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Efeito de vernizes fluoretados suplementados com trimetafosfato de sódio na desmineralização e remineralização do esmalte bovino – estudos in vitro e in situ

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Doutor em Ciência Odontológica - Área de Concentração: Saúde Bucal da Criança.

Orientador: Prof. Dr. Juliano Pelim Pessan

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Dedicatória

Michele Mauricio Manarelli

Dedico este trabalho,

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“Plante seu jardim e decore sua alma, ao invés de esperar que alguém lhe traga flores. E você aprende que realmente pode suportar, que realmente é forte, e que pode ir muito mais longe depois de pensar que não se pode mais. E que realmente a vida tem valor e que você tem valor diante da vida!”

(William Shakespeare)

Epígrafe

Resumo Geral

Michele Mauricio Manarelli

Manarelli MM. Efeito de vernizes fluoretados suplementados com trimetafosfato de sódio na desmineralização e remineralização do esmalte bovino – estudos in vitro e in situ [tese]. Araçatuba: Universidade Estadual Paulista; 2014.

O objetivo do presente estudo foi avaliar o efeito de vernizes fluoretados suplementados com Trimetafosfato de Sódio (TMP) sobre a desmineralização e remineralização do esmalte dentário. Para tanto, o estudo foi dividido em 4 capítulos. Inicialmente foi avaliada a liberação de fluoreto (F) e fosfato (P) em soluções de saliva artificial (24 h) após aplicação de vernizes contendo 2,5% NaF, 5% NaF, 5% TMP, 2,5% NaF/5% TMP, 5% NaF/5% TMP, além de uma formulação placebo (sem NaF e TMP) e um verniz comercial (Duraphat), descrito no Capítulo 1. Os efeitos destes vernizes sobre a remineralização de lesões de cárie artificial, bem como sobre a desmineralização do esmalte hígido foram avaliados, respectivamente, nos Capítulos 2 e 3, em modelos de ciclagem de pH. Por fim, o potencial remineralizador dos vernizes placebo, 5% NaF e 5% NaF/5% TMP foi avaliado em um protocolo *in situ* (Capítulo 4). Os espécimes foram avaliados quanto a dureza de superfície (SH), porcentagem de recuperação de SH (%SHR), dureza em secção longitudinal (ΔKHN), microscopia de luz polarizada, bem como a quantidade de CaF_2 e fluoreto fortemente ligado ao esmalte. Uma relação dose-resposta entre a quantidade de NaF e TMP nos vernizes e a liberação de F e P nas soluções de saliva artificial foi observada. Embora um efeito inibitório parcial na liberação de F e P tenha sido observado na presença dos dois sais, um efeito sinérgico foi observado na %SHR e ΔKHN na remineralização de lesões de cárie, bem como na redução da desmineralização (SH e ΔKHN) do esmalte hígido para vernizes contendo NaF e TMP. Em ambas as situações, o efeito protetor do verniz contendo 5% NaF/5% TMP foi significativamente maior que os demais grupos *in vitro*. Este padrão foi confirmado em um protocolo *in situ* sobre a remineralização do esmalte cariado. A formação de CaF_2 e incorporação de fluoreto no esmalte foi, contraditoriamente, menor para vernizes contendo TMP em comparação aos vernizes contendo mesma concentração de F, sem TMP. Com base no exposto, concluiu-se que os vernizes suplementados com TMP foram capazes de promover uma maior remineralização de lesões de cárie artificiais e uma menor desmineralização no esmalte hígido do que os vernizes

com apenas NaF. Considerando que o maior efeito protetor de vernizes suplementados com TMP foi acompanhado por uma redução na concentração de CaF_2 e F fortemente ligado ao esmalte, um possível mecanismo de ação para estes produtos foi proposto.

Palavras-chaves: Remineralização. Desmineralização. Fluoreto. Verniz Fluoretado. Polifosfatos. Esmalte Dentário.

General Abstract

Manarelli MM. Effect of fluoride varnishes supplemented with sodium trimetaphosphate on the demineralization and reminieralization of bovine tooth enamel – in vitro and in situ studies [thesis]. Araçatuba: Universidade Estadual Paulista; 2014.

General Abstract

The aim of the study was to assess the effects of fluoridated varnishes supplemented with sodium trimetaphosphate (TMP) on enamel demineralization and remineralization. For this purpose, the study was divided into 4 chapters. First, the amount of fluoride (F) and phosphate (P) released in artificial saliva solutions (24 h) were measured after the application of varnishes containing 2.5% NaF, 5% NaF, 5% TMP, 2.5% NaF/5% TMP, 5% NaF/5% TMP, besides a PLACEBO formulation (no NaF or TMP) and a commercial formulation (Duraphat), as described in Chapter 1. The effects of these varnishes on the remineralization of caries-like lesions, as well as on the demineralization of sound enamel were assessed on Chapters 2 and 3, respectively, using pH-cycling models. Finally, the remineralizing effect of PLACEBO, 5% NaF and 5% NaF/5% TMP was evaluated using and *in situ* protocol (Chapter 4). Specimens were analyzed by surface hardness (SH), percentage of SH recovery (%SHR), cross-sectional hardness (ΔKHN), polarized light microscopy, as well as firmly and loosely (CaF_2) bound fluoride. A dose-response relationship was observed between NaF and TMP concentrations in the formulations and the amount of F and P released into artificial saliva. Although a partial inhibitory effect on F and P release was observed in the presence of the two salts, a synergistic effect was observed in %SHR and ΔKHN on the remineralization of caries-like lesions, as well as on the reduction of sound enamel demineralization (SH e ΔKHN) for varnishes containing NaF e TMP. Under both conditions, the protective effect of the 5% NaF/5% TMP varnish was significantly higher than the other groups *in vitro*. The same pattern was confirmed *in situ* on the remineralization carious enamel. Firmly and loosely bound fluoride were contradictorily lower for varnishes containing TMP when compared with their counterparts without TMP. Based on the above, it was concluded that TMP-supplemented fluoride varnishes were able to promote a significantly higher

remineralization rate of caries-like lesions and to significantly reduce sound enamel demineralization when compared with varnishes containing the same concentration of NaF, but without TMP. Considering that the higher protective effect of TMP-supplemented varnishes was accompanied by a significant reduction on the amount of firmly and loosely enamel fluoride, a possible mechanism of action for these products was also proposed.

Keywords: Remineralization. Demineralization. Fluoride. Topical Fluoride. Polyphosphates. Dental Enamel.

Lista de Abreviaturas

LISTAS DE ABREVIATURAS

ºC	Graus Celsius
Ca	Cálcio
Ca^{++}	Íon cálcio
CaF^{+}	Íon Fluoreto de cálcio
$CaHPO_4^{0}$	Fosfato de cálcio neutro
$Ca(NO_3)_2.4H_2O$	Nitrato de cálcio tetra hidratado
DP	Desvio padrão
F	Fluoreto
g	Grama
h	Hora
HA	Hidroxiapatita
HCl	Ácido clorídrico
HF^{0}	Fluoreto de hidrogênio neutro
$H_2PO^{-}_4$	Íon fosfato dihidrogênio
KCl	Cloreto de potássio
kgf/mm^2	Quilograma-força por milímetro quadrado
KHN	Unidade de dureza Knoop (Knoop hardness number)
KOH	Hidróxido de Potássio
L	Litro
M	Molar
n	Número de amostra
Na^{+}	Íon sódio
NaF	Fluoreto de sódio
NaOH	Hidróxido de Sódio
$NaH_2PO_4.2H_2O$	Fosfato de sódio monobásico hidratado
P	Fósforo
pH	Potencial hidrogeniônico
s	Segundo
SH	Dureza de superfície (surface hardness)
SHi	Dureza de superfície inicial
SHf	Dureza de superfície final

