conselho Regional de Odontologia de São Paulo. SBPqO – Sociedade Brasileira de Pesquisa IADR – International Association for Dental Research.

COMISSÃO EXAMINADORA

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR

Prof. Dr. Juliano Pelim Pessan- Orientador, Professor Adjunto do Departamento de Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista Júlio de Mesquita Filho-UNESP.

Prof. Dr. Célio Percinoto- Professor Titular do Departamento de Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria, da Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista Júlio de Mesquita Filho-UNESP.

Dr. Douglas Roberto Monteiro- Doutor, Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria, da Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista Júlio de Mesquita Filho-UNESP.

Dra. Cleide Cristina Rodrigues Martinhon- Doutora em Odontologia, com ênfase em Odontopediatria pela Universidade de São Paulo, USP, Brasil. Prática particular.

Prof. Heitor Marques Honório- Professor Associado do Departamento de Odontopediatria, Ortodontia e Saúde Coletiva, Disciplina de Metodologia de Pesquisa e Estatística, Faculdade de Odontologia de Bauru, Universidade de São Paulo.

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agora, apesar da distância. Obrigada, por fazer parte de minha vida.

Aos meus amigos *Pilar Rodriguez, Rosse Falcon e Rodrigo Oliveria,*
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rapidamente surgiu uma amizade desinteressada que permanece até
agora, apesar da distância. Obrigada, por fazer parte de minha vida.

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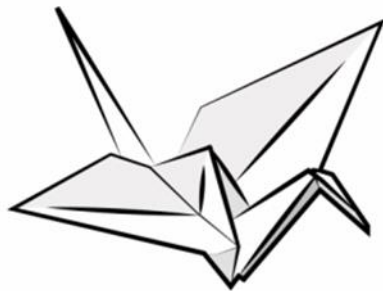
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Paulo Freire

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RESUMO

O presente estudo avaliou o efeito de vernizes fluoretados contendo nanopartículas de trimetafosfato de sódio (TMP) sobre a remineralização de lesões de cárie e erosão de esmalte dental *in vitro*. Assim como o padrão de liberação de F e TMP a partir destas formulações. Os vernizes testados foram: Placebo (sem flúor ou TMP, controle negativo), 2,5% NaF, 5% NaF (controle positivo), 5% NaF + 5% TMP microparticulado, 5% NaF + 2,5% TMP nanoparticulado, 5% NaF + 5% TMP nanoparticulado, além de uma formulação comercial (Duraphat), doravante denominados PLA, 2.5%F, 5%F, 5%F+5%TMPmicro, 5%F+2,5%TMPnano, 5%F+5%TMPnano e Duraphat, respectivamente. Na 1ª fase, lesões de cárie artificiais foram induzidas em blocos de esmalte bovino ($n=168$), os quais foram selecionados por dureza de superfície (DS). Os blocos receberam uma aplicação dos vernizes supracitados, permanecendo em contato durante 6 h. Metade dos blocos ($n=12$ /grupo) foi utilizada para determinação da concentração de CaF_2 e FA formado após o tratamento com os vernizes. A outra metade foi submetida um modelo de ciclagem de pH (6 dias). Os blocos foram analisados quanto a porcentagem de recuperação de dureza de superfície (%RDS), dureza em secção longitudinal (ΔKHN), CaF_2 e FA retidos após a ciclagem de pH. Os vernizes contendo TMP promoveram %RDS significativamente maior em comparação ao verniz contendo 5%F, sem diferenças significativas entre os vernizes com TMP. Um padrão semelhante foi observado para ΔKHN , embora os valores obtidos para 5%F+5%TMPnano tenham sido 25% menores que os obtidos para 5%F+5%TMPmicro. As maiores concentrações de CaF_2 foram promovidas pelos vernizes 5%F, 5%F+5%TMPmicro e Duraphat. A 2ª fase avaliou o efeito protetor dos vernizes PLA, 5%F, 5%F+5%TMPmicro, 5%F+2,5%TMPnano e 5%F+5%TMPnano sobre a erosão de esmalte bovino. Os espécimes ($n=8$), selecionados por DS, receberam uma única aplicação dos vernizes, permanecendo em contato durante 6 h. Em seguida, os vernizes

foram removidos e os blocos, submetidos a quatro desafios erosivos individuais (1 minuto, ácido cítrico, 0,75%, pH = 3,5, sob agitação), sendo analisados por DS após cada desafio. Em geral, observou-se a maior porcentagem de alteração de DS para PLA, seguido de 5%F, 5%F+5%TMPmicro e ambos os vernizes contendo TMPnano, sem diferenças significativas entre 5%F+2,5%TMPnano e 5%F+5%TMPnano. Por fim, a 3ª fase avaliou o padrão de liberação de flúor e fosfato dos vernizes PLA, 2.5%F, 5%F, 5%F+5%TMPmicro, 5%F+2,5%TMPnano, 5%F+5%TMPnano e Duraphat ao longo de 24 h, em um modelo de ciclagem de pH. Os vernizes foram aplicados em tiras de poliéster ($n=8$ /grupo), as quais foram alternadamente imersas em soluções remineralizadora e desmineralizadora aos 30, 60, 90, 120, 180, 240, 300, 360, 420, 540, 600, 720, 780, 960, 1200 e 1440 min após a primeira imersão. As soluções foram analisadas quanto às concentrações de flúor e fosfato. Os vernizes contendo TMP apresentaram um padrão crescente exponencial quanto a liberação cumulativa de flúor até 6 horas, atingindo um platô nos tempos seguintes. Em acréscimo, os vernizes liberaram maiores quantidades de flúor quando imersos em solução desmineralizadora. De forma geral, os vernizes contendo TMPnano liberaram quantidades significativamente maiores de flúor em comparação ao TMPmicro. Os resultados da 1ª fase permitem concluir que a adição de TMP a vernizes fluoretados aumenta significativamente seu potencial remineralizador em lesões de cárie artificiais, com um efeito adicional com o uso de TMPnano em relação ao TMPmicro, embora este acréscimo não seja estatisticamente significativo. Quanto ao efeito contra desafios erosivos (2ª fase), um padrão semelhante foi observado, tendo o maior efeito protetor sido observado para os vernizes contendo TMPnano, o qual foi significativamente maior que os demais grupos. O maior efeito dos vernizes suplementados com TMP parece estar relacionado à maior liberação de flúor destes (3ª fase), especialmente TMPmicro, associada à liberação constante de TMP a partir destas formulações.

Palavras-chave: Fluoretos tópicos. Polifosfatos. Cárie dentária. Erosão dentária. Nanopartículas.

Báez-Quintero LC. **Effect of fluoride varnishes supplemented with nano-sized sodium trimetaphosphate on the remineralization of artificial caries lesions and enamel erosion *in vitro***. 2017. 104 f. Tese (Doutorado), Universidade Estadual Paulista, Araçatuba, 2017.

ABSTRACT

The present study evaluated the effect of fluoride varnishes containing nano-sized sodium trimetaphosphate (TMP) on the remineralization of artificial caries lesions and erosion of dental enamel *in vitro*. As well as the pattern of F and TMP release from these formulations. The varnishes tested were: Placebo (without fluoride or TMP, negative control), 2.5% NaF, 5% NaF (positive control), 5% NaF + 5% TMP micrometric, 5% NaF + 2.5% nano-sized TMP, 5% NaF + 5% nano-sized TMP, besides a commercial formulation (Duraphat), hereafter referred to as PLA, 2.5%F, 5%F, 5%F+5%TMPmicro, 5%F+2.5%TMPnano, 5%F+5%TMPnano and Duraphat, respectively. In the first phase, artificial caries lesions were induced on bovine enamel blocks ($n=168$), which were selected by surface hardness (SH). Blocks received a single application of the aforementioned varnishes, remaining in contact for 6 h. Half of the blocks ($n=12$ /group) were used to determine the concentration of CaF_2 and FA fluoride formed on the specimens after the treatment with the varnishes. The other half was subjected to a pH cycling model (6 days). The blocks were analyzed for the percentage of SH recovery (%SHR), cross-sectional hardness (ΔKHN), CaF_2 and FA retained after pH cycling. The varnishes containing TMP promoted significantly higher %SHR compared to 5%F, without significant differences among the TMP-containing varnishes. A similar pattern was observed for ΔKHN , although the values obtained for 5%F+5%TMPnano were 25% lower than those obtained for 5%F+5%TMPmicro. The highest CaF_2 concentrations were promoted by 5%F, 5%F+5%TMPmicro and Duraphat varnishes. The second phase evaluated the protective effect of PLA, 5%F, 5%F+5%TMPmicro, 5%F+2.5%TMPnano and 5%F+5%TMPnano varnishes on initial erosion of bovine enamel. Specimens ($n=8$), selected by SH, received a single application of the varnishes, remaining in contact for 6 h. Varnishes were then removed and the blocks, submitted to four individual erosive challenges

(1 minute, citric acid, 0.75%, pH = 3.5, under stirring) and analyzed by SH after each challenge. Overall, the highest percentage of SH change was observed for PLA, followed by 5%F, 5%F+5%TMPmicro and both varnishes containing TMPnano, with no significant differences between 5%F+2.5%TMPnano, 5%F+5%TMPnano. Finally, the third phase evaluated the pattern of fluoride and phosphate release from PLA, 2.5%F, 5%F, 5%F+5%TMPmicro, 5%F+2.5%TMPnano, 5%F+5%TMPnano and Duraphat varnishes over 24 h, in a pH cycling model. The varnishes were applied on polyester sheets (n=8/group), which were alternately immersed in remineralizing and demineralizing solutions at 30, 60, 90, 120, 180, 240, 300, 360, 420, 540, 600, 720, 780, 960, 1200 and 1440 min after the first immersion. The solutions were analyzed for fluoride and phosphate concentrations. The varnishes containing TMP promoted an increasing, exponential pattern for the cumulative fluoride release up to 6 hours, reaching a plateau afterwards. In addition, higher quantities of fluoride were released when varnishes were immersed in the demineralizing solution. In general, varnishes containing TMPnano released significantly higher amount of fluoride compared to TMPmicro. The results of the 1st phase allow to conclude that the addition of TMP to fluoride varnishes significantly increases its remineralizing potential in artificial caries lesions, with an additional effect with the use of TMPnano in relation to TMPmicro, this increment was not statistically significant. As for the effect against erosive challenges (2nd phase), a similar pattern was observed, with the highest protective effect observed for both varnishes containing TMPnano, which was significantly higher than the other groups. The higher effect of varnishes supplemented with TMP seems to be related to the higher fluoride release from these products (3rd phase), especially TMPmicro, associated to the constant release of TMP from these formulations.

Key words: Topical fluorides. Polyphosphates. Dental caries. Tooth erosion. Nanoparticles.

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1. Introdução Geral

1. Introdução Geral

A cárie dentária é uma doença biofilme-dependente, resultado de um processo dinâmico de desmineralização e remineralização do esmalte dental decorrente de flutuações no PH [Whitford et al., 2002; Vogel, 2011]. A causa principal é o metabolismo microbiano de carboidratos em biofilmes presentes sobre as superfícies dentárias, o que pode levar à perda mineral ao longo do tempo e, conseqüentemente, à formação da cavidade [Kidd e Fejerskov, 2004]. Este processo é passível de intervenção, enfatizando a necessidade de diagnóstico e tratamento precoce das lesões iniciais, bem como o adequado controle de fatores moduladores, como higiene bucal, dieta, fluxo salivar e exposição ao fluoreto [Kidd, 2011].

Apesar da redução na prevalência da cárie dentária observada em todo o mundo, esta doença continua sendo um desafio de saúde pública, uma vez que é influenciada por condições socioeconômicas, estilo de vida, acesso a serviços de saúde e a programas preventivos de saúde bucal [Petersen, 2005], tornando-a fortemente polarizada e apresentando uma distribuição bimodal [Narvai *et al.*, 2006; Salas *et al.*, 2015].

A redução na prevalência da carie dentária possibilitou o diagnóstico de outras alterações não cariosas que afetam os tecidos dentários duros. Entre elas, destaca-se a erosão dentária, que é definida como a dissolução química do tecido dentário sem envolvimento bacteriano [West e Joiner, 2014]. O processo leva ao amolecimento da superfície dentária seguido de sua dissolução, tornando-a mais propensa aos impactos mecânicos [Wiegand e Attin, 2003; Lussi, *et al.*, 2011]. Entre as medidas usadas para prevenir ou minimizar os efeitos da erosão envolvem orientações sobre a necessidade de reduzir a frequência de ingestão de alimentos e bebidas ácidas e a aplicação de fluoretos tópicos [Ganss, 2008; Salas *et al.*, 2015]. O diagnóstico do desgaste dentário erosivo em estágios precoces é difícil, razão pela qual as lesões são diagnosticadas em estágio moderado ou avançado. Estudos epidemiológicos apresentam ampla variação entre os dados, o que possivelmente se deve a diferentes critérios de diagnóstico, idade dos

pacientes, dentre outros fatores. No Brasil, a prevalência entre crianças e adolescentes varia entre (20% a 78%) [Rios et al., 2007; Gurgel et al., 2011].

A terapia com fluoretos tem sido uma estratégia para o controle da cárie dentária e o desgaste dental erosivo. O flúor (F) tem a capacidade de retardar o prevenir o desenvolvimento de lesões de cárie dentária por reduzir a solubilidade do esmalte em meio ácido, inibir a obtenção e utilização de glicose por bactérias e promover a remineralização do esmalte [Buzalaf *et al.*, 2011]. Quanto à erosão dentária, as terapias que contem só F têm promovido um efeito limitado na redução do desgaste dental, sendo efetivas quando é combinado com outros agentes protetores como o estanho e fosfatos [Lussi e Carvalho, 2015; Ganss *et al.*, 2011]. No entanto, considera-se que a aplicação tópica de F a altas concentrações fornece minerais adicionais à superfície dos dentes (CaF_2), que são dissolvidos durante exposições futuras a ácidos, o que reduz a taxa de dissolução do esmalte dental [Ganss et al., 2001; Largerweij et al., 2006].

Dentre os métodos de aplicação profissional de F, os vernizes fluoretados apresentam como principais vantagens a facilidade de aplicação, tempo de contato prolongado com a superfície dentária, segurança e boa aceitabilidade pelos pacientes [Pessan *et al.*, 2005, 2011; Marinho *et al.*, 2013]. Os vernizes são produtos viscosos que endurecem quando em contato com a saliva, formando uma película aderida à superfície dentária, a qual libera F para a superfície do esmalte, biofilme dental e saliva. Desta forma, os vernizes tanto reduzem a desmineralização, como aceleram o processo de remineralização do esmalte [Pessan *et al.*, 2011]. Uma revisão sistemática recente concluiu que a redução nos índices ceo-s e CPO-S associada a seu uso foi 37% e 43% respectivamente [Marinho et al., 2013].

Alternativas para potencializar o efeito do F em veículos de uso caseiro e profissional têm sido estudadas, as quais incluem a redução do pH do veículo [Øgaard et al., 2001; Vilhena et al., 2010] e a suplementação com sais de cálcio e/ou de fosfato [Schemohorn et al., 1999 a,b]. A adição de trimetafosfato de sódio (TMP) a produtos fluoretados demonstrou aumentar significativamente seus efeitos protetores e terapêuticos contra a cárie dentaria [Takeshita *et*

al.,2009,2015; Danelon *et al.*,2013, 2014; Favretto 2013] e desgaste dental erosivo [Moretto *et al.*, 2010; Manarelli *et al.*, 2011; Pancote *et al.*, 2014; Cruz *et al.*, 2015]. Esta associação também foi avaliada para vernizes fluoretados, em uma série de estudos que demonstram um efeito sinérgico na desmineralização e remineralização do esmalte, bem como no desgaste erosivo do esmalte usando protocolos *in vitro* e *in situ* [Manarelli *et al.*, 2013, 2014, 2015, 2017; Moretto *et al.*, 2013].

A fim de aumentar ainda mais os efeitos preventivos e terapêuticos de dentifrícios contendo TMP, nanopartículas deste fosfato foram adicionadas a um dentifrício de 1100 ppm F, o que demonstrou reduzir significativamente a desmineralização do esmalte *in vitro* [Danelon *et al.*, 2017a] e aumentar seu efeito remineralizador *in situ* [Danelon, 2015]. Esta formulação também se mostrou eficaz em aumentou os efeitos protetores sobre o desgaste dental erosivo quando comparada a formulações de mesma concentração de F, contendo TMP microparticulado ou sem TMP [Danelon *et al.*, 2017b].

Considerando as vantagens anteriormente citadas da suplementação de dentifrícios fluoretados com nanopartículas de TMP, é possível que a adição de TMP nanoparticulado a vernizes fluoretados promova um aumento do potencial preventivo e terapêutico destas formulações. Assim o objetivo do presente estudo foi avaliar o efeito de vernizes fluoretados contendo nanopartículas de TMP sobre a remineralização de lesões de cárie artificiais e sobre a erosão de esmalte dental, em protocolos *in vitro*. Em acréscimo, este estudo objetivou avaliar o padrão de liberação de F e TMP a partir destas formulações, a fim de melhor compreender os mecanismos envolvidos na interação entre F e TMP liberados a partir destes produtos em função da concentração de TMP e tamanho da partícula adicionada.

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

- Capítulo 1: **“In vitro remineralization of caries-like lesions with fluoride varnishes containing nano-sized sodium trimetaphosphate”** (artigo submetido ao periódico Journal of Dentistry);

- Capítulo 2: **“Nano-sized sodium trimetaphosphate enhances the protective effect of fluoridated varnishes on initial enamel erosion”** (artigo formatado nas normas do periódico Journal of Dentistry);

- Capítulo 3: **“Fluoride and phosphate release from fluoride varnishes supplemented with nano-sized sodium trimetaphosphate”** (artigo formatado nas normas do periódico Caries Research).

2. Capítulo 1

***In vitro* remineralization of caries-like lesions with fluoride varnishes containing nano-sized sodium trimetaphosphate¹**

LC Báez-Quintero¹, ACB Delbem¹, ME Nagata¹, MM Manarelli¹, ER Camargo²,
VT Sakai³, M Danelon¹, JP Pessan¹

¹Department of Pediatric Dentistry and Public Health, School of Dentistry, Araçatuba, São Paulo State University (UNESP), Araçatuba, SP, Brazil

² LIEC-Department of Chemistry, Federal University of São Carlos (UFSCar), São Carlos, SP, Brazil

³Department of Clinic and Surgery, School of Dentistry, Universidade Federal University of Alfenas (UNIFAL-MG), Alfenas, MG, Brazil

Short title: F varnish containing nanosized TMP on enamel remineralization.

Keywords: Fluoride varnish. Polyphosphates. Dental caries. Topical fluorides. Dental enamel.

Corresponding author:

Juliano Pelim Pessan

School of Dentistry, Araçatuba, São Paulo State University (UNESP)

Department of Pediatric Dentistry and Public Health

Rua Jose Bonifacio 1193

16015-050 Araçatuba - SP - Brazil

Tel: (+55) 18 3636 3314

Email: jpessan@foa.unesp.br

Declaration of interest

A patent was requested for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3.

¹ Artigo formatado de acordo com as normas do periódico *Journal of Dentistry* (Anexo B)

2.1. Abstract

Objective to evaluate *in vitro* the effect of fluoride varnishes supplemented with nano-sized TMP on the remineralization of caries-like lesions using bovine enamel. **Materials and Methods:** enamel blocks ($n=168$) were submitted to induction of caries-like lesions and randomly divided into 7 experimental groups ($n=24$ /group): Placebo (no fluoride or TMP), 2.5% NaF, 5% NaF, 5% NaF + 5% micrometric TMP, 5% NaF + 2.5 or 5% nano-sized TMP, and a commercial varnish (Duraphat™). Varnishes remained in contact with the blocks during 6 h; half of the blocks were used for analysis of loosely- (CaF_2) and firmly-bound (FA) fluoride, while the remaining blocks were submitted to a pH-cycling regimen (6 days). The percentage of surface hardness recovery (%SHR), cross-sectional hardness (ΔKHN), and CaF_2 and FA on/in enamel were determined. Data were analyzed by ANOVA and Student-Newman-Keuls' test ($p<0.05$). **Results:** A dose-response relationship was observed between the fluoride concentration in the varnishes without TMP and %SHR ($p<0.05$). TMP-containing varnishes promoted significantly higher %SHR when compared to TMP-free products, without significant differences among varnishes with TMP. A similar pattern was found for ΔKHN , despite the values found for 5% NaF + 5% nano-sized TMP were 25% lower when compared with 5% NaF + 5% micrometric TMP. Varnishes containing 5% NaF, 5% NaF + 5% TMPmicro and Duraphat promoted the highest CaF_2 concentrations compared to the other groups ($p<0.05$). **Conclusion:** the supplementation of fluoride varnishes with TMP enhances the remineralizing effect of these products, without a significant additional benefit of the use of nano-sized particles.

Clinical Significance: the enhanced remineralizing effect promoted by the TMP-containing varnishes suggest that these products could be a viable alternative for the treatment of patients with non-cavitated carious lesions. The reduction in ΔKHN also indicates a higher effect on the subsurface when compared with a conventional formulation.

Key words: Nanotechnology, Tooth Remineralization, fluoride varnish, pH-cycling, polyphosphate.

2.2. Introduction

Dental caries is the result of a dynamic process of de- and remineralization, caused by microbial metabolism of carbohydrates at the tooth surfaces, which can lead to mineral loss (critical pH around 5.5) over time and, subsequently, to cavity formation [1, 2]. Adequate oral hygiene, diet, salivary flow and fluoride exposure are modulating factors that should be evaluated and/or controlled in order to prevent or reverse the caries process [3]. Among these factors, the use of topical fluorides is known to directly interfere with the balance between de- and remineralization [4], being used worldwide in community-based, self-applied and professionally applied fluoride schemes.

Regarding professionally applied fluoridated vehicles, fluoridated varnishes are widely recommended for the remineralization of initial caries lesions, since they are viscous products containing high concentrations of fluoride that harden upon contact with saliva, forming a thin film that adheres to the surface of the carious teeth, releasing F to the oral cavity for prolonged periods of time [5]. A recent meta-analysis evaluating the effect of fluoride varnishes on the prevention of caries in children and adolescents concluded that the reduction in the DMFS and dmfs indexes associated with their use are 43% and 37%, respectively [6].

The addition of sodium trimetaphosphate (TMP) to fluoridated products has been shown to increase their protective and therapeutic effects against dental caries. The supplementation of toothpastes at varying fluoride concentrations with TMP was shown to significantly enhance the effects against enamel demineralization [7], reduce enamel demineralization [8], promote significant changes in the dental biofilm regarding mineral composition and the production of extracellular polysaccharides [9], and to reduce the progression of caries lesions in children, in a randomized clinical trial [10]. This association has also been assessed for fluoridated varnishes, in in a series of studies demonstrating a synergistic effect on enamel demineralization and remineralization, as well as on enamel erosive wear using *in vitro* and *in situ* protocols [11-15].

In order to further enhance the effects of TMP-containing toothpaste, recent studies have demonstrated that the addition of nano-sized TMP to a

1100 ppm F toothpaste significantly reduced enamel demineralization *in vitro* [16] and enhanced the remineralizing effect *in situ* [17] when compared to its counterpart containing micrometric TMP, what has been attributed to the greater reactivity of nano-sized TMP (due to the higher ratio of surface area to volume). This higher reactivity of nano-sized TMP was also recently shown to enhance the protective effect of low-fluoride toothpaste (250 ppm F) against enamel demineralization, achieving superior levels when compared to an 1100 ppm F formulation without TMP [18].

Considering the advantages of fluoride varnishes in the clinical practice and given that the above-mentioned effects of nano-sized TMP have not yet been tested for these formulations, the objective of this work was to evaluate *in vitro* the effect of fluoride varnishes supplemented with nano-sized TMP on the remineralization of caries-like lesions using bovine enamel. The null hypothesis was that fluoride varnishes supplemented with nano-sized TMP have a similar ability to promote enamel remineralization compared to their counterparts supplemented with micrometric TMP.

2.3. Materials and Methods

Experimental Design

Bovine enamel blocks (n = 168) were selected after surface hardness analysis (SH), and caries-like lesions were induced (SH1). The blocks were then randomly divided into 7 experimental groups (n = 24 / group) according to the varnishes to be tested: Placebo (no fluoride or TMP); 2.5% NaF; 5% NaF; 5% NaF/5% micrometric TMP; 5% NaF/5% nanosized TMP; 5% NaF/2.5% nanosized TMP, and commercial varnish (Duraphat, 5% NaF), hereafter abbreviated as PLA, 2.5%NaF, 5%NaF, 5%NaF/5%TMPmicro, 5%NaF/5%TMPnano, 5%NaF/2.5%TMPnano and Duraphat, respectively. Blocks were treated once with the varnishes and immersed in a remineralizing solution (4 h), followed by a demineralizing solution (2 h). Varnishes were then removed from the enamel surface and the blocks, subjected to a pH-cycling for 6 days. Finally, specimens were analyzed for SH (allowing the calculation of SH recovery (% SHR)), integrated area of subsurface lesion (Δ KHN), firmly- and

loosely-bound formed (6 h after the application of the varnishes) and retained in/on enamel (after pH-cycling).