SH_1	Dureza de superfície pós-desmineralização
SH_2	Dureza de superfície pós-remineralização
%SH	Porcentagem de dureza de superfície
$\%SH_R$	Porcentagem de recuperação de dureza de superfície
TISAB	Tampão ajustador de força iônica total (total ionic strenght adjustment buffer)
TMP	Trimetafosfato de sódio
mg	Miligrama
mL	Mililitro
mL/mm^2	Mililitro por milímetro ao quadrado
mm	Milímetro
mm^2	Milímetro quadrado
mol/ L	Mol por litro
mmol/ L	Milimol por litro
mV	Milivolts
nm	nanômetro
PO_4^-	Íon fosfato
SD	Desvio padrão (standard deviation)
vol% min	Porcentagem de volume mineral
µg	Micrograma
$\mu g/mm^3$	Micrograma por milímetro cúbico
µg F/mL	Micrograma de fluoreto por mililitro
µm	Micrômetro
ΔKHN	Perda integrada de dureza de subsuperfície

Sumário

SUMÁRIO

Introdução Geral

1 INTRODUÇÃO GERAL

A cárie dentária é considerada um problema de saúde pública em todo o mundo, afetando a maioria dos adultos e crianças, em variados graus de severidade. A prevalência da doença varia entre diferentes países, mas geralmente crianças em grupos socioeconômicos mais baixos são mais acometidas, tanto em número de lesões como em severidade.[1] A cárie causa destruição dentária progressiva, muitas vezes acompanhada de dor e sofrimento. Em acréscimo, a reparação e substituição de dentes cariados é extremamente dispendiosa e um grande sorvedouro de recursos dos sistemas de saúde.[2]

A prevenção da cárie dentária em crianças e adolescentes é prioridade para os serviços odontológicos, pois tem uma melhor relação custo-benefício que o tratamento das lesões dentárias e suas consequências. Dentre as estratégias disponíveis para o controle da doença, a terapia com fluoretos tem sido uma estratégia utilizada há mais de 6 décadas, desde a introdução de sistemas de fluoretação da água.[3] Resultados de estudos laboratoriais e epidemiológicos sobre o mecanismo de ação do fluoreto no controle da cárie indicam que seu efeito é predominantemente tópico, o qual ocorre principalmente por meio do aumento da velocidade de remineralização de lesões de cárie iniciais e redução da taxa de desmineralização do esmalte dentário.[4,5]

Por definição, “fluoretos tópicos” se referem a produtos aplicados às superfícies expostas da dentição, em concentrações elevadas, promovendo um

[1]Marinho VCC, Worthington HV, Walsh T, Clarkson JE. Fluoride varnishes for preventing dental caries in children and adolescents. Cochrane Database of Systematic Reviews. In: The Cochrane Library, Issue 11, Art. No. CD002279. DOI: 10.1002/14651858.CD002279.pub3, 2013a.

[2] Marinho VCC, Higgins JPT, Logan S, Sheiham A. Topical fluoride (toothpastes, mouthrinses, gels or varnishes) for preventing dental caries in children and adolescents. Cochrane Database of Systematic Reviews. In: The Cochrane Library, Issue 11, Art. No. CD002782. DOI: 10.1002/14651858.CD002782.pub4, 2013b.

[3] Sampaio FC, Levy SM. Systemic fluoride. Monogr Oral Sci 2011; 22:133-145.

[4] Featherstone JDB, Cate JM. Physicochemical aspects of fluoride-enamel interactions. In: Ekstrand J, Fejerskov O, Silverstone LM, editor(s). Fluoride in Dentistry Copenhagen: Munksgaard, 1988:125-149.

[5] Buzalaf MAR, Pessan JP, Honário HM, Ten Cate JM. Mechanisms of action of fluoride for caries control. Monogr Oral Sci 2011; 22:97-114.

efeito de proteção local e, portanto, não são destinados para ingestão.[2] Os dentifrícios, enxaguatórios, géis, espumas e vernizes fluoretados são as modalidades mais utilizadas atualmente, tanto sozinhas ou em diferentes combinações.[6] Géis, espumas e vernizes são veículos típicos de aplicação profissional e têm sido extensivamente utilizados em programas de prevenção, enquanto que enxaguatórios e dentifrícios são as principais formas de auto-aplicação de fluoreto. A eficácia de dentifrícios, enxaguatórios, géis e vernizes foi avaliada em uma série de revisões sistemáticas[7,8,9], cujos efeitos médios na redução da incidência de cárie (em comparação ao tratamento com placebo ou nenhum tratamento) foram, respectivamente 24%, 26%, 28% e 46%. A associação de veículos fluoretados mostrou ter efeito adicional na redução da incidência de cárie em comparação com veículos isoladamente.[6]

Dentre os produtos de aplicação profissional, os vernizes apresentam boa aceitação por parte dos pacientes e são o veículo de escolha para uso em crianças de pouca idade.[10,11] Vernizes fluoretados foram introduzidos no mercado na década de 1960, para uso exclusivamente profissional. As principais vantagens destes são o tempo de contato prolongado do fluoreto com a superfície dentária, o que aumenta a incorporação de fluoreto pelos tecidos dentais duros, bem como a formação de reservatórios de CaF_2. Em acréscimo, a possibilidade de se utilizar pequenas quantidades do produto

[6] Marinho VCC, Higgins JPT, Sheiham A, Logan S. Combinations of topical fluoride (toothpastes, mouthrinses, gels, varnishes) versus single topical fluoride for preventing dental caries in children and adolescents. Cochrane Database of Systematic Reviews. In: The Cochrane Library, Issue 11, Art. No. CD002781. DOI: 10.1002/14651858.CD002781.pub1, 2013c.

[7] Marinho VCC, Higgins JPT, Logan S, Sheiham A. Fluoride gels for preventing dental caries in children and adolescents. Cochrane Database of Systematic Reviews. In: The Cochrane Library, Issue 11, Art. No. CD002280. DOI: 10.1002/14651858.CD002280.pub2 2013d.

[8] Marinho VCC, Higgins JPT, Logan S, Sheiham A. Fluoride mouthrinses for preventing dental caries in children and adolescents. Cochrane Database of Systematic Reviews. In: The Cochrane Library, Issue 11, Art. No. CD002284. DOI: 10.1002/14651858.CD002284.pub3 2013e.

[9] Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev. 2010;CD007868.

[10] Hawkins R, Locker D, Noble J, Kay EJ. Prevention. Part 7: Professionally applied topical fluorides for caries prevention. Br Dent J 2003; 195:313-317.

[11] Weintraub JA, Ramos-Gomez F, Jue B, Shain S, Hoover CI, Featherstone JDB, Gansky AS. Fluoride varnishes efficacy in preventing early childhood caries. J Dent Res 2006; 85:172-176.

minimiza o risco de ingestão excessiva de fluoreto.[12] Para se maximizar o efeito do produto no controle da cárie, estes devem ser aplicados de 2 a 4 vezes por ano, dependendo do risco e atividade de cárie do indivíduo.[13] O efeito do uso de vernizes no controle da cárie tem sido extensivamente documentado em um grande número de pesquisas clínicas e revisões de literatura, com resultados variando expressivamente entre os estudos. Meta-análises recentes, no entanto, indicam que a redução média na incidência de cárie é de 46% em dentes permanentes e de 33%, em dentes decíduos.[1] Mesmo considerando o grande benefício obtido pelo uso de veículos fluoretados no controle da cárie dentária em crianças e adolescentes, sabe-se que tais produtos apresentam um limite de ação, o que está relacionado a fatores individuais quanto à dieta, microbiota bucal, hábitos de higiene, frequência de consumo de carboidratos fermentáveis, além de outros aspectos relacionados ao hospedeiro. Desta forma, a busca por formulações que aumentem a efetividade de veículos fluoretados tem se intensificado nos últimos anos. A adição de sais de cálcio e/ou fosfatos orgânicos ou inorgânicos pode aumentar a eficácia dos produtos fluoretados.[14,15,16,17] Dentre os fosfatos inorgânicos, o trimetafosfato de sódio (TMP) apresenta alta afinidade com a hidroxiapatita (HA), produzindo um efeito contra a desmineralização do esmalte.[14,15,16,18,19] Estudos recentes

[12] Pessan JP, Toumba KJ, Buzalaf MAR. Topical use of fluorides for caries control. Monogr Oral Sci 2011; 22:115-132.

[13] Weyant RJ, Tracy SL, Anselmo TT, Beltrán-Aguilar ED, Donly KJ, Frese WA, Hujoel PP, Iafolla T, Kohn W, Kumar J, Levy SM, Tinanoff N, Wright JT, Zero D, Aravamudhan K, Frantsve-Hawley J, Meyer DM; American Dental Association Council on Scientific Affairs Expert Panel on Topical Fluoride Caries Preventive Agents. Topical fluoride for caries prevention: Executive summary of the updated clinical recommendations and supporting systematic review. J Am Dent Assoc. 2013;144:1279-1291.

[14] Takeshita EM, Castro LP, Sassaki KT, Delbem ACB: In vitro evaluation of dentifrice with low fluoride content supplemented with trimetaphosphate. Caries Res 2009; 43: 50-56.

[15] Takeshita EM, Exterkate RAM, Delbem AC, Ten Cate JM. Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization in vitro. Caries Res 2011;45:494–497.

[16] Delbem AC, Bergamaschi M, Rodrigues E, Sassaki KT, Vieira AE, Missel EM. Anticaries effect of dentifrices with calcium citrate and sodium trimetaphosphate. J Appl Oral Sci. 2012;20:94-98.

[17] Amaral JG, Sassaki KT, Martinhon CC, Delbem AC. Effect of low-fluoride dentifrices supplemented with calcium glycerophosphate on enamel demineralization in situ. Am J Dent. 2013; 26:75-80.

[18] Barbour ME, Parker DM, Allen GC, Jandt KD. Human enamel erosion in constant composition citric acid solutions as a function of degree of saturation with respect to hydroxyapatite. Journal of Oral Rehabilitation 2005;32: 16–21.

demonstraram que a suplementação de veículos fluoretados com trimetafosfato de sódio (TMP) promove um aumento na efetividade de dentifrícios, géis e enxaguatórios contra a cárie[14,20,21] e erosão dentária.[22,23,24]

As evidências da associação do TMP ao fluoreto em vernizes, entretanto, são escassas e se limitam a estudos avaliando o desgaste erosivo do esmalte[22,24], não havendo, até o momento, nenhuma evidência desta associação na dinâmica da cárie nos processos de des- e remineralização do esmalte dentário. Uma vez que os vernizes fluoretados são amplamente utilizados na prática odontológica para pacientes de todas as idades com o objetivo de reverter lesões iniciais de mancha branca, bem como fortalecer o esmalte hígido contra futuros desafios cariogênicos, o presente estudo teve como objetivo avaliar a capacidade de vernizes fluoretados suplementados com TMP em diminuir a desmineralização do esmalte hígido e promover a remineralização de lesões de cárie artificiais, em protocolos in vitro e in situ.

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

- Capítulo 1: **"Fluoride and phosphate release from fluoride varnishes supplemented with sodium trimetaphosphate"** (artigo submetido ao periódico Brazilian Oral Research)

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- Capítulo 2: **“In vitro remineralizing effect of fluoride varnishes containing sodium trimetaphosphate”** (artigo aceito para publicação no periódico Caries Research);

- Capítulo 3: **“In vitro effect of fluoride varnishes containing sodium trimetaphosphate on enamel demineralization”**(artigo submetido ao periódico International Journal of Paediatric Dentistry);

- Capítulo 4: **“In situ remineralizing effect of fluoride varnishes containing sodium trimetaphosphate”** (artigo submetido ao periódico Archives in Oral Biology).

Capítulo1

Pediatric Dentistry

2 Fluoride and phosphate release from fluoride varnishes supplemented with sodium trimetaphosphate

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De acordo com as instruções aos autores do periódico Brazilian Oral Research (Anexo A)

2.1 Abstract

This study evaluated the amount of fluoride and phosphate released into artificial saliva from varnishes containing fluoride and/or sodium trimetaphosphate (TMP). Varnishes contained 0, 2.5 and 5% NaF, supplemented or not with 5% TMP, besides a commercial formulation (Duraphat, 5% NaF). A thin layer of varnish was applied on polyester sheets (*n*=8/group), which were transferred to vials containing fresh artificial saliva solutions for up to 24 hours. Fluoride and phosphate were analyzed with an ion-specific electrode and colorimetrically, respectively. Data was analyzed by ANOVA and Tukey's test ($p<0.05$). A dose-response relationship was observed between fluoride and phosphate in the varnishes and the amount released into artificial saliva. A markedly higher fluoride release was seen for Duraphat, 5% NaF, 5%NaF/5%TMP within the first hour, while higher amounts of phosphate were released from 5%TMP. Although a partial mutual inhibitory effect was seen when fluoride and TMP were used in combination, high amounts of ions were released from the varnishes containing these salts. The results suggest that fluoride varnishes supplemented with TMP may be effective in preventing dental caries, since both ions released take part in de/remineralization processes.

Keywords: fluoride varnish, polyphosphates, fluoride release.

2.2 Introduction

Fluoride varnishes have been the standard of practice for professional application of topical fluoride for many years, with effectiveness and safety documented in more than 50 clinical trials.[1] The ease of application of fluoride varnishes has led to its popularity in the practice of pediatric dentistry, which involves its use for pre-cooperative children, children with special health care needs, children exposed to head and neck radiation and children who have an exaggerated gag reflex.[2,3]

Recent studies have shown the use of fluoride varnishes to be effective in the prevention of early childhood caries, and reduce caries by 33% and 46%, respectively for the primary and permanent dentitions.[4] They have been shown to decrease the incidence of root caries, and to be better than other topical fluoride agents.[5,6] Duraphat is the most widely used and extensively studied fluoride varnish on the market today, which contains sodium fluoride in a colophony-based resin.[7] However, it is known that fluoride has a limited action in interfering with the de- and remineralization processes occurring on and in dental surfaces.

In order to increase the anticaries action of fluoridated products, the supplementation with salts containing phosphates and/or calcium has been proposed.[8-10] The addition of sodium trimetaphosphate (TMP) has been proven to be a viable alternative for increasing the effectiveness of fluoridated gels, dentifrices and rinses against dental caries.[9-11] More recently, the effects of the association between F and TMP in varnishes on enamel remineralization was tested in an *in vitro* study, in which a synergistic effect was seen for varnishes containing both TMP and F when compared with their counterparts without TMP.[12]

Considering the complex interactions between F and TMP and the scarcity of studies assessing the association of these compounds in varnish formulations, it would be interesting to evaluate the pattern of fluoride and phosphate release from these products, since interactions between NaF and TMP salts, as well as among other components of when added to varnishes are still unknown and could potentially affect the efficacy of the products. Moreover, the knowledge of the release pattern of F and TMP could contribute for a better understanding of the mechanisms of action of TMP-supplemented fluoride vehicles, which have not yet been fully elucidated. Therefore, the present study assessed fluoride and phosphate released from varnishes containing NaF and/or TMP in artificial saliva solutions for a period of 24 hours.

2.3 Materials and Methods

Experimental design

All test varnishes contained the same basic formulation, differing regarding fluoride and sodium trimetaphosphate concentrations, as follows: placebo (no fluoride or TMP), 2.5% NaF, 5% NaF, 5% TMP, 2.5% NaF plus 5% TMP, 5% NaF plus 5% TMP, and a commercial formulation (Duraphat™, lot 02, expiration date February 2015), hereafter abbreviated as PLA, 2.5%NaF, 5%NaF, 5%TMP, 2.5%NaF/5%TMP, 5%NaF/5%TMP, 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 artificial saliva (3 mL). Polyester sheets were transferred to new vials containing fresh artificial saliva solutions at 30, 60, 90, 120, 180, 240, 300, 360, 540, 720, 900, 1080 and 1440 minutes after first immersion.[25] Fluoride and phosphate were analyzed with an electrode and colorimetrically, respectively.