Synthesis and characterization of nano-sized TMP particles

Commercial micrometric sodium trimetaphosphate (70 g, Na₃O₉P₃, Aldrich Chemistry, China, purity ≥ 95% CAS 7785-84-4) were ball milled using 500 g of sintered zirconia spheres of 2 mm diameter in 1 liter of isopropanol in a polypropylene bottle. After 48 h, at a grinding speed of 1200 rpm, powders were separated from the alcoholic medium, dried at 60 °C, and ground in a mortar. Histograms were constructed counting more than 100 particles from images obtained with a transmission electron microscope (Philips XL-30 FEG) that were treated using the public domain ImageJ image processing software. Powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC diffractometer in the 2θ range from 10 to 80° with a scanning rate of 2°/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation ($L = K\lambda / B\cos\theta_B$), where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9. [17]

Varnish formulation and determination of fluoride in products

The varnishes were produced by SS White Dental Products (Rio de Janeiro, RJ, Brazil), containing the following components: colophony, ethyl cellulose, tolu balsam, beeswax, toluene sulfonamide, vanillin, saccharin and ethanol. F concentrations were 0 (negative control), 2.5% and 5% NaF (Merck, Germany). To the 5% NaF varnish (Fluorniz, SS White Dental Products, Brazil), sodium trimetaphosphate (Aldrich Chemistry, China) was added at 5% (micrometric or nanosized) or 2.5% (nanosized). F concentrations in the varnishes were determined using a fluoride ion specific electrode (9609 BN, Orion, USA) coupled to an ion analyzer (Orion 720 A+), and calibrated with standards containing 2.0–32.0 µg fluoride/mL, as previously described [19,11].

Preparation of enamel blocks and induction of subsurface lesions

Enamel blocks (4mm x 4mm) were obtained from bovine incisors, previously stored in 2% formaldehyde solution pH 7.0 for 30 days at room temperature [20]. The enamel surface of the blocks was serially polished and selected on the basis of their SH (320.0–380.0 KHN). All surfaces of each block, except the enamel surface, were coated with acid-resistant varnish (Risqué®-Brazil), and subsurface enamel demineralization was produced by immersing each enamel block in 32 mL of a solution with 1.3 mmol/L calcium nitrate tetrahydrate (Zigma), 0.78 mmol/L sodium dihydrogen phosphate monohydrate (Zigma) in 0.05 mol/L acetate buffer, pH 5.0; 0.8 mL F; for 16 h at 37 °C [21]. Thereafter, the SH1 was determined.

Treatment with the varnishes and pH-cycling

The varnishes were applied with a microbrush on each block (n=24/group) only once, which were immersed in 4 mL of a remineralizing solution (1.5 mmol/L Ca, 0.9 mmol/L P, 0.15 mol/L KCl in 0.02 mol/L cacodylate buffer, 0.4 mL F, pH 7.0) during 4 h; followed by immersion in 12 mL of a demineralizing solution (2.0 mmol/L Ca and P in 0.075 mol/L acetate buffer, 0.45 mL F, pH 4.7) for 2 h. The varnishes were then gently removed with a blade and acetone and twelve blocks from each group were subjected a to pH-cycling at 37 °C for 6 days [22, 11]. The other twelve blocks were stored for subsequent analysis of loosely- bound fluoride (CaF_2) and firmly-bound fluoride (FA).

Analysis of enamel hardness

The hardness of the enamel surface was determined using a microhardness tester (Shimadzu HMV-2000), with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations, separated by a distance of 100 μm , were made in the center of each block to analyze initial SH. After the induction of artificially demineralized lesions and after the pH cycling, SH was measured again (SH1 and SH2, respectively), 100 μm from the initial indentations (SH) [22]. The percentage of SH recovery (%SHR = $\{[\text{SH2} -$

$\frac{SH_1}{[SH - SH_1]} \times 100$) was calculated. 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 Micromet 5114 (Buehler, Lake Bluff, USA) and Buehler Omi Met software (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load, for 10 s. Integrated hardness ($\text{KHN} \times \mu\text{m}$) for the lesion into the 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 lesion in the enamel, which was named integrated loss of subsurface hardness (ΔKHN ; $\text{KHN} \times \mu\text{m}$) [23].

Analysis of loosely-bound fluoride (CaF_2) on enamel

The concentration of CaF_2 on enamel was analyzed 6 h after the application of the varnishes (CaF_2 formed; $n=12/\text{group}$) and after the pH cycling (CaF_2 retained; $n=12/\text{group}$). A digital caliper (Mitutoyo CD-15B, Mitutoyo Corporation, Japan) was used to measure the surface area of enamel blocks. Assessment of loosely-bound fluoride (alkali-soluble fluoride – CaF_2 formed and retained) was performed following the methodology of Caslavská *et al.*, 1975[24]. The surfaces of each specimen, except the enamel surface, were coated with wax. Specimens were then immersed in 0.5 ml of 1.0 mol/l KOH solution for 24 h, under constant agitation. The solution was neutralized and buffered with 0.5 ml of TISAB II modified with HCl 1.0 mol/l. An ion analyzer (720A+) and a combined ion-selective electrode (9609 BN), previously calibrated with the standards 0.0625, 0.125, 0.250, 0.500 and 1.0 μg fluoride/ml were used. [25]

Analysis of firmly-bound fluoride (FA) in enamel

After extraction of CaF_2 , enamel biopsy was performed, to provides data on firmly bound fluoride, also 6 h after varnish application (name “formed) and after the pH cycling (named “retained”). Blocks measuring 2 mm x 2 mm were

obtained from half of the longitudinally sectioned blocks, and fixed with adhesive glue on a mandrel for straight. 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, São Paulo, SP, Brazil). One layer of $57.0 \pm 0.06 \mu\text{m}$ each was removed from the enamel block. To the resulting enamel powder, 500 μL of 1.0 mol/l HCl was added. The tubes were agitated for 1 h, and an aliquot of 250 μL of this solution was used for F analysis, after buffering with 250 μL of TISAB II modified with 1.0 mol/l NaOH [8, 25]. An ion-specific electrode (Orion 9609) was connected to an ion analyzer (Orion 720+) were used for the analysis.

Statistical analysis

Analyses were performed using the SigmaPlot software (version 12.0) and the level of statistical significance was established at 5%. The variables %SHR (\log_{10} transformed) and ΔKHN (natural outcomes), showed normal (Shapiro-Wilk test) and homogeneous (Cochran test) distributions and were submitted to one-way ANOVA. Data for enamel fluoride concentrations (\log_{10} transformed) showed normal (Shapiro-Wilk test) and homogeneous (Cochran test) distributions and were submitted to two-way ANOVA, considering the type of varnish and CaF_2 and FA formed and retained. Student-Newman-Keuls' test was used for multiple comparisons for all conditions above.

2.4. Results

The mean (SD) fluoride concentrations (micrograms of fluoride per gram) in the varnishes were 433.6 (33.5), 10,758.4 (302.0), 21,378.8 (708.1), 20,154.0 (326.9), 20,400.2 (262.2), 19,827.8 (316.8) and 23,702.5 (1748.0), respectively for Placebo, 2.5% NaF, 5% NaF, 5% NaF + 5% TMPmicro, 5% NaF + 2.5% TMP nano, 5% NaF + 5% TMPnano and Duraphat. The mean (SD) surface hardness of all the bovine tooth blocks was 355.2 (11.6) KHN. After induction of artificial caries lesions in half of the blocks, the mean (SD) percentage of hardness loss was 85.1 (4.0) %, without significant differences among the groups ($p=0.62$).

A dose-response relationship was observed between the fluoride concentrations in the varnishes without TMP and %SHR ($p < 0.05$). Groups treated with varnishes containing TMP showed significantly higher %SHR when compared to those without TMP, without significant differences among the TMP-containing varnishes (Table 1). A similar pattern was observed for Δ KHN, with the highest values obtained for the Placebo, and the lowest observed for the TMP-containing varnishes (Table 1; Figure 1).

The highest concentration of CaF_2 formed on enamel was found for the groups Duraphat, 5% NaF, 5% NaF + 5% TMPmicro, without significant differences among them. The 5% NaF + 2.5% TMPnano group presented similar values to the 2.5% NaF group. After pH-cycling, the concentration of CaF_2 was lower for all groups, except for the Placebo group. The varnishes Duraphat, 5% NaF, 5% NaF + 5%TMPmicro and 5% NaF + 5%TMPnano had the highest values of CaF_2 retained, without significant differences among them. (Table 2)

No significant differences were observed for groups 5% NaF, 5% NaF + 5% TMPnano, 5% NaF + 5% TMPmicro and Duraphat, regarding firmly bound fluoride after varnish application (6h) or after pH-cycling (6 days). Groups treated with varnishes containing nano-sized TMP had lower firmly-bond F values formed in enamel than the other groups, except for the Placebo. Regarding firmly-bond fluoride retained in enamel, groups treated with varnishes containing TMPnano were not significantly different from 5%NaF, 5%NaF + 5%TMPmicro and Duraphat (Table 2).

2.5. Discussion

Previous studies evaluating the effectiveness of fluoride varnishes supplemented with TMPmicro have shown a significantly greater remineralization of caries lesions *in vitro* [11] and *in situ* [12] compared to TMP-free varnishes with the same fluoride content (5% NaF). The present study confirmed the above-mentioned findings, despite the additional remineralizing effect observed for varnishes containing nano-sized TMP was not statistically significant compared with micrometric particles. Therefore, the study's null

hypothesis was accepted. Some aspects of these outcomes need to be discussed for a better comprehension of the actual benefits of nano-sized TMP when added to varnishes.

Despite the differences between TMPmicro and TMPnano were not significant, the formulation containing 5%NaF/5%TMPnano promoted a ~10% increase in %SHR in comparison with 5% NaF/5%TMPmicro (25% increase when compared with 5%NaF), indicating an additional benefit of this formulation over 5%NaF/5%TMPmicro. It is noteworthy, however, that this modest benefit was achieved using a short-term caries model, what cannot be fully extrapolated to *in vivo* conditions, in which the caries dynamics (de- and remineralization cycles) occur over much longer periods of time. Nonetheless, even a small benefit on enamel surface could be regarded as a positive aspect of the formulation, as it may be associated with an increased resistance to acids upon cariogenic challenges, consequently affecting ion mobility from and to the subsurface.

A second – and most important – aspect of the present data refers to the additional benefit of 5%NaF/5%TMPnano in the subsurface (Δ KHN), which was 25% lower in comparison with the 5%NaF/5%TMPmicro (~40% when compared with 5%NaF). Despite not statistically significant, such effect represents a substantial reduction in the lesion body, which is the most relevant parameter when considering the nature of caries lesions (*i.e.*, subsurface lesions). Given that dental caries is the net result of the cumulative effect of successive de- and remineralizing cycles, it might be hypothesized that the differences among the three varnishes (5%NaF, 5%NaF/5%TMPmicro and 5%NaF/5%TMPnano) in the present short-term study under *in vitro* testing conditions would become significant over time under clinical conditions, in which varnishes are usually applied 4 times at weekly intervals for the remineralization of white spot lesions (therefore producing a cumulative effect) and the patients are simultaneously exposed to other sources of fluoride (including toothpastes and water). In this sense, it should be noted that the dynamics of de- and re-mineralization in shallow lesions (as in the present study) is much faster when compared with larger, more porous lesions (as may be found *in vivo*) [26], what supports the above-mentioned hypothesis.

It has been reported that an ideal F:TMP molar ratio is paramount for achieving optimum results on enamel de- and remineralization. Considering the different particle sizes assessed in the present study and based on previous observations with toothpastes [17], it was expected that a lower concentration of nano-sized TMP would be required to promote a similar remineralizing effect to that achieved with micrometric TMP. While this was indeed achieved for %SHR and Δ KHN when considering 5%NaF/2.5%TMPnano and 5%NaF/5%TMPmicro, respectively, it was surprising that the use of higher concentrations of TMPnano (5%NaF/5%TMPnano) further enhanced the remineralizing effect of the varnish. This raises important questions on the reactivity of nano-sized TMP embedded in the varnish matrix after hardening. The smaller size of TMPnano might be associated with a higher interaction with the varnish matrix, which may result in a lower mobility from it to the oral environment (saliva and tooth surfaces). Therefore, increasing the amount of TMPnano on the formulation (from 2.5 to 5%) seems to have overcome this limitation, ultimately leading to a higher release of TMP from the varnish, thus achieving a higher remineralizing effect when compared to 5%NaF/2.5%TMPnano. The above-mentioned considerations would not be applicable to other topical formulations (such as toothpastes and gels), due to the higher mobility of active ingredients (F and TMP) within the vehicle [16-18].