Varnish formulation and fluoride assessment

All varnishes were manufactured by SS White Dental Products (Rio de Janeiro, RJ, 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, Germany), with or without the addition of TMP at concentration 5% (Sigma, China). A varnish without F and TMP was also prepared (PLA).

Fluoride concentrations in the varnishes were determined using a fluoride ion specific electrode (9609 BN – Orion, USA), coupled to an ion analyzer (Orion 720 A+, Orion, USA), calibrated with standards containing 2.0 to 32.0 µg F/mL. The varnishes were weighed (0.015 to 0.020 g) from each sample separately in a 500 mL Erlenmeyer, in triplicate. Chloroform (15 mL) was added to dissolve the resin, followed by the addition of 285 mL of distilled water to recover fluoride. The vials were then vigorously stirred for 15 seconds (3 times) to allow water and chloroform to

[25] Anexo H

separate.[13] 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 phosphate release from the varnishes

A thin layer of varnish was applied on each side of a polyester sheet (20 × 40 mm) (Mylar, DuPont, Wilmington, Del.), which was weighed before and after the varnish application, providing information on the amount of varnish applied (*n*=8/group).[13] Sheets were then placed inside polystyrene vials and allowed to set for 24 hours. Following, 3 mL of artificial saliva[14] (1.5 mmol.l^{-1} $Ca(NO_3)_2 \cdot 4H_2O$; 0.9 mmol.l^{-1} $NaH_2PO_4 \cdot 2H_2O$; 150 mmol.l^{-1} KCl; 0.1 mol.l^{-1} Tris buffer; 0.03 ppm F; pH 7.0; unstirred, 37 ºC) was added to the tubes, so that the varnish coating became completely immersed in the solution. Each polyester sheet was transferred to new polystyrene vials containing fresh artificial saliva solutions at 30, 60, 90, 120, 180, 240, 300, 360, 540, 720, 900, 1080 and 1440 minutes after first immersion. To prevent the varnish coating from peeling off, efforts were made not to bend the polyester sheets when transferring then into new tubes.

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. For phosphorus determination, solutions were hydrolyzed by adding HCl 1 mol/L and heating at a temperature just below the boiling point. Total phosphorus was determined colorimetrically.[15]

Statistical Analysis

Data on fluoride and phosphorus released into the artificial saliva accumulated over 24 hours were analyzed by 1-way ANOVA, followed by Tukey's test ($p<0.05$).

2.4 Results

Mean (SD) fluoride concentrations (µg F/g) in the varnishes were 405.2 (84.9), 22,788.5 (2,553.1), 13,470.2 (911.2), 13,845.5 (703.5), 22,861.6 (1,701.5), 405.6 (74.8) and 21,187.6 (168.3), respectively for PLA, 5%NaF, 2.5%NaF, 2.5%NaF/5%TMP, 5%NaF/5%TMP, 5%TMP and Duraphat.

Time-course release of fluoride and phosphate from the varnishes into the artificial saliva solution is shown in Figures 1A and 1B, respectively. Fluoride release from the

fluoridated varnishes without TMP was significantly higher than the other varnishes, being dose-dependent. A markedly higher fluoride release was seen for Duraphat, 5%NaF, 5%NaF/5%TMP within the first hour, decreasing exponentially afterwards. A more constant pattern of fluoride release into the solutions was seen for 2.5%F/5%TMP. No phosphate was released from the varnishes not supplemented with TMP. Among the supplemented varnishes, significantly higher amounts of phosphate were released from 5%TMP when compared to the other concentrations, which occurred mainly within the first hour.

2.5 Discussion

Different fluoride release profiles were observed, despite all varnishes contained the amount of fluoride specified by the manufacturer. Hence, there seems to be inherent differences in the carrier for the sodium fluoride in the fluoride varnishes, which in turn affects the rate of the ion release into the artificial saliva solutions. This variation in fluoride release by different varnishes has been previously reported.[13]

According to the present study protocol, two factors seemed to play an important role in fluoride release from the products: the varnish composition and the addition of TMP. As for the composition, it can be noticed that fluoride release from Duraphat was 2-fold higher than that observed for the experimental varnish containing 5% NaF, even considering that both were manufactured using the same fluoridated salt (NaF) and same natural resin (colophony). Therefore, it seems reasonable to assume that the differences in fluoride release patterns between the 2 formulations are due to other ingredients and/or ratios used in the manufacturing process.[16]

Regarding the presence of TMP, it can be observed that the total fluoride release from the 2.5% NaF and 5% NaF varnishes supplemented with TMP were around 60% and 20% lower than their counterparts without TMP. A similar pattern was seen for phosphate release from the varnishes containing TMP, in which reductions of 30% and 40% in the ion release were seen for the 2.5% and 5% NaF varnishes, respectively, when compared to the 5% TMP varnish. These data taken together indicate that both fluoride and phosphate release are influenced by the appropriate TMP:fluoride ratio in the product. It is noteworthy, however, that despite the reductions in fluoride and phosphate release from the varnishes containing both NaF and TMP salts, no intense inhibitory effect was seen for any of the combinations,

indicating that the formulations were successful in releasing fluoride and phosphate release into the artificial saliva solutions.[13]

The partial and mutual inhibitory effect on fluoride and phosphate release observed when TMP and F were combined could initially suggest a reduction on CaF_2 deposition, leading ultimately in a reduction on the anticaries effects of the TMP-supplemented varnishes. Although the study by Manarelli *et al.*[12] actually showed a reduction in CaF_2 deposition on enamel, no direct effect was observed given that the reductions in fluoride release from the TMP-supplemented varnishes (60% and 20%, respectively for 2.5% and 5% NaF products) caused similar reductions on CaF_2 deposition (around 57%) regardless of the fluoride concentration in the varnishes. Most importantly, the TMP-supplemented varnishes promoted a significantly higher percentage of enamel surface hardness recovery and higher remineralization of the lesion body when compared with their counterparts without TMP. The present results on fluoride and phosphate release from varnishes taken together with the above-mentioned data of CaF_2 formation and remineralizing effects clearly suggest that the mechanism of action of TMP-supplemented varnishes follow a different pattern of that described for conventional varnishes.[17] Instead, the mechanisms involved seem to be related to the high affinity of TMP to enamel and intraoral calcium species, which take part in the formation of more reactive remineralizing compounds after cariogenic challenges.[12,18]

From a clinical perspective, it is important to know the scientific time frame for resuming tooth brushing after application of fluoride varnish. Clinical recommendations by authors on resuming tooth brushing after fluoride varnish application varies from 12 hours[3] to 24 hours[7], which might be unfeasible due to patient compliance, or even misinterpreted and lead to failure of compliance in tooth brushing for a longer period of time. This ultimately would prove counterproductive to oral health.[19] Therefore, it is important to assess when the rate of fluoride release plateaus after application of fluoride varnishes. The rate of fluoride release from 5%NaF, 2.5%NaF, 5%NaF/5%TMP and Duraphat reached a plateau at 2 hours, while the plateau of phosphate release from 2.5%NaF/5%TMP, 5%NaF/5%TMP and 5%TMP varnishes was seen at 4 hours. These differences in fluoride and phosphate release rates could have clinical implications when considering instructions on resuming oral hygiene practices and/or consuming solid foodstuff after professional fluoride applications.

The limitations of the present study should be considered when interpreting the results from a clinical perspective. This *in vitro* study only measured the rate of fluoride and phosphate release, with no data on fluoride and phosphate uptake by enamel. Moreover, since an *in vitro* protocol was used, the dynamics of human saliva affecting the rate of release were not evaluated. Further, it is not known how much fluoride and phosphate are required for caries prevention, or what the appropriate rate of fluoride and phosphate release ought to be for caries prevention when a varnish is used. However, the data from this study provides useful information about the minimum lag time to be recommended to patients for resuming regular oral hygiene practices, based on the type of fluoride varnish used. In summary, as only a partial inhibitory effect was seen between fluoride and TMP on the release of fluoride and phosphate into artificial saliva, along with previous *in vitro* data on enamel remineralization, the TMP-supplemented fluoride varnishes can be considered as a promising alternative for reversing initial caries lesions.