One of the main advantages of professionally applied fluoridated vehicles is the formation of large quantities of CaF_2 , which act as a slow-release F reservoir controlled by the intraoral pH, able to interfere in the processes of de- and remineralization [27]. For the TMP-free formulations, a clear dose-response relationship between F content in the varnishes and the resulting CaF_2 concentrations formed on enamel, what is in line with previous observations [11-12]. For the TMP-containing varnishes, however, the amount of CaF_2 formed varied depending on the particle size (micro or nano) and concentration (2.5 or 5%), with the highest values observed for the 5%NaF/5%TMPmicro (similar to 5%NaF), followed by 5%NaF/5%TMPnano and 5%NaF/2.5%TMPnano (2- and 4-fold lower than 5%NaF, respectively). These data provide interesting information on the mechanisms of TMP-containing fluoridated varnishes on the dynamics of de- and remineralization. Given that

the highest remineralizing effect (5%NaF/2.5%TMPnano) was associated with a reduced formation of CaF_2 , the present study reinforces the concept that the main effect of this formulation is not related to the deposition of high quantities of loosely-bound fluoride [11]. Instead, previous observations indicate that the main effect of TMP is related to a reduction of acid diffusion into enamel and to the retention of ions on its molecule, which result in more reactive remineralizing species upon acid challenges [28,29, 17].

Differences between present CaF_2 data and those obtained in a previous study using the same varnish formulations (except for TMPnano) also deserve comment. First, the present CaF_2 data are, overall, substantially lower than those reported by Manarelli et al. [11]. Second, while the results of the 5%NaF/5%TMPmicro in the present study were similar to those found for 5%NaF, these were 2-fold lower when compared with 5%NaF in the above-mentioned study. Third, CaF_2 data resulting from Duraphat were virtually identical to those seen for 5%NaF in the present study, but 40% lower in the study by Manarelli et al. [11]. Considering that both studies were conducted by the same research group, and that the test varnishes were produced by the same manufacturer, the differences above seem to be related to the natural resin used in the production of the varnishes. Shen and Autio-Gold [19] reported significant differences between tubes (of the same batch) of colophony-based varnishes containing 5% NaF, and that fluoride content can vary between doses dispensed from the same tube. Nonetheless, despite these differences inherent to the non-therapeutic component of the formulation, it must be emphasized that both in the study by Manarelli et al. [11] and in present work, a clear dose-response relationship was observed between fluoride content in the varnishes without TMP and the rate of enamel remineralization, and that TMP significantly enhanced this effect. This clearly indicates that differences in the natural resin used for the manufacturing produce negligible effects on the main outcomes assessed (hardness data).

Similarly to the findings of CaF_2 described above, the effect of TMP-containing varnishes does not seem to be related to enamel fluoride uptake (firmly-bound) in the outermost enamel layers, especially TMPnano, which promoted the lowest uptake among the varnishes containing 5%NaF (6 h after

varnish application). A different pattern, however, has been described for fluoridated toothpastes supplemented with TMPnano, what seem to be related to the different caries model used (demineralization), type of substrate (sound enamel), fluoride concentration (1100 ppm F) and frequency of use (2x/day, during 5 days) [16]. Another important aspect might be related to the differences between the two vehicles (toothpaste and varnish), what might affect ion mobility within the formulation, thus leading to different outcomes. Also, it is important to point out that the amount of enamel removed for firmly-bound fluoride determination in the present study was $\sim 57\mu\text{m}$, so that any possible changes in fluoride concentrations in deeper enamel layers would not, therefore, be detected by the analytical method employed. Regardless of the above, the absence of a linear correlation between firmly-bound fluoride and enamel remineralization after the use of fluoride varnishes [30] suggest that enamel fluoride uptake should not be considered as a main response variable in *in vitro* models assessing the remineralizing effect of such formulations.

Some methodological implications related to the *in vitro* model used might also have impacted the results obtained. The removal of the varnishes 6 h after application could possibly underestimated the effect of the products, given that F and TMP release from the products was stable for up to 24 h after varnish application. However, the varnishes were removed in order to assess the chemical effect of the products (*i.e.*, F and TMP release) instead its mechanical protection [31]. Under clinical conditions, the varnishes would be removed by mechanic forces (mastication or brushing), at varying times after application, opposed to *in vitro* conditions in which the varnishes would remain over the specimens for several days. Other *in vitro* limitations such as absence of self-applied and/or community-based fluoride sources commonly used by the patients, the short-term nature of the protocol and the lesion depth have already been discussed and might also have played an important role on the outcomes. Therefore, the investigation of the remineralizing potential of the test varnishes under *in situ* and *in vivo* conditions could provide important data on the real benefit of such formulations in caries prevention and control.

In conclusion, fluoride and TMP present a synergistic effect on the remineralization of artificial caries lesions *in vitro*. Despite the differences

between micrometric and nano-sized particles of this polyphosphate were not significant, the marked reduction of the subsurface lesion (~25%) when compared with TMPmicro suggest that the addition of nano-sized TMP could be an interesting alternative to further enhance the remineralizing effect of TMP-containing fluoridated varnishes. Studies with different protocols would be instructive in this regard.

2.6. Acknowledgments

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Table 1. Percentage of surface hardness recovery (%SHR) and integrated loss of subsurface hardness (Δ KNH) according to the varnishes

Groups	%SHR	Δ KNH
Placebo	14,6 (3,2) ^A	6876,7 (827,5) ^A
2,5% NaF	35,8 (3,7) ^B	5794,8 (752,0) ^B
5% NaF	41,1 (2,6) ^C	4400,2 (1266,6) ^C
5% NaF + 5% TMP micro	47,2 (5,4) ^D	3709,5 (929,5) ^D
5% NaF + 5% TMP nano	51,4 (3,2) ^D	2787,7 (781,9) ^D
5% NaF + 2,5% TMP nano	48,2 (3,3) ^D	3181,2 (742,3) ^D
Duraphat	37,6 (4,8) ^{B,C}	4783,1 (1184,3) ^C

Values are presented as means (SD). Different uppercase superscript letters indicate significant differences among the groups (Student –Newman Keuls' method, $n=12$, $p<0.05$). %SHR data were \log_{10} -transformed for the statistical analysis.

Table 2. CaF₂ and firmly bound fluoride 6 h after varnish application and after pH-cycling according to groups

Groups	CaF ₂ , µg/cm ²		Fluoride, µg/cm ²	
	Formed	Retained	Formed	Retained
Placebo	0.24(0.04) ^{A,a}	0.26(0.04) ^{A,a}	0.40(0.10) ^{A,a}	0.60(0.11) ^{B,a}
2,5% NaF	5.30(2.9) ^{A,b}	0.35(0.11) ^{B,bc}	0.91(0.15) ^{A,bcd}	0.73(0.11) ^{B,bcd}
5% NaF	22.84(7.7) ^{A,cd}	0.45(0.14) ^{B,def}	1.09(0.38) ^{A,d}	0.97(0.30) ^{A,e}
5% NaF + 5% TMP micro	25.51(8.3) ^{A,d}	0.58(0.09) ^{B,ef}	0.91(0.21) ^{A,bcde}	0.94(0.39) ^{A,ef}
5% NaF + 5% TMP nano	10.86(4.4) ^{A,e}	0.47(0.10) ^{B,def}	0.64(0.09) ^{A,fg}	0.77(0.17) ^{A,defg}
5% NaF + 2,5% TMP nano	5.70 (1.8) ^{A,b}	0.38(0.09) ^{B,bcd}	0.64(0.18) ^{A,g}	0.80(0.19) ^{B,defg}
Duraphat	21.39(6,8) ^{A,cd}	0.59(0.15) ^{B,f}	0.96(0.28) ^{A,cd}	0.95(0.25) ^{A,efg}

Values are presented as mean (SD). Different lowercase superscript letters show significant difference among groups in each analysis. Different uppercase superscript letters indicate differences between CaF₂ formed and retained, as well as between fluoride formed and retained within each group. Data (log₁₀-transformed) were submitted the Student-Newman-Keuls' method, $n = 12$ ($p < 0.05$).

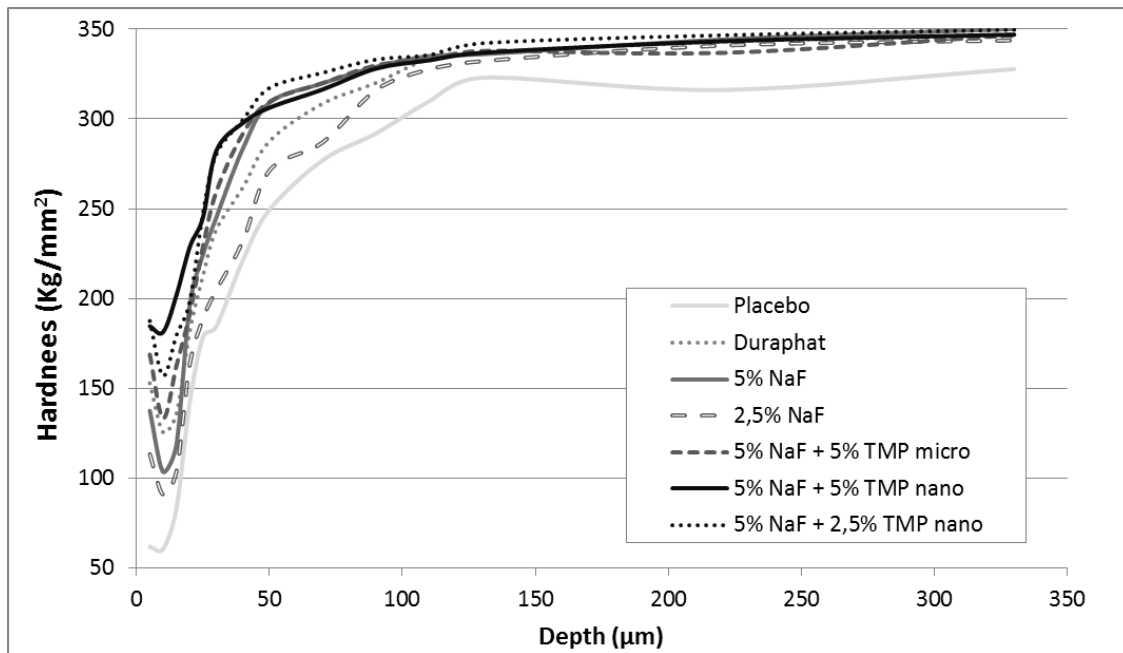


Figure 1. Differential hardness profile as a function of depth according to the groups.

3. Capítulo 2

Sodium trimetaphosphate enhances the protective effect of fluoridated varnishes against initial enamel erosion (short communication)²

Liliana Carolina Báez-Quintero¹, Alberto Carlos Botazzo Delbem¹,
Mariana Emi Nagata¹, Marcelle Danelon¹, Diego Felipe Mardegan Gonçalves¹,
Daniela Rios², Juliano Pelim Pessan¹

¹Department of Pediatric Dentistry and Public Health, School of Dentistry, Araçatuba, São Paulo State University (UNESP), Araçatuba, SP, Brazil

²Department of Pediatric Dentistry, Orthodontics and Public Health, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil

Short title: F varnishes containing TMP reduce enamel erosion

Corresponding author:

Juliano Pelim Pessan

School of Dentistry, Araçatuba, São Paulo State University (UNESP)

Department of Pediatric Dentistry and Public Health

Rua Jose Bonifacio 1193

16015-050 Araçatuba - SP - Brazil

Tel: (+55) 18 3636 3314

Email: jpessan@foa.unesp.br

Declaration of interest

A patent was requested for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3.

² Artigo formatado de acordo com as normas do periódico *Journal of Dentistry* (Anexo B)

3.1. Abstract

This study assessed the protective effect of fluoridated varnishes supplemented with micrometric or nanosized sodium trimetaphosphate (TMPmicro or TMPnano, respectively) on initial enamel erosion. Bovine enamel blocks were selected by surface hardness (SH) and randomly assigned ($n=8/\text{group}$) into the groups: Placebo (no F/TMP), 5% NaF, 5% NaF+5%TMPmicro, 5% NaF+2.5%TMPnano and 5% NaF+5%TMPnano. Blocks received a single application of the varnishes and were immersed in artificial saliva (6 h). Following, the varnishes were removed and the blocks, subjected to four individual erosive challenges (1-min, citric acid, 0.75%, pH=3.5, under agitation); SH was determined after each challenge. Data were analyzed by ANOVA and Student-Newman-Keuls' test ($p<0.05$). Overall, the highest %SH loss was observed for Placebo, followed by 5% NaF, 5% NaF+5% TMPmicro and both varnishes containing TMPnano, without significant differences between 2.5% and 5% TMPnano. It was concluded that TMP increases the protective effect of fluoridated varnishes against erosive challenges, with an additional benefit from the use of TMPnano.

Keywords: Fluoride varnish. Polyphosphates. Tooth erosion. Topical fluorides. Dental enamel.