The results suggest that fluoride varnishes supplemented with sodium trimetaphosphate might be effective in preventing dental caries and erosion, as both components take part in de- and remineralization processes.

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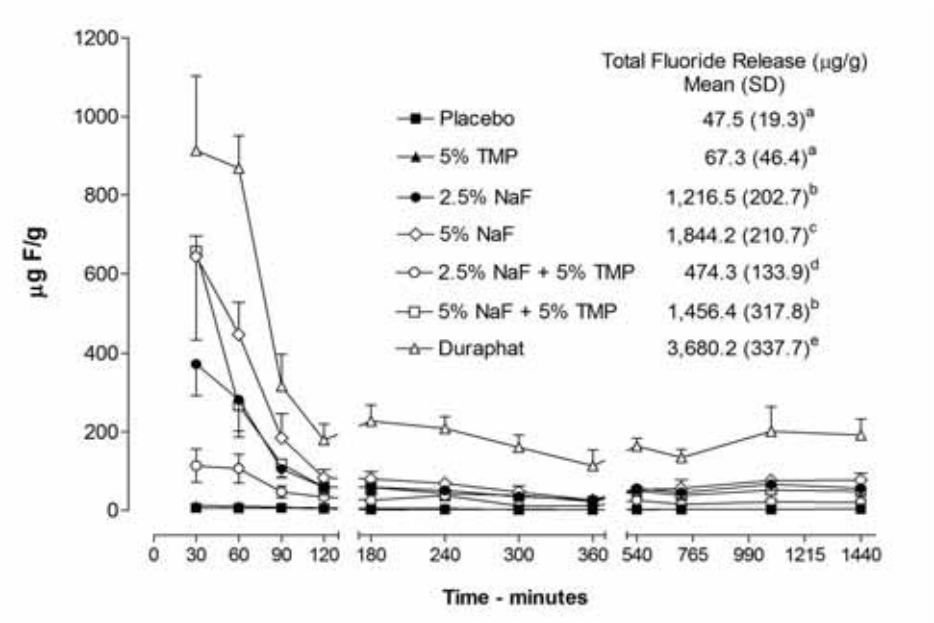
Figure captions:

Figure 1. Time-course fluoride (A) and phosphorus (B) release from varnishes into artificial saliva solutions (24 h). Vertical bars indicate the standard error of means.



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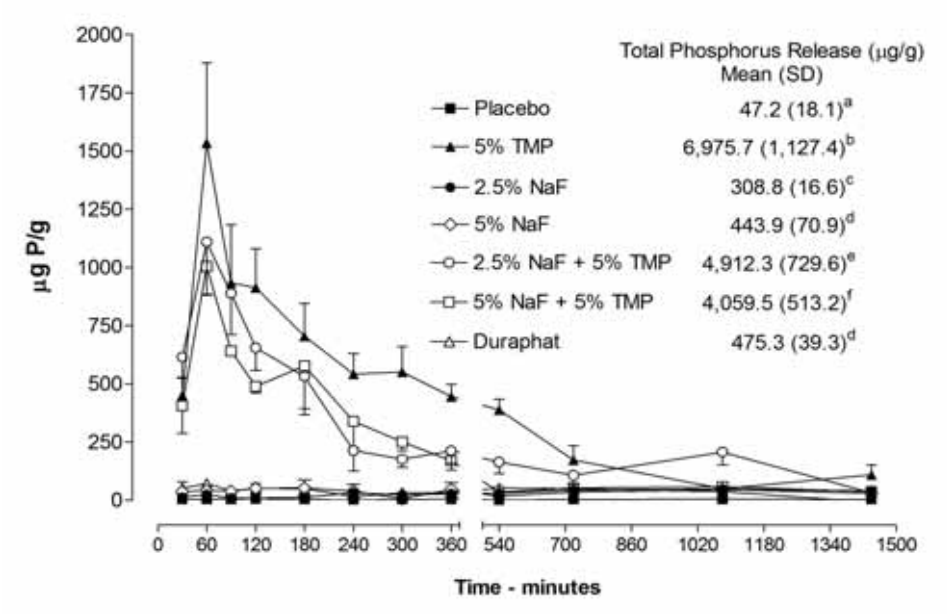

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Figure 1.

Capítulo 2

3 In vitro remineralizing effect of fluoride varnishes containing sodium trimetaphosphate

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Short title: Remineralizing effect of varnishes with TMP and F

Key words: Fluoride varnish. Remineralization. Polyphosphates. pH-cycling.

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Declaration of Interests

There is no conflict of interest that might introduce bias or affect the manuscript's judgment.

*** De acordo com as instruções aos autores do periódico Caries Research (Anexo B)**

3.1 Abstract

This study analyzed the effects of fluoride (F) varnishes supplemented with sodium trimetaphosphate (TMP) on the remineralization of caries-like lesions *in vitro*. Bovine enamel discs were selected through surface hardness (SH) and caries-like lesions were induced. SH was again determined and the blocks were divided into 7 experimental groups (n=24/group): Placebo (no F or TMP), 5%TMP, 2.5%NaF, 2.5%NaF/5%TMP, 5%NaF, 5%NaF/5%TMP and commercial formulation (Duraphat, 5%NaF), following a blind protocol. Discs were treated with the varnishes and kept in a remineralizing solution for 4 h and demineralizing solution for 2 h. Following, varnishes were removed and half discs were used for analysis of loosely (CaF_2) and firmly bound F. The remaining discs were submitted to a pH-cycling regimen during 6 days. The percentage of surface hardness recovery (%SH), cross-sectional hardness (ΔKHN) and enamel CaF_2 and F were determined. Data were analyzed by ANOVA and Student-Newman-Keuls test ($p<0.05$). A dose-response relationship was observed between F concentrations in the varnishes and %SHR. The 5%TMP varnish led to %SRH similar to that obtained for the placebo. When TMP was used in association with F, however, significantly higher %SHR were observed in comparison to their counterparts without TMP. Moreover, ΔKHN obtained for the 5%NaF/5%TMP was significantly lower among all groups tested. Higher concentrations of CaF_2 and F were observed for Duraphat and 5%NaF, followed by 5%NaF/5%TMP, 2.5%NaF, 2.5%NaF/5%TMP ($P<0.05$). It was concluded that the supplementation of fluoride varnishes with TMP leads to enhanced remineralizing effect of artificial caries lesions *in vitro*.

3.2 Introduction

Dental caries has multiple consequences for individuals, societies and health systems and its effects have been discussed by scientific and professional bodies, as well as policy makers [Casamassimo *et al.*, 2009]. White spot lesions (WSL) represent the first clinical observation of demineralization in enamel and can be regarded as a sign of dental caries [Zero, 1999; Fejerskov, 2004]. A WSL is generally characterized by enamel demineralization of the subsurface, with increasing porosity due to the removal of minerals into the outer surface [Kidd and Fejerskov, 2004]. It may be active, with a rough and opaque enamel surface, or inactive, presenting a smooth and shiny enamel surface [Zero, 1999]. Many reports support that the early diagnosis or detection of WSL and the use of non-invasive therapies such as fluoride are important strategies for controlling the development of a carious lesion [Stahl and Zandona, 2007].

Professionally applied topical fluoride treatments have been used for controlling dental caries in patients at high risk of developing cavities [Øgaard *et al.*, 1994]. Among the options available, fluoride varnishes are one of the most widely used. The simplicity of the technique, its good acceptance by patients, the possibility of longer contact with the demineralized enamel, and the lower index of acute intoxication in relation to the other vehicles justifies its indication for young patients [Marinho *et al.*, 2002; Pessan *et al.*, 2011]. Studies indicate that fluoride varnishes can reverse or arrest carious lesions, as well as prevent the demineralization process of an incipient carious lesion, when combined with other preventive measures such as diet control and dental biofilm control [Weintraub *et al.*, 2006; Olympio *et al.*, 2009].

The addition of a phosphate with anticaries action may increase the effectiveness of fluoridated products. The supplementation of fluoridated vehicles with sodium trimetaphosphate (TMP) has been shown to increase the effectiveness of dentifrices, gels and mouth rinses against dental caries [Takeshita *et al.*, 2009; Danelon *et al.*, 2013; Favretto *et al.*, 2013] and dental erosion [Moretto *et al.*, 2010; Manarelli *et al.*, 2011; Manarelli *et al.*, 2013]. Given that fluoridated varnishes are widely used in the dental practice for patients of all ages aiming to reverse WSL, and considering that the association of fluoride and TMP has not been yet tested in varnishes against enamel

demineralization in a caries model, this study evaluated the ability of fluoridated varnishes supplemented with TMP in promoting the remineralization of artificial caries lesions *in vitro*. It was hypothesized that TMP and fluoride would have a synergistic effect, being more effective in promoting enamel remineralization when compared with varnishes with same fluoride concentration without TMP.

3.3 Materials and Methods

Bovine enamel discs were selected through surface hardness (SH) and caries-like lesions were induced. SH was again determined and the discs were divided into 7 experimental groups (n=24/group), following a blind protocol. Discs were treated with the varnishes and immediately kept in solutions for 6 hours (remineralizing solution for 4 h and demineralizing solution for 2 h). Following, varnishes were removed and half of the discs were removed for analysis of the loosely (CaF_2) and firmly bound fluoride (F). The remaining discs were submitted to a pH-cycling regimen for six days [Vieira *et al.*, 2005]. The percentage of surface hardness recovery (%SH), cross-sectional hardness (ΔKHN), polarized light microscopy (PLM), enamel CaF_2 and F were determined after pH-cycling.

Experimental Groups

All test varnishes contained the same basic formulation, differing regarding fluoride and sodium trimetaphosphate concentrations, as follows: placebo (no fluoride or TMP), 2.5% NaF, 5% NaF, 5% TMP, 2.5% NaF plus 5% TMP, 5% NaF plus 5% TMP, and a commercial formulation (Duraphat™, batch 02, expiration date February 2015), hereafter abbreviated as PLA, 2.5%NaF, 5%NaF, 5%TMP, 2.5%NaF/5%TMP, 5%NaF/5%TMP and Duraphat, respectively.

Varnish formulation and determination of fluoride in products

All varnishes were manufactured by SS White Dental Products (Rio de Janeiro, RJ, 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, Germany), with or without the addition of TMP at concentration 5% (Sigma,

China). A varnish without F and TMP was also prepared (PLA). Fluoride concentrations in the varnishes were determined using a fluoride ion specific electrode (9609 BN – Orion, USA), coupled to an ion analyzer (Orion 720 A+, Orion, USA), calibrated with standards containing 2.0 to 32.0 µg F/mL. The varnishes were weighed (0.015 to 0.020 g) from each sample separately in a 500 mL Erlenmeyer, in triplicate. Chloroform (15 mL) was added to dissolve the resin, followed by the addition of 285 mL of distilled water to recover fluoride. The vials were then vigorously stirred for 15 seconds (3 times) to allow water and chloroform to separate. Two samples of 0.5 mL were collected from the water phase and buffered with 0.5 mL of TISAB II [Shen and Autio-Gold , 2002].

Preparation of enamel discs and subsurface enamel demineralization[26]

Enamel discs were obtained from bovine incisors, previously stored in 2% formaldehyde solution pH 7.0 for 30 days at room temperature [Delbem and Cury, 2002]. The enamel surface of the discs was then serially polished and selected on the basis of their SH (320.0 to 380.0 KHN). All surfaces of each specimen, except the enamel surface, were coated with acid-resistant varnish and the subsurface enamel demineralization was produced by immersing each enamel block in 32 mL of a solution with 1.3 mmol/L Ca, and 0.78 mmol/L P in 0.05 mol/L acetate buffer, pH 5.0; 0.03 ppm F, for 16 hours at 37°C [Queiroz *et al.*, 2008; Spiguel *et al.*, 2009].

Treatment with F varnishes and pH-cycling [27]

The discs were subjected to 6 days of a pH-cycling regimen, described in details by Vieira *et al.* [2005]. Initially, the enamel was treated with the varnishes and placed in a remineralizing solution (1.5 mmol L^{-1} calcium, 0.9 mmol L^{-1} phosphate, 150 mmol L^{-1} KCl in 0.1 mol L^{-1} cacodylic buffer, pH 7.0; 0.05 mg F/mL, 1.1 mL/mm^2) for 4 h and a subsequent demineralizing solution (2.0 mmol L^{-1} calcium and phosphate in 75 mmol L^{-1} acetate buffer, pH 4.7; 0.04 µg F/mL, 2.2 mL/mm^2) for 2 h. Following, the varnishes were removed with a blade and acetone [Bruun and Givskov, 1991] and the discs were transferred to new remineralizing solutions for 18 h.

[26] Anexo E
[27] Anexo H

Analysis of enamel hardness and polarized light microscopy[28]

Enamel SH was determined using a microhardness tester (Buehler, Lake Bluff, USA) and a Knoop diamond under a 25 g load for 10 seconds [Vieira *et al.*, 2005]. Five indentations spaced 100 µm apart were made at the center of the enamel surface (SH_1). After pH-cycling, there were five other indentations (SH_2) spaced 100 µm apart and from SH_1 [Vieira *et al.*, 2005]. The percentage of surface hardness recovery (%SH) was calculated using the following formula: $\%SH = [(SH_2 - SH_1)/(SH - SH_1)] \times 100$. For cross-sectional hardness measurements, the enamel discs were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and polished. A sequence of 14 indentations at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel were created in the central region, spaced 100 µm apart. This was achieved using a hardness tester (Micromet 5114) and the Buehler OmniMet software, with a Knoop diamond indenter under a 5 g load for 10 seconds (Buehler, Lake Bluff, USA) [Delbem *et al.*, 2010]. Following this, the integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated using the trapezoidal rule (GraphPad Prism, version 3.02) in each depth (µm) from the lesion up to sound enamel. This value was subtracted from the integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named integrated recovery of subsurface hardness (ΔKHN; KHN × µm) [Spiguel *et al.*, 2009]. To analyze the patterns of remineralization, differential hardness profiles were calculated by subtracting the hardness values of each group at each depth from the hardness values of subsurface enamel demineralization.

Following, a diamond saw was used to prepare sections approximately 600 µm thick from the half of each disc embedded in acrylic resin. These were then ground and polished to a thickness of 100 µm by using a BETA grinder polisher. Enamel sections were prepared on slides in distilled/deionized water and covered with a glass cover, the edges of which were sealed with synthetic resin (Entellan) [Takeshita *et al.*, 2009]. The sections were examined by

[28] Anexo F, G e J

polarized light microscopy (PLM) at ×400 magnification (Zeiss, Oberkochen, Germany). Three areas in the central regions of the slices were analyzed using Axiovision Software Rel. 4.3 to verify the presence and thickness (µm) of the superficial enamel layer (superficial PLM) and the demineralization depth (depth PLM).

Calcium fluoride analysis in enamel [29]

The amount of loosely bound fluoride (CaF_2) on enamel was quantified 6 h after varnish application and after 6 days of pH-cycling in two different sets of specimens. A digital caliper (Mitutoyo CD-15B) was used to measure the surface area of each of the all specimens. Assessment of CaF_2 uptake by enamel was performed following the methodology of Caslavska *et al.* [1975]. 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 hours under constant agitation. The solution was neutralized and buffered with 0.5 mL of TISAB II + 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 F/mL were used. The data were obtained in mV and converted to µg F/cm^2 using Microsoft Excel.