3.2. Introduction

The limited action of conventional fluoride therapies on the prevention of tooth erosion has prompted to studies assessing the effects of novel therapeutic agents used in association with fluoride [1, 2]. Among the options available, sodium trimetaphosphate (TMP) has shown to promote a synergistic protective effect against enamel erosion, followed or not by abrasion by toothbrushing, when added to fluoridated toothpastes, gels, mouthrinses and varnishes, using *in vitro* and *in situ* protocols [3-6].

The use of nano-sized TMP has been shown to further enhance the protective effects of fluoridated toothpastes against enamel erosive wear using an *in vitro* protocol [7]. No evidence, however, is available for other topically applied fluoridated vehicles. Given the promising results obtained for fluoridated varnishes containing nano-sized TMP on enamel remineralization in an *in vitro* caries model (unpublished data), the present study assessed the effects of fluoridated varnishes supplemented with micrometric or nano-sized TMP on initial enamel erosion. The null hypothesis was that the effect of the fluoridated varnish would not be affected by the addition of TMP, regardless of the particle size.

3.3. Material and Methods

Experimental design

Bovine enamel blocks (n = 40), were selected after surface hardness analysis (SH). The blocks were then randomly divided into 5 experimental groups (n= 8 / group) according to the varnishes to be tested: Placebo (no fluoride or TMP); 5% NaF; 5% NaF/5% micrometric TMP; 5% NaF/5% nanosized TMP and 5% NaF/2.5% nanosized TMP, hereafter abbreviated as PLA, 5%NaF, 5%NaF/5%TMPmicro, 5%NaF/5%TMPnano and 5%NaF/2.5%TMPnano, respectively. Blocks were treated once with the varnishes and immersed in artificial saliva (6 h). Varnishes were then removed from the enamel surface and the blocks, subjected to individual erosive challenges (four erosive challenges/1 min each); SH was determined after each challenge.

Synthesis and characterization of nano-sized TMP particles

Commercial micrometric sodium trimetaphosphate (70 g, Na₃O₉P₃, Aldrich Chemistry, China, purity ≥ 95% CAS 7785-84-4) were ball milled using 500 g of sintered zirconia spheres of 2 mm diameter in 1 liter of isopropanol in a polypropylene bottle. After 48 h, at a grinding speed of 1200 rpm, powders were separated from the alcoholic medium, dried at 60 °C, and ground in a mortar. Histograms were constructed counting more than 100 particles from images obtained with a transmission electron microscope (Philips XL-30 FEG) that were treated using the public domain ImageJ image processing software. Powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC diffractometer in the 2θ range from 10 to 80° with a scanning rate of 2°/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation ($L = K\lambda / B\cos\theta_B$), where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9 [8].

Varnish formulation and determination of fluoride in products

The varnishes were produced by SS White Dental Products (Rio de Janeiro, RJ, Brazil), containing the following components: colophony, ethyl cellulose, tolu balsam, beeswax, toluene sulfonamide, vanillin, saccharin and ethanol. F concentrations were 0 (negative control), 2.5% and 5% NaF (Merck, Germany). To the 5% NaF varnish, sodium trimetaphosphate (Aldrich Chemistry, China) was added at 5% (micrometric or nanosized) or 2.5% (nanosized). F concentrations in the varnishes were determined using a fluoride ion specific electrode (9609 BN, Orion, USA) coupled to an ion analyzer (Orion 720 A+), and calibrated with standards containing 2.0–32.0 µg fluoride/mL, as previously described [9,10].

Preparation of enamel blocks

Enamel blocks (4mm X 4mm) were obtained from bovine incisors and was serially polished. Surface hardness (SH) was determined in the following way: one indentation was made at a distance of 1000 μm (Knoop diamond, 500 g, 10 seconds, Shimadzu HMV-2000) [11] from the central region of the blocks' surface, to facilitate indentation localization on subsequent measurement. At a distance of 200 μm from the right vertex of this greater indentation, five indentations, separated by a distance of 100 μm , were made (Knoop diamond, 25 g, 10 seconds, Shimadzu HMV-2000).

Sample size was calculated based on a pilot study, according to which 7 blocks would be required to detect significant differences in mean percentage of surface hardness change of blocks treated with a Placebo (no F or TMP) and a 5% NaF varnish (mean difference = 5.79, standard deviation = 1.5), considering a power of 80% ($\alpha=0.05$). Due to the possibility of losses during the processing of the specimens, 8 blocks were included in each group.

Treatment with the varnishes and erosion cycles

The varnishes were applied with a microbrush on each block only once, which were immersed in 4 mL of artificial saliva (1.5 mmol.l⁻¹ Ca(NO₃)₂·4H₂O; 0.9 mmol.l⁻¹ NaH₂PO₄·2H₂O; 150 mmol.l⁻¹ KCl; 0.1 mol.l⁻¹ Tris buffer; pH 7.0; unstirred, 37 °C) for 6h. The varnishes were then gently removed with a blade and acetone [12]. The erosive challenge consisted of individually immersing each enamel block in 4 mL of citric acid (0.75%, pH=3.5; Synth, Brazil) under agitation (100 rpm) for 1 min at room temperature, followed by washing with deionized water for 20 seconds. In total four erosive challenges were performed. Surface hardness (SH_f) measurements were carried after each erosive challenge to calculate the percentage of surface hardness change ($\text{SHC} = [(\text{SH}_f - \text{SH}_i) / (\text{SH}_i)] \times 100$) after 1, 2, 3 and 4 min of challenge [13, 14].

Statistical analysis

For the statistical analysis, SigmaPlot software for Windows (version 12.0) was used and the significance limit was set at 5%. Data did not present normal (Shapiro-Wilk) and homogeneous (Cochran) distribution. For surface hardness at baseline (prior to the first acid challenge), data were submitted to Kruskal-Wallis test. Data on the percentage of surface hardness change were submitted to 2-way, repeated-measures ANOVA, considering the type of varnish and time of exposure to acid as variation factors. Student-Newman-Keuls' test was used as the *post hoc* test for ANOVA.

3.4. Results

The median (range) of surface hardness at baseline was 354.4 (349.3-366.6) Kg/mm² considering all groups, without significant differences among the groups prior the first acid challenge ($H = 0.439$, $p=0.979$).

Significant differences were observed among the varnishes ($p<0.001$), times of exposure to acid ($p<0.001$), and for the interaction between these variables ($p<0.001$). At 1-min of exposure to acid, the highest surface hardness change was observed for Placebo, followed by 5% NaF and the TMP-containing varnishes ($p<0.05$), without differences among the latter regardless of the type of particle (micro or nano) or TMP concentration (2.5 or 5%). At 2-, 3- and 4-min significant differences were observed among Placebo, 5% NaF, 5% NaF/5% TMP micro and both varnishes containing TMP nano, however, without significant differences between 2.5% and 5% TMP nano. As for the times of exposure to acid, significant differences were observed among all times, for all varnishes tested. (Figure 1)

3.5. Discussion

Several strategies have been investigated in order to prevent or minimize enamel erosive lesions, among which topical fluoride application at high concentrations has been shown to be beneficial, despite of a limited effect [15]. The present study demonstrated that TMP significantly increases the protective

effect of fluoridated varnishes against enamel erosion, and that the use of nanoparticles further enhances this additional benefit, in a short-term, *in vitro* model, thus leading to the rejection of the study's null hypothesis.

The protective effect of TMP when added to fluoride varnishes against enamel erosive wear had already been reported, in studies with *in vitro* and *in situ* protocols, but using a 2.5% NaF formulation produced with an artificial resin as the varnish base [6,12]. In the present study, the additional benefit of TMP was assessed in 5% NaF, colophony-based varnishes, as this is the most widely available formulation. Despite the effect of 5% NaF varnishes containing TMP had not yet been investigated against enamel erosion, an additional protective effect was somehow expected, considering the synergistic action of this formulation reported on the reduction of enamel demineralization and on the remineralization of artificial caries lesions under *in vitro* and *in situ* conditions [10,16,17]. In the present study, the benefit of conventional (micrometric) TMP was shown to be around 43% when compared with 5% NaF (after the last erosive challenge), and such effects further increased to 60-67% when nano-sized TMP was added to the varnishes.

Although this additional protective effect of nano-sized particles over micrometric TMP (30-40%) cannot be directly extrapolated to *in vivo* conditions, it indicates that such formulations might be a promising alternative for preventing enamel mineral loss upon erosive challenges. In this sense, it is noteworthy that despite all test formulations demonstrated immediate (first challenge) and sustained (second to fourth challenges) protective effects, the TMP-containing varnishes (especially those supplemented with nano-sized particles) were more effective in sustaining the protective effects against surface hardness change when compared with 5% NaF. In fact, the percentage of surface hardness change promoted by the 5% NaF/5% TMPnano after the fourth acid challenge (10%) was very similar to that already seen for the 5% NaF after the first challenge (~8%), what seems to be attributed the greater reactivity of nano-sized TMP, ought to the higher ratio of surface area to volume when compared with conventional (micrometric) particles. This aspect is paramount from a clinical perspective, given that vehicles for professional application are used at much lower frequency than products for home use.

Furthermore, for enamel erosion the desirable predominant effect of fluoride-based preventive measures is the formation of an acid-resistant mechanical barrier rather than mineral precipitation. Given that the formation of CaF_2 is significantly reduced for TMP-containing varnishes [10,16,17], the enhanced protective effect of these formulations can be regarded as a result of the strong interactions of TMP with tooth enamel, limiting acid diffusion [10].

This short-term erosion model provides valuable information on the effects of preventive agents against enamel softening after exposure to acidic challenges, given that surface hardness has been shown to be suitable for analyzing minor changes in surface enamel, without bulk enamel loss [18]. Nonetheless, in order to further investigate the potential application of the TMP-containing varnishes used in the present study, it would be instructive to assess the effect of these formulations in promoting the remineralization of enamel with initial erosion lesions, as well as the effects of this therapy against prolonged erosive challenges. Also, given the complex interplay among chemical, physical and behavioral aspects involved in enamel erosive wear, the assessment of the anti-erosive potential of the TMP-containing varnishes under *in situ* conditions, in subjects regularly using fluoridated toothpastes, could bring relevant information related to the real benefit of these varnishes for the clinical practice.

To sum up, this study demonstrated that the addition of micrometric TMP to 5% NaF varnishes significantly reduces initial enamel erosion *in vitro*, and that nano-sized TMP further enhances the protective effect of the varnishes.

3.6 Acknowledgements

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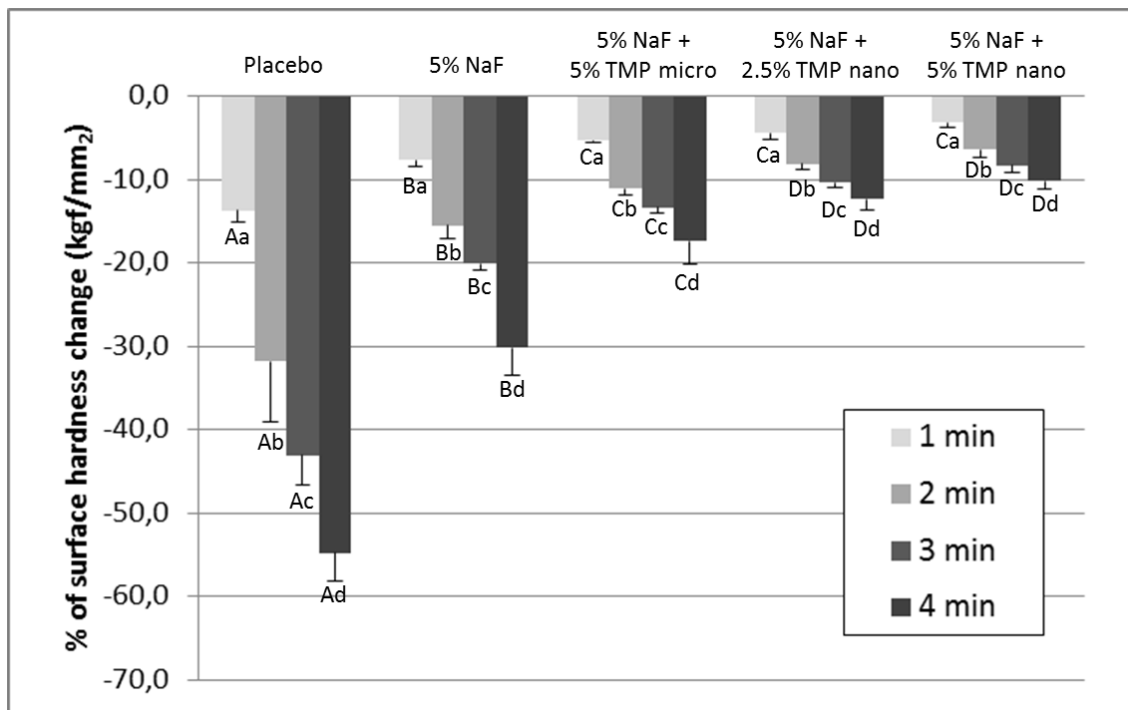


Figure 1. Mean percentage of surface hardness change according to the varnishes applied and the time of exposure to citric acid. Upper- and lower-case letters indicate significant differences among the varnishes (at each individual time point) and among the times after exposure to acid (within each varnish), respectively. Two-way, repeated-ANOVA.