Firmly bound fluoride analysis in enamel[29]

After extraction of CaF_2, enamel biopsy was performed according to the method of Weatherell *et al.* [1985], modified by Alves *et al.* [2007]. Blocks for measuring fluoride were obtained from one half of each of the longitudinally sectioned discs. The discs were fixed to a mandrel and attached to a handpiece (N 270), which was fixed to the top of a modified microscope with a micrometer to measure depth. Self-adhesive polishing discs (13 mm diameter) and 400 grit silicon carbide were fixed to the bottom of polystyrene crystal tubes (J 10). A 100 µm layer was removed from each enamel disc. The discs were stored in tubes, and their surfaces were washed with 0.5 mL of 0.5 mol/L HCl. The tubes were agitated for 1 hour, and 0.5 mL of 0.5 mol/L NaOH was then added [Takeshita *et al.*, 2009]. For F analysis, a specific electrode (Orion 9609) was

[29] Anexo I

connected to an ion analyzer (Orion 720+) and TISAB II. A 1:1 ratio (TISAB: sample) was used.

Statistical analysis

Analyses were performed using the SigmaPlot software (version 12.0) and the level of statistical significance was established at 5%. The statistical power was calculated considering all the differences among groups of each primary outcome (%SH and ∆KHN). The variables %SH, ∆KHN, superficial PLM and depth PLM showed normal (Shapiro-Wilk) and homogeneous (Cochran test) distributions. One-way ANOVA was then performed, followed by the Student-Newman-Keuls test. The CaF_2 and F data, after verifying homogeneity (cubic root transformation), were subjected to a two-way ANOVA followed by Student-Newman-Keuls test.

3.4 Results

Mean (SD) fluoride concentrations (µg F/g) in the varnishes were 302.2 (62.8), 22,187.5 (1,053.1), 12,470.2 (712.2), 12,645.5 (603.5), 22,061.6 (1,101.5), 215.6 (54.8) and 21,107.6 (138.3), respectively for PLA, 5%NaF, 2.5%NaF, 2.5%NaF/5%TMP, 5%NaF/5%TMP, 5%TMP and Duraphat.

A dose-response relationship was observed between fluoride concentration in the varnishes and %SHR. The 5% TMP varnish led to %SRH similar to that obtained for the PLA (Table 1). When TMP was used in association with fluoride, however, significantly higher %SHR were observed in comparison to their counterparts without TMP. The highest %SHR was obtained for the 5%NaF/5%TMP, which was significantly different from all the other groups.

An inverse pattern was observed for ΔKHN, with the highest values found for the PLA, followed by the 5% TMP and by the 2.5% NaF (Table 1 and Figure 1). No significant differences were observed for ΔKHN between the 2.5%NaF/5%TMP and the 5% NaF. Moreover, ΔKHN obtained for the 5%NaF/5%TMP was significantly lower among all groups tested, including the commercial formulation Duraphat (Table 1 and Figure 1).

The polarized light microscopy data showed subsurface lesions in all of the groups, but these were present to a lesser extent in the groups treated with

F varnishes (with or without TMP) (Table 1). The thickest superficial enamel layer (superficial PLM) was found for the 5%NaF/5%TMP, 5% NaF, 2.5%NaF/5%TMP and Duraphat varnishes, without significant differences among them (Table 1).

The highest concentration of CaF_2 formed after varnish application was observed for the 5% NaF group when compared to other varnishes (Table 2). After 6 days of pH-cycling, the CaF_2 concentration was reduced for all groups (Table 2), remaining significantly higher for the Duraphat and 5% NaF groups in comparison with the remaining varnishes. Also, Duraphat and 5% NaF varnishes led to the highest concentration of F present in enamel among all groups, both after varnish application (6 h) and after pH-cycling (6 days). Unlike CaF_2, the F concentration present in enamel increased after pH-cycling in all groups.

3.5 Discussion

Fluoride varnishes were introduced into the market in the 1960s, there being substantial evidence attesting the clinical efficacy of these products in both the permanent and deciduous dentitions [Marinho *et al.*, 2002]. Since dental caries has become a polarized disease and conventional fluoride therapy seems to have little effect for high-risk groups, alternatives have been proposed to increase the effectiveness of fluoridated vehicles [Alves *et al.*, 2009; Buzalaf *et al.*, 2009; Takeshita *et al.*, 2009; Vilhena *et al.*, 2010; Amaral *et al.*, 2013]. All fluoride varnishes tested in the present study were shown to significantly increase remineralization of enamel carious lesions, following a dose-response relationship. Also, confirming our hypothesis, when fluoride varnishes were supplemented with TMP, a marked effect on the percentage of surface hardness recovery (%SH), cross-sectional hardness (ΔKHN) and PLM was seen.

The varnish containing 5% TMP and 5% NaF was 25% more effective in promoting enamel remineralization (%SHR) when compared its counterpart without TMP. Its effects on the lesion body (ΔKHN) were even more pronounced, since the association of 5% TMP with 5% NaF resulted in a lesion area that was reduced by 63% when compared to the 5% NaF and Duraphat varnishes. Moreover, the low-fluoride formulation tested (2.5% NaF)

supplemented with TMP was 30% more effective in reducing the lesion body when compared to its counterpart without TMP, and this effect was not statistically different from that obtained with the conventional formulation (5% NaF). Finally, the results of ΔKHN were confirmed by PLM, which showed that the highest remineralizing effect was obtained after using the 5%NaF/5%TMP varnish.

The synergistic effect of F and TMP in varnishes had already been shown *in vitro* using an erosion model, which demonstrated that fluoride varnishes with TMP promoted significantly lower wear and hardness loss when compared to varnishes without TMP [Manarelli *et al.*, 2013]. This is noteworthy given that the effects of TMP were sustained even after the superficial enamel layers were removed after the erosive and abrasive challenges. Therefore, the enhanced remineralizing effect of varnishes containing both TMP and F in the present study, taken together with the erosive data described by Manarelli *et al.* [2013] indicates that the remineralization capacity of these varnishes is associated with an action in the depth of the enamel lesion produced by TMP. This is an extremely desirable aspect from a clinical perspective, considering that fluoride alone is associated with hypermineralization of outer enamel layers, with a reduced effect in deeper regions [Altenburger *et al.*, 2008; Lagerweij *et al.*, 2006].

The results of CaF_2 formation on enamel after varnish application seem to confirm the above-mentioned theory, since the addition of TMP significantly reduced the deposition of CaF_2 on enamel by 50% and 65%, respectively for varnishes 2.5%NaF/5%TMP and 5%NaF/5%TMP in comparison with varnishes with same fluoride concentrations, without TMP. Similarly, the amount of fluoride incorporated by enamel was also reduced by 35% and 53%, respectively for varnishes 2.5%NaF/5%TMP and 5%NaF/5%TMP in comparison with their counterparts without TMP. Therefore, the enhanced performance of TMP supplemented varnishes cannot be regarded as the result of increased amounts of loosely or firmly bound fluoride.

Although the mechanism of action of TMP associated with F in dental formulations has not yet been established, it is possible that TMP might act in a similar pattern as described for CPP-ACP, according to the mechanism proposed by Cochrane *et al.* [2008]. Given that TMP molecules are not

dissolved in physiological conditions [Takeshita *et al.*, 2009], this salt cannot be regarded as a phosphate source for enamel remineralization. On the contrary, when TMP is in the oral environment, it becomes negatively charged (due to release of Na^+ ions), and can adsorb to enamel by binding to Ca^{++} in the outermost layers. Other negatively charged sites of TMP are then available for Ca^{++} and CaF^+ binding. Following this hypothesis, under acidic conditions TMP would then release CaF^+ species to saliva, which can react with $H_2PO_4^-$, leading to the formation of $CaHPO_4^0$ and HF^0, following an equilibrium equation. The formation of neutrally charged $CaHPO_4^0$ is paramount when considering enamel remineralization, since the diffusion coefficient of this compound into enamel lesion is thousandfold higher when compared to ionic calcium [Cochrane *et al.*, 2008].