4. Capítulo 3

**Fluoride and phosphate release from fluoride varnishes supplemented
with nano-sized sodium trimetaphosphate³**

Liliana Carolina Báez-Quintero, Alberto Carlos Botazzo Delbem,
Mariana Emi Nagata, Malena Morais Castro e Silva, Robson Frederico Cunha,
José Antonio Santos Souza, Juliano Pelim Pessan

Department of Pediatric Dentistry and Public Health, School of Dentistry,
Araçatuba, São Paulo State University (UNESP), Araçatuba, SP, Brazil

Short title: Fluoride and TMP release from varnishes

Corresponding author:

Juliano Pelim Pessan

School of Dentistry, Araçatuba, São Paulo State University (UNESP)

Department of Pediatric Dentistry and Public Health

Rua Jose Bonifacio 1193

16015-050 Araçatuba - SP - Brazil

Tel: (+55) 18 3636 3314

Email: jpessan@foa.unesp.br

Declaration of interest

A patent was requested for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 10/17/2014 under number BR 10 2014 025902 3.

³ Artigo formatado de acordo com as normas do periódico *Caries Research* (Anexo C)

4.1. Abstract

This study evaluated the amount of fluoride and phosphate released from fluoride varnishes containing micrometric or nano-sized sodium trimetaphosphate (TMP). The experimental varnishes included a placebo formulation (no fluoride or TMP), 2.5% NaF, 5% NaF, 5% NaF + 5% micrometric TMP, 5% NaF + 5% nano-sized TMP, 5%NaF + 2.5% nano-sized TMP, and commercial varnish (Duraphat, 5% NaF). Varnishes were applied on polyester sheets (n=8/group) and alternately immersed in remineralizing or demineralizing solutions at 30, 60, 90, 120, 180, 240, 300, 360, 420, 540, 600, 720, 780, 960, 1200 and 1440 min after first immersion. Fluoride and phosphate were analyzed with an ion-selective electrode and colorimetrically, respectively. Data were analyzed by 2-way, repeated measures ANOVA and Student-Newman-Keuls' test ($p < 0.05$). A dose-response relationship was observed between the fluoride content in the test varnishes without TMP and the amount of fluoride released. Regarding the TMP-containing varnishes, an exponential cumulative release was observed up to 6 h, reaching a plateau afterwards. Also, the amount of fluoride released from the varnishes increased when immersed in demineralizing solutions. Overall, varnishes containing TMPnano released significantly higher amounts of fluoride in comparison with TMPmicro. As for cumulative phosphate release from the varnishes, the general trend from the TMP-containing varnishes showed a constant increase up to 12 h, becoming less marked afterwards, while phosphate release remained fairly constant at low levels for the TMP-free products. Particle size and TMP concentration did not influence the pattern and amount of phosphate released from the varnishes. It was concluded fluoride release was significantly increased in TMP-containing formulations, and that the nanosized TMP further enhanced such effects, without affecting phosphate release from the varnishes.

Key-words: Fluorine; Polyphosphates; Sodium Fluoride; Nano-sized.

4.2. Introduction

Fluoride varnishes are professionally applied vehicles considered as slow fluoride release products, due to their ability to adhere to tooth surfaces and to release fluoride to the oral environment for prolonged periods of time [Pessan et al., 2011]. The advantages of fluoride varnishes regarding ease of application, safety, patient's acceptability, and clinical efficacy are the main reasons for their widespread use in individuals of all ages [Marinho et al., 2013].

In vitro and *in situ* data have shown that the addition of sodium trimetaphosphate (TMP) to fluoridated varnishes promote a synergistic effect on enamel remineralization [Manarelli et al., 2014, 2015], as well as on the reduction of enamel demineralization [Manarelli et al., 2017] and erosive wear [Manarelli et al., 2013; Moretto et al., 2013]. Also, recent data demonstrated that the use of nano-sized TMP further enhanced the protective and therapeutic effects of fluoridated varnishes on enamel remineralization and against erosive challenges, respectively (unpublished observations).

In order to better understand the mechanisms of action of TMP-containing fluoridated varnishes, especially containing nano-sized particles, it is essential to determine the pattern of fluoride and TMP release from the varnishes over time. It has been previously reported that the amount of fluoride released from commercial varnishes are influenced by several factors, including the immersion medium [Lippert, 2014] and type of resin [Shen and Autio-Gold, 2002; Ritwik et al., 2012]. Regarding formulations containing phosphate salts, conflicting evidence is available depending on the type of phosphate, in studies showing that varnishes containing calcium glycerophosphate [Carvalho et al., 2015] or TMP [Manarelli et al., 2016] released higher and lower amount of fluoride, respectively, in comparison with varnishes without any phosphate added.

Considering the above-mentioned variables that influence fluoride release from varnishes, the greater reactivity of nano-sized particles compared with conventional (micrometric) ones, and the need to assess the pattern of fluoride release from varnishes in protocols that better resemble clinical conditions regarding intraoral pH fluctuations, the present study assessed pattern of fluoride and phosphate release from varnishes containing micrometric

or nano-sized TMP, in a pH-cycling model. The null hypothesis were that the pattern of fluoride and phosphate release from the varnishes would not be influenced by the presence of TMP, and that TMP particle size would not affect the results.

4.3. Materials and Methods

Experimental design

The varnishes contained the same basic formulation, differing with respect to the concentrations of fluoride and TMP as follows: placebo (no fluoride or TMP), 2.5% NaF, 5% NaF, 5% NaF + 5% micrometric TMP, 5% NaF + 5% nano-sized TMP, 5%NaF + 2.5% nano-sized TMP and commercial varnish (Duraphat, 5% NaF), hereafter abbreviated as PLA, 2.5%NaF, 5%NaF, 5%NaF/5%TMPmicro, 5%NaF/5%TMPnano, 5%NaF/2.5%TMPnano and Duraphat, respectively. The total fluoride concentration in the varnishes was measured prior to the beginning of the study. Following, a thin layer of varnish was applied on polyester sheets (n=8/group) and subsequently immersed in remineralizing (RE) or demineralizing (DE) solutions (3 mL) in the following sequence: 30 (RE), 60 (RE), 90 (DE), 120 (DE), 180 (RE), 240 (RE), 300 (DE), 360 (RE), 420 (DE), 540 (RE), 600 (DE), 720 (RE), 780 (DE), 960 (RE), 1200 (RE) and 1440 (RE) minutes after first immersion. Fluoride and phosphate were analyzed with an ion-selective electrode and colorimetrically, respectively.

Varnish formulation and fluoride assessment

The experimental varnishes were manufactured by SS White Dental Products (SS White Dental Products, Rio de Janeiro, Brazil) and contained the following components: colophony, ethyl cellulose, tolu balsam, beeswax, toluene sulfonamide, vanillin, saccharin and ethanol. Fluoride concentrations were 0%, 2.5% and 5% of NaF (Merck, Darmstadt, Germany), with or without the addition TMP (Aldrich Chemistry, China) at 5% (micrometric or nano-sized) or 2.5% (nano-sized). A varnish without F and TMP was also prepared (PLA). Fluoride concentrations in the varnishes were determined according to the protocol described by Shen and Autio-Gold [2002] and Manarelli et al. [2013].

Two samples of 0.5 mL were collected from the water phase and buffered with 0.5 mL of TISAB II.

Assessment of fluoride and TMP release from the varnishes

A thin layer of varnish was applied on each side of a polyester sheet (0.5 × 120 mm) (K Dent, Quimidrol, Brazil), which was weighed before and after the varnish application, providing information on the amount of varnish applied (n=8/group) [Manarelli et al., 2016]. Sheets were then placed inside polystyrene vials containing 3 mL of remineralizing solution (1.5 mmol/L Ca, 0.9 mmol/L P, 0.15 mol/L KCl in 0.02 mol/L cacodylate buffer, 0.4 mL F, pH 7.0) or 3 mL of demineralizing solution (2.0 mmol/L Ca and P in 0.075 mol/L acetate buffer, 0.45 mL F, pH 4.7) unstirred, at 37 °C, allowing the varnish to be completely immersed in the solution. Each polyester sheet was transferred to new polystyrene vials in the following sequence: 30 (RE), 60 (RE), 90 (DES), 120 (DES), 180 (RE), 240 (RE), 300 (DES), 360 (RE), 420 (DES), 540 (RE), 600 (DES), 720 (RE), 780 (DES), 960 (RE), 1200 (RE) and 1440 (RE) minutes after first immersion. Fluoride released from all varnishes at each time interval was determined as previously described, calibrated with standards containing 0.5 to 16.0 µg F/mL. Two samples of 1 mL were collected from of the water phase and buffered with 1 mL of TISAB II.

The amount of phosphorus (P) released was determined according to Anderson et al. [1977]. For this, 40 µL of 0.05 mol/L sulfuric acid and 40 µL of 1% periodic acid were added to 200 µL of the DE and RE solutions, and the resulting mixture was kept in boiling bath for 1 h. After cooling, 160 µL of deionized water was added, and an aliquot of 55 µL was transferred to a 96-well polystyrene plate (Costar, Tewksbury, MA, USA). Afterwards, 10 µL of 8% sodium sulfite and 5 µL of 1% sodium molybdate were added. After homogenization, 5 µL of 1% hydroquinone was added, and the plate was kept at 37° C during 30 min. Following, the volume of each well was adjusted to 250 µL with deionized water. Next, the absorbance readings were recorded at 640 nm by using a plate reader (PowerWave 340, Biotek, and Winooski, VT, USA). The amount of P in the DE and RE solutions was then subtracted from that

originally present in the DE and RE solutions, allowing to calculate the amount of TMP present in the solutions.

Statistical Analysis

Statistical analyzes were performed using the SigmaPlot (version 12.0), the level of statistical significance was established at 5% (SigmaPlot, Systat Software Incorporation, San Jose, USA). Data of fluoride and phosphate release showed normal distribution (Shapiro-Wilk test). The cumulative release value was calculated over the 24 hour period. Data obtained on the release of fluoride and phosphate in the DE and RE solutions as a function of time and varnish were analyzed by 2-way, repeated measures ANOVA and Student-Newman-Keuls' test ($p < 0.05$). In order to simplify the interpretation of the data, 0.5 h, 2 h, 6 h, 12 h and 24 h were chosen to present the results.

4.4. Results

Mean (SD) fluoride concentrations ($\mu\text{g F/g}$) in the varnishes were 433.6 (33.5), 10,758.4 (302.0), 21,378.8 (708.1), 20,154.0 (326.9), 20,400.2 (262.2), 19,827.8 (316.8) and 23,702.5 (1748.0), respectively for PLA, 2.5%NaF, 5%NaF, 5%NaF/5%TMPmicro, 5%NaF/2.5%TMPnano, 5%NaF/5%TMPnano and Duraphat.

Figure 1 shows the time-course fluoride release from the varnishes into remineralizing and demineralizing solutions determined at 0.5, 2, 6, 12 and 24 h after immersion into the solutions, as well as the cumulative release over time. The highest amount of fluoride was released by DuraphatTM. Also, a dose-response relationship was observed between the fluoride content in the test varnishes without TMP (PLA, 2.5%NaF and 5%NaF) and the amount of fluoride released. Regarding the TMP-containing varnishes, an exponential cumulative release was observed up to 6 h, reaching a plateau afterwards.

Overall, the amount of fluoride released from the varnishes increased when immersed in demineralizing solutions (Fig. 1A). At 2h (demineralizing solution), F release from varnishes containing TMPnano was significantly higher when compared with 5%NaF and 5%NaF/5%TMPmicro ($p < 0.05$). At 6h (remineralizing solution), no significant differences were observed among the

TMP-containing products, regardless of the particle size (Table 1). As for the cumulative release (Fig. 1B), varnishes containing TMPnano released significantly higher amount of fluoride in comparison with TMPmicro, except for the initial release (0.5 h).

As for phosphate release from the varnishes (Figure 2), a less defined pattern was observed when considering the release at each time point (Fig. 2A). On the other hand, the overall trend seen for the cumulative phosphate release (Fig. 2B) from the TMP-containing varnishes showed a constant increase up to 12 h, becoming less marked afterwards. For the varnishes without TMP, however, phosphate release remained fairly constant at low levels. Cumulative phosphate release from the TMP-containing varnishes was significantly higher than the other products from at 6 h and afterwards (Table 2), without significant differences among the varnishes supplemented with TMP, regardless of the particle size.

4.5. Discussion

The present study was conducted in order to provide additional data on the mechanisms by which TMP, in micrometric or nano-sized particles, interfere in the dynamics of de- and re-mineralization of dental enamel. Given that the pattern of fluoride and phosphate release from the varnishes was influenced by the presence of TMP, and that its particle size significantly influenced only the fluoride release from the products, the study's null hypothesis was partially rejected.

A clear dose-response relationship was observed between F concentration in the test varnishes without TMP and the amount of F released into the media. Nonetheless, when comparing the two varnishes containing 5% NaF (test and commercial formulations), a significant difference was observed, since Duraphat released ~40 to 1700% more fluoride when compared with 5% NaF, depending on the time after first immersion. Given that both varnishes were manufactured with the same salt (NaF) and with the same natural resin (colophony), such differences are likely to be due to inherent properties of the carrier for NaF, including its viscosity. Similar data have been previously

reported in studies assessing fluoride release into artificial saliva (instead of a pH-cycling regimen) [Virupaxi et al., 2016; Shen and Autio Gold, 2002; Al Dehailan et al., 2016], thus reinforcing the above-mentioned observations. However, it must be emphasized that the amount of fluoride released from different varnish formulations cannot be considered as a direct indicator of effect. A study conducted with the same varnishes as those used in the present study (unpublished data) showed no differences between the two 5% NaF formulations (Duraphat and the experimental varnish) regarding their remineralizing effect. Previous observations with different study protocols also support this observation [Maas et al., 2013; Jablonowski et al., 2012; Manarelli et al., 2016; Carvalho et al., 2015; Bolis et al., 2015].

The amount of fluoride released was also shown to be influenced by the addition of micrometric or nano-sized TMP. Despite all TMP-containing varnishes released significantly higher amounts of fluoride when compared with 5% NaF, the additional effect of 5% NaF/5% TMPmicro was ~17%, while the corresponding rate for both formulations containing nano-sized TMP was around 60%. Since the only difference between the varnishes supplemented with 5% TMP is the particle size of TMP (micrometric or nano-sized), one possible explanation for the higher fluoride release from the 5% NaF/5% TMPnano might be related to physico-chemical characteristics of this formulation. Considering a constant dissolution of colophony and other inactive ingredients of the formulations, the lower fluoride release from the 5% NaF (compared with the TMP-containing varnishes) is plausible when taking into account that carrier accounts for 95% of the total mass of the product, while the corresponding rate for the varnishes containing 5% TMP is 90%. This could explain – at least in part – the lower fluoride release from the TMP-free varnish, as the higher proportion of carrier in the formulation would require a larger time for its dissolution, with a consequent impact on fluoride release from the varnish matrix. Regarding the differences in fluoride release from varnishes containing TMPmicro and TMPnano, it is possible the higher particle size of TMPmicro would imply in a lower mobility of this salt from the varnish matrix, consequently affecting fluoride mobility.

It is noteworthy that the pattern observed for fluoride release from the 5% NaF/5% TMPmicro varnish in the present study was the opposite of that previously reported for the same formulation [Manarelli *et al.*, 2016]. Considering that the varnishes used in the present study were produced by the same manufacturer and with the same ingredients as in the study conducted by Manarelli *et al.* [2016], the different protocols for fluoride release is believed to be the main responsible for the discrepant results. While in the above-mentioned study fluoride release was assessed by immersion of the varnishes into artificial saliva solutions at neutral pH, the present protocol used a pH-cycling model (alternating from pH 7.0 to 4.7), in order to mimic pH fluctuations occurring in the oral cavity after varnish application. Furthermore, a previous study showed that fluoride release from varnishes under acidic conditions during 5 min (exposure to citric acid) was significantly higher than the corresponding release in artificial saliva during 30 min [Lippert F, 2014], what is in line with the release observed when the varnishes were immersed in the demineralizing solutions in the present study (Table 1 and Figure 1). All the above taken together suggest that the choice of a protocol for fluoride release from varnish formulations may have important implications related to the patterns observed, as discussed below.

In addition to varnish composition and TMP particle size, time was shown to also influence the pattern of fluoride release from the varnishes. The amount of fluoride released in the first 30 minutes from experimental varnishes was very low, with values not significantly different among the 5% NaF experimental varnishes, regardless of the addition of TMP. Significant differences among the TMP-containing varnishes first were detected at 2 h (considering cumulative fluoride release, Table 1) and the pattern of release exponentially rose up to 6 hours, before reaching a plateau. These results suggest that contact time between the varnishes and the tooth surfaces is paramount for optimizing the protective and/or therapeutic effects of varnishes [Fernandez *et al.*, 2014], what has clinical implications regarding the professional recommendations and patient compliance. Nonetheless, taking into account the limitations inherent to a short-term, *in vitro* protocol, the study of fluoride release from these formulations under *in vivo* conditions could provide important data regarding the

resulting fluoride levels in some biomarkers of exposure, including saliva, dental biofilm and biofilm fluid.

Regarding phosphate release from the varnishes, two aspects deserve comment. Firstly, despite the addition of nano-sized TMP significantly enhanced fluoride release when compared with TMPmicro, such effects were not observed for phosphate release. Considering both varnishes containing 5% TMP (for comparison purposes), the lack of significant differences in phosphate release seem to confirm the above-mentioned hypothesis that the rate of dissolution of the varnish matrix is a determining aspect for the release of the active ingredients of the formulations. Following this rationale, the amount of phosphate released from both varnishes containing 5% TMP would, therefore, not be influenced by the particle size, what is in accordance with the present data. The second aspect is related to the lack of significant differences between the two varnishes supplemented with TMPnano. Due to the two-fold difference in TMP concentrations between the two formulations, it would be expected that the differences in the resulting phosphate release from the formulations would, therefore, be around 100%. The reasons for such a trend are not apparent, and since this is the first study assessing phosphate release from TMP-containing fluoridated varnishes, any hypothesis raised on these results would be speculative, so that future studies with different research protocols would be instructive.

Taking the results of fluoride and phosphate release from the varnishes together, along with data on the effects of these formulations on the remineralization of artificial caries lesions and on the protection of enamel against erosive challenges (unpublished data), it seems logical that the constant release of TMP and the enhanced fluoride release from the varnishes supplemented with TMPnano are the reasons for the higher preventive and therapeutic effects of these products. Further studies, however, are still required in order to confirm the additional effect of TMPnano on the dynamics of dental caries and erosion, especially employing different *in vitro* models and subsequent *in situ* protocols that better resemble clinical conditions.

4.6. Conclusion

The results indicate that the supplementation of fluoride varnishes with TMP significantly increases the amount of fluoride released from the formulations, with an additional effect achieved by the use of nano-sized particles. Phosphate release from the TMP-containing varnishes was not affected by particle size.

4.7 Acknowledgements

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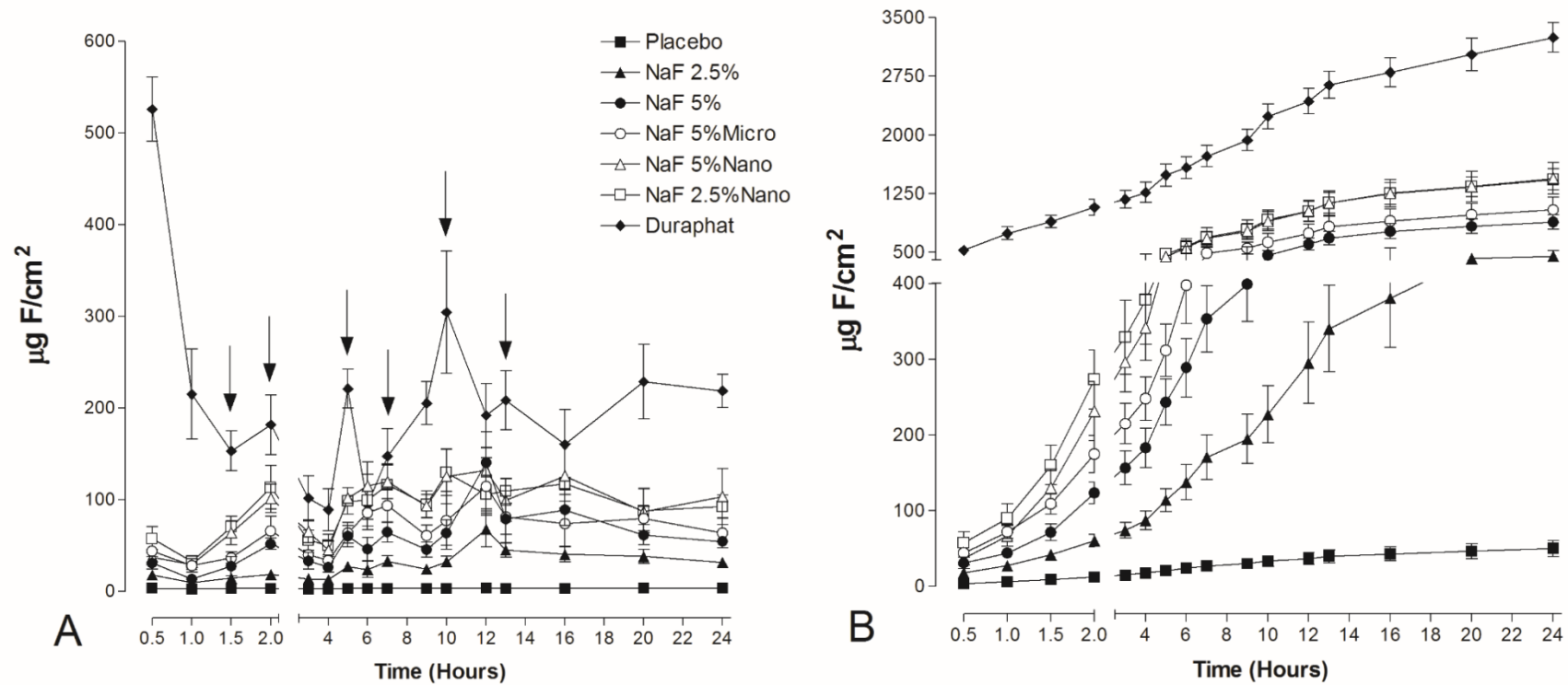


Figure 1. Time-course fluoride release from the varnishes into remineralizing and demineralizing solutions over 24 h. Vertical bars represent standard error of means, while the arrows indicate immersion in the demineralizing solution. A: fluoride release determined at each point. B: cumulative release.

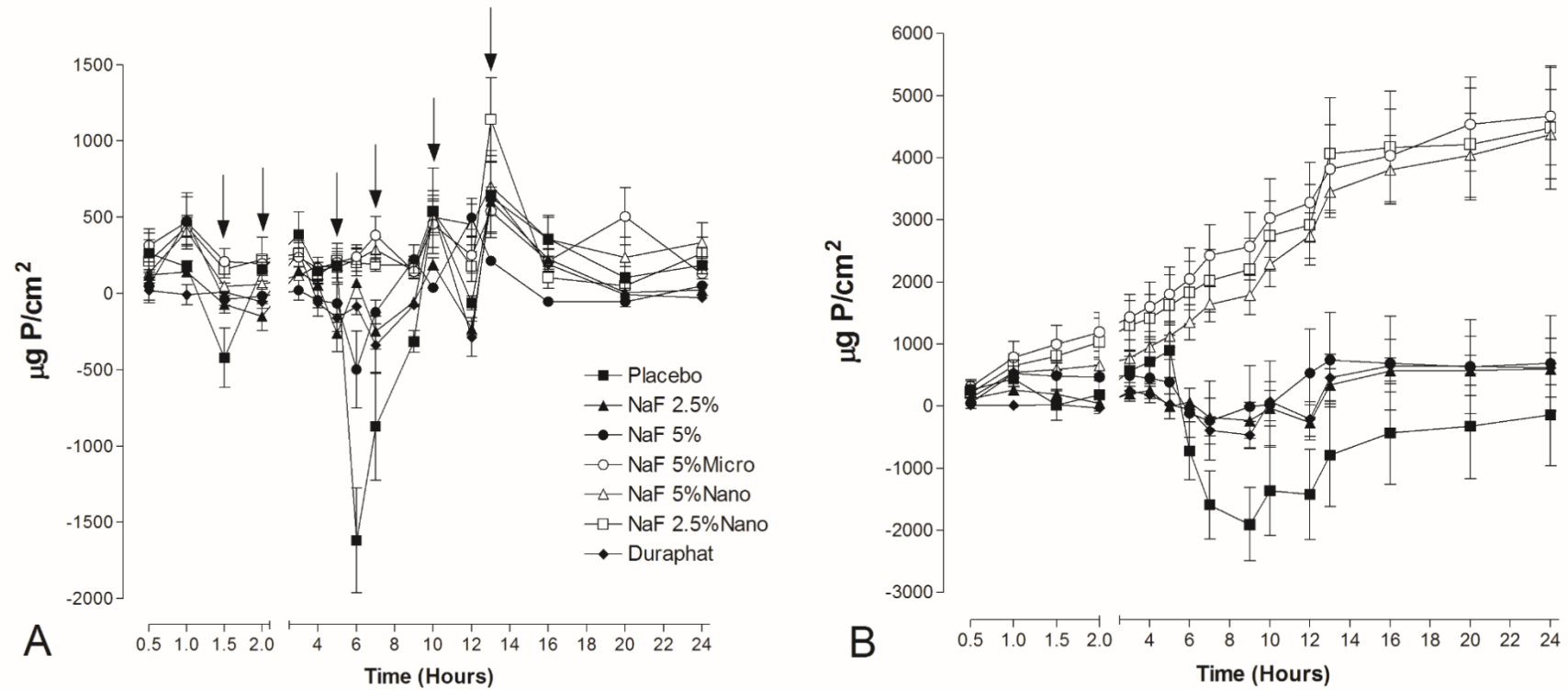


Figure 2. Time-course phosphate release from the varnishes into remineralizing and demineralizing solutions over 24 h. Vertical bars represent standard error of means, while the arrows indicate immersion in the demineralizing solution. A: phosphate release determined at each point. B: cumulative release.

Table 1. Fluoride released from the varnishes at each individual time point and cumulative release as a function of time and varnish composition

Groups	$\mu\text{g F/cm}^2$									
	Released					Cumulative				
	0.5 h	2 h	6 h	12 h	24 h	0.5 h	2 h	6 h	12 h	24 h
Placebo	3.4 ^{a,A} (0.6)	3.2 ^{a,A} (0.6)	3.0 ^{a,A} (0.6)	3.4 ^{a,A} (0.7)	3.4 ^{a,A} (0.7)	3.4 ^{a,A} (0.7)	12.3 ^{a,B} (2.6)	24.0 ^{a,B,C} (5.1)	36.8 ^{a,C,D} (8.0)	50.0 ^{a,D} (10.7)
2.5% NaF	18.0 ^{b,A} (4.2)	18.4 ^{b,A} (3.0)	23.7 ^{b,A} (8.8)	67.9 ^{b,B} (19.3)	31.7 ^{b,C} (3.1)	18.0 ^{b,A} (4.6)	60.3 ^{b,B} (8.2)	137.8 ^{b,C} (23.1)	295.1 ^{b,D} (53.4)	450.6 ^{b,E} (68.3)
5% NaF	30.9 ^{c,A} (6.8)	51.9 ^{c,B} (6.3)	45.8 ^{c,B} (12.6)	140.5 ^{c,C} (15.9)	54.4 ^{c,B} (4.2)	30.9 ^{b,c,A} (7.5)	123.4 ^{c,B} (14.3)	288.9 ^{c,C} (38.6)	603.1 ^{c,D} (74.5)	886.5 ^{c,E} (95.2)
5% NaF + 5% TMP micro	44.0 ^{d,A} (4.6)	65.9 ^{c,B} (16.0)	85.9 ^{d,B,C} (18.9)	114.9 ^{c,C} (31.3)	63.5 ^{c,B} (16.4)	44.0 ^{c,A} (5.0)	174.7 ^{d,B} (24.8)	397.7 ^{d,C} (50.9)	743.7 ^{d,D} (113.1)	1,041.1 ^{d,E} (159.2)
5% NaF + 5% TMP nano	37.4 ^{c,A} (8.6)	101.3 ^{d,B} (15.7)	115.0 ^{d,B} (26.2)	132.0 ^{c,B} (42.0)	103.2 ^{d,B} (30.8)	37.4 ^{b,c,A} (9.5)	231.5 ^{e,B} (38.9)	558.2 ^{e,C} (72.0)	1,028.0 ^{e,D} (137.7)	1,443.6 ^{e,E} (202.1)
5% NaF + 2.5% TMP nano	57.4 ^{d,A} (13.3)	113.5 ^{d,B} (23.9)	99.3 ^{d,B} (28.4)	105.8 ^{c,B} (20.6)	92.6 ^{d,B} (12.4)	57.4 ^{c,A} (14.6)	273.3 ^{e,B} (39.1)	576.5 ^{e,C} (97.5)	1,022.6 ^{e,D} (126.9)	1,429.8 ^{e,E} (135.7)
Duraphat	525.7 ^{e,A} (35.0)	181.6 ^{e,B} (32.5)	94.3 ^{d,C} (15.8)	191.8 ^{d,B} (34.8)	218.7 ^{e,B} (18.1)	525.7 ^{d,A} (38.4)	1,075.5 ^{f,B} (105.5)	1,581.6 ^{f,C} (136.9)	2,430.6 ^{f,D} (161.1)	3,246.5 ^{f,E} (190.7)

Values are presented as mean (SD). Different lowercase letters show significant difference among groups within each column. Different uppercase superscript letters indicate difference among the times of analysis within the same row (two-way ANOVA, Student-Newman-Keuls' test, $n=8$, $p<0.05$).

Table 2. Phosphate released from the varnishes at each individual time point and cumulative release as a function of time and varnish composition

Groups	$\mu\text{g P/cm}^2$									
	Released					Cumulative				
	0.5 h	2 h	6 h	12 h	24 h	0.5 h	2 h	6 h	12 h	24 h
Placebo	265.1 ^{a,c,A} (132.5)	160.5 ^{a,A} (41.8)	-1,618.7 ^{a,B} (343.0)	-60.9 ^{a,C} (46.1)	185.5 ^{a,A} (55.3)	265.1 ^{a,A} (132.5)	181.8 ^{a,c,A} (230.1)	-719.2 ^{a,B} (463.3)	-1,423.3 ^{a,C} (723.7)	-139.7 ^{a,A} (819.6)
2.5% NaF	121.5 ^{a,b,A} (34.2)	-147.1 ^{b,B} (96.1)	72.3 ^{b,A} (29.3)	-228.6 ^{b,B} (45.3)	22.6 ^{b,A} (36.6)	121.5 ^{a,A,B} (34.2)	46.3 ^{a,c,A,B} (129.6)	68.4 ^{b,A,B} (215.2)	-264.3 ^{b,c,A} (280.3)	599.0 ^{b,c,B} (257.7)
5% NaF	49.7 ^{b,A} (92.9)	-15.6 ^{b,A} (53.3)	-498.5 ^{c,A} (250.9)	499.8 ^{a,A} (123.7)	52.4 ^{b,A} (29.2)	49.7 ^{a,A} (92.9)	471.8 ^{a,c,A,B} (279.3)	-111.9 ^{b,A,B} (393.0)	530.5 ^{b,A} (704.9)	690.8 ^{b,B} (768.1)
5% NaF + 5% TMP micro	315.0 ^{c,A} (108.6)	195.9 ^{a,A,B} (51.6)	242.5 ^{d,A,B} (73.9)	251.8 ^{c,A,B} (118.0)	129.5 ^{a,B} (34.2)	315.0 ^{a,A} (108.6)	1,193.4 ^{b,c,B} (311.4)	2,045.7 ^{c,C} (498.5)	3,278.2 ^{d,D} (642.6)	4,668.4 ^{d,E} (787.1)
5% NaF + 5% TMP nano	135.1 ^{a,A,B} (46.5)	60.6 ^{a,A} (29.2)	221.9 ^{d,B,D} (77.5)	452.3 ^{d,C} (134.7)	335.1 ^{c,D} (130.8)	135.1 ^{a,A} (46.5)	655.0 ^{c,B} (132.1)	1,351.7 ^{d,C} (280.3)	2,743.3 ^{d,D} (366.3)	4,375.2 ^{d,E} (718.3)
5% NaF + 2.5% TMP nano	211.7 ^{a,b,c,A} (142.6)	225.1 ^{a,A} (144.5)	202.8 ^{d,A} (87.5)	177.9 ^{c,A} (98.0)	263.2 ^{c,A} (105.0)	211.7 ^{a,A} (142.6)	1,034.8 ^{b,c,B} (380.9)	1,828.6 ^{c,d,C} (502.2)	2,918.9 ^{d,D} (651.0)	4,482.3 ^{d,E} (992.7)
Duraphat	20.9 ^{b,A} (81.7)	-54.4 ^{b,A} (44.4)	-85.0 ^{c,A} (53.1)	-285.6 ^{b,B} (127.6)	-26.7 ^{b,A} (13.4)	20.9 ^{a,A} (81.7)	-28.9 ^{a,c,A} (93.8)	-55.2 ^{b,A} (84.0)	-209.5 ^{c,B} (276.3)	617.3 ^{a,c,A} (476.7)

Values are presented as mean (SD). Different lowercase letters show significant difference among groups within each column. Different uppercase superscript letters indicate difference among the times of analysis within the same row (two-way ANOVA, Student-Newman-Keuls' test, $n=8$, $p<0.05$).

5. Considerações finais

5. CONSIDERAÇÕES FINAIS

O estudo apresentado no Capítulo 1 permite concluir que:

1. A adição de trimetafosfato de sódio (TMP) a formulações de vernizes fluoretados promove um efeito sinérgico sobre a remineralização de lesões de cárie artificial *in vitro*, sem diferença significativa entre partículas convencionais (micrométricas) e nanopartículas deste polifosfato. No entanto, a diferença numérica de ~ 25% na redução da lesão de subsuperfície promovida pela formulação que contém 5% TMP nanoparticulado em comparação ao verniz suplementado com 5% TMP microparticulado sugere que este efeito adicional pode ser de grande importância *in vivo*, considerando a progressão lenta das lesões de cárie e o efeito cumulativo dos vernizes em protocolos clínicos para remineralização de lesões de mancha branca (4 aplicações semanais);
2. Uma vez que o efeito sinérgico do F e TMP nano em formulações de vernizes foi acompanhado de uma menor formação de CaF_2 (F fracamente ligado) sobre o esmalte, resultados reforçam o conceito de que o principal efeito do TMP está relacionado à redução na difusão de ácidos no esmalte, assim como a retenção de íons e moléculas adsorvidas ao TMP ligado à superfície do esmalte, as quais desempenham importante papel remineralizador durante desafios ácidos.

Quanto ao estudo do Capítulo 2, concluiu-se que:

1. Os vernizes suplementados com TMP nanoparticulado (2,5% ou 5%) promovem um maior efeito protetor contra a formação de lesões erosivas iniciais comparado com vernizes sem TMP ou com TMP microparticulado, uma vez que o esmalte tratado com essas formulações é mais resistente a desafios ácidos (aferido indiretamente por dureza de superfície).;

Finalmente, os dados do capítulo 3 indicam:

1. As diferenças na liberação de flúor (F) e fosforo (P) dos vernizes estudados foram influenciadas por características inerentes à produção dos mesmos e ao meio de imersão, tendo sido observada maior liberação sob condições ácidas. Este padrão de liberação parece explicar os resultados obtidos no Capítulo 2, no qual os vernizes suplementados com TMP promoveram os melhores resultados quanto à redução da erosão do esmalte;
2. Os vernizes suplementados com TMP nanoparticulado (5% ou 2,5%) promoveram uma liberação cumulativa (24h) de F maior que o verniz contendo TMP microparticulado, diferentemente da liberação cumulativa de P, a qual não foi influenciada pelo tamanho da partícula deste fosfato. A maior liberação de F a partir dos vernizes contendo TMP nanoparticulado pode ser uma das razões pelo maior efeito destes produtos sobre a remineralização de lesões de cárie *in vitro* (Capítulo 1), bem como na proteção contra a erosão do esmalte (Capítulo 2);
3. A avaliação de liberação de F e P usando soluções de remineralização e desmineralização permitiu obter resultados mais próximos do que ocorre na cavidade bucal após sua aplicação. Estes resultados sugerem que o consumo de alimentos e a escovação dos dentes após a aplicação de vernizes pode ter efeito direto sobre o padrão de liberação de F e P a

6. Anexos

ANEXO A

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ANEXO B

JOURNAL OF DENTISTRY

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ANEXO C

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Submission

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Should you experience problems with your submission, please contact:

Prof. David Beighton

(Editor-in-Chief, Caries Research)

Department of Microbiology

The Henry Wellcome Laboratories for Microbiology and Salivary Research

KCL Dental Institute, Floor 17, Guys Tower

London Bridge SE1 9RT (UK)

Tel. +44 2071887465

Fax +44 2071887466

david.beighton@kcl.ac.uk

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(c) *Monographs*: Matthews DE, Farewell VT: *Using and Understanding Medical Statistics*. Basel, Karger, 1985.

(d) *Edited books*: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): *COX-2*. *Prog Exp Tum Res*. Basel, Karger, 2003, vol 37, pp 124-137.

(e) *Patents*: Diggins AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

(f) *World Wide Web*: Chaplin M: Water structure and behavior. www.lsbu.ac.uk/water, 2004.

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