Although this theory remains to be proved, it seems to explain why fluoridated varnishes supplemented with TMP are more effective in promoting remineralization of deeper enamel layers when compared with their counterparts without TMP. Moreover, given the reduced amount of CaF_2 and firmly bound fluoride, it can also be hypothesized that TMP acts as a partial barrier not only to CaF_2 deposition on enamel, but also to ionic fluoride penetration into enamel. An important factor that promotes such effects is the ability of TMP to remain bound to enamel for a longer period than other polyphosphates do. It is noteworthy, however, that the effects of TMP alone are negligible, thus corroborating our theory that the main mechanism of action of TMP is not to release phosphate to enamel, but to retain fluoride compounds (i.e. CaF^+) that will be then released during subsequent cariogenic challenges.

In summary, within the limitations of this *in vitro* methodology, it can be concluded that the supplementation of fluoride varnishes with TMP leads to enhanced remineralizing effect of artificial caries lesions when compared to varnishes with same fluoride concentration without TMP. Given that this improved effectiveness is not related to increased fluoride retention on and into enamel, it is possible that the main action of TMP is related to its ability to retain positively charged species, which are released under cariogenic challenges to form more reactive compounds.

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Legends

Table 1. Mean (SD) percentage of surface hardness recovery (%SHR), integrated recovery of subsurface hardness (ΔKHN) and polarized light microscopy (PLM) according to groups. (n=12/group)

Table 2. Mean (SD) enamel calcium fluoride (CaF_2) and firmly bound fluoride (F) 6 h after varnish application and after pH-cycling according groups. (n=24/group)

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

Table 1.

	Analysis			
	Hardness		PLM	
Groups	%SHR	∆KHN	Superficial	Depth
Placebo	12.1[a] (1.7)	14,820.4[a] (5,577.3)	2.4[a] (0.5)	39.9[a] (7.9)
5% TMP	12.4[a] (1.8)	7,157.5[b] (582.2)	3.3[b] (0.5)	49.9[b] (9.2)
2.5% NaF	33.1[b] (2.0)	4,234.4[c] (337.9)	5.7[c] (0.5)	40.9[a] (6.4)
5% NaF	42.5[c] (1.5)	3,248.5[d] (155.9)	7.3[d] (0.3)	38.6[a] (3.8)
2.5%NaF/5%TMP	42.9[c] (2.2)	3,240.8[d] (438.1)	7.0[d] (0.4)	46.3[b] (7.8)
5%NaF/5%TMP	53.7[d] (1.9)	1,997.2[e] (272.1)	10.6[e] (1.0)	48.5[b] (9.6)
Duraphat	42.5[c] (2.3)	3,702.8[d] (299.8)	7.9[d] (0.7)	46.9[b] (10.8)

%SH: Percentage of surface hardness recovery.

∆KHN: integrated recovery of subsurface hardness.

Superficial PLM: thickness of the superficial enamel layer.

Depth PLM: thickness of the demineralization depth.

*Distinct superscript lowercase letters indicate statistical significance in each rows (Student-Newman-Keuls Method, *n* = 12, p< 0.05).

Table 2.

Groups	CaF_2 (µg/cm^2)		FA (µg/mm^3)	
	Formed	Retained	Formed	Retained
Placebo	$^{A}2.8^{a}$ (0.6)	$^{B}0.3^{a}$ (0.1)	$^{A}0.25^{a}$ (0.03)	$^{B}0.37^{a}$ (0.05)
5% TMP	$^{A}1.5^{b}$ (0.3)	$^{B}0.3^{a}$ (0.1)	$^{A}0.26^{a}$ (0.06)	$^{B}0.39^{a}$ (0.08)
2.5% NaF	$^{A}111.2^{c}$ (5.0)	$^{B}1.4^{b}$ (0.2)	$^{A}0.71^{b}$ (0.06)	$^{B}1.43^{b}$ (0.20)
5% NaF	$^{A}252.6^{d}$ (15.0)	$^{B}3.0^{c}$ (0.3)	$^{A}1.13^{c}$ (0.08)	$^{B}3.52^{c}$ (0.38)
2.5%NaF/5%TMP	$^{A}54.9^{e}$ (4.9)	$^{B}0.8^{d}$ (0.1)	$^{A}0.57^{d}$ (0.05)	$^{B}0.93^{d}$ (0.05)
5%NaF/5%TMP	$^{A}87.7^{f}$ (6.6)	$^{B}1.7^{b}$ (0.2)	$^{A}0.80^{b}$ (0.06)	$^{B}1.66^{e}$ (0.27)
Duraphat	$^{A}155.1^{g}$ (11.2)	$^{B}3.1^{c}$ (0.5)	$^{A}1.10^{c}$ (0.10)	$^{B}3.58^{c}$ (0.49)

Different letters show significant difference between groups in each analysis and when comparing the CaF_2 formed and retained within the group (Student-Newman-Keuls, n = 12, $p < 0.05$).

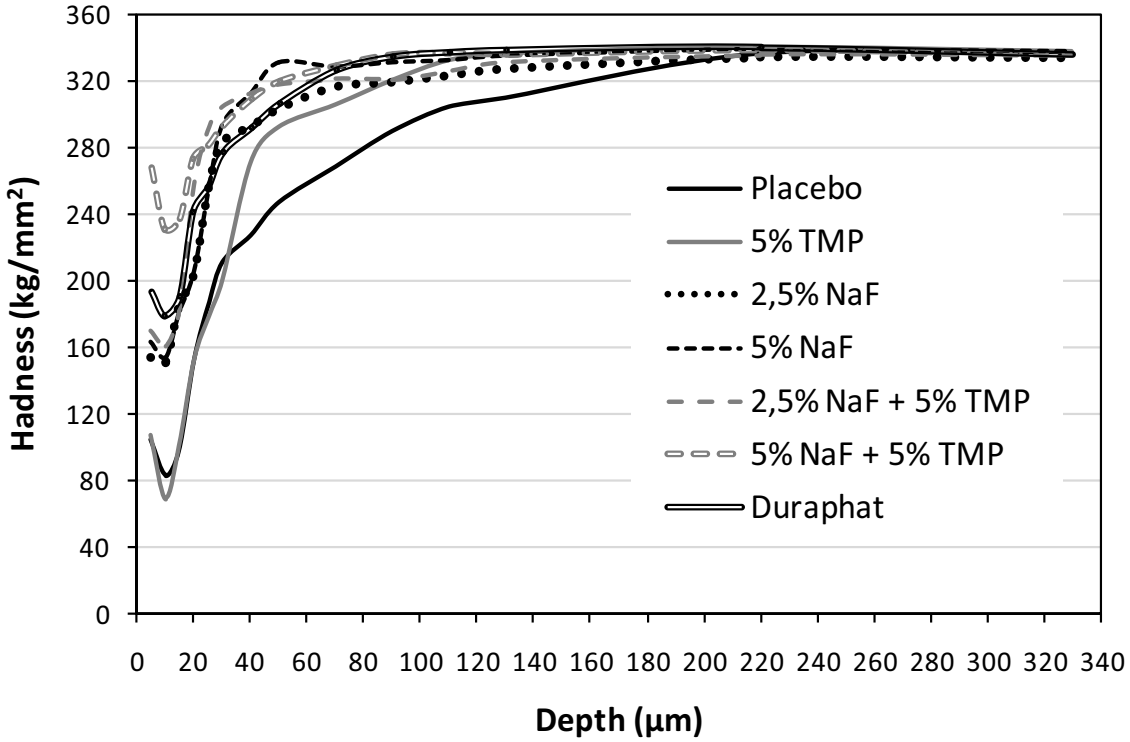
Hadness (kg/mm²)
360
320
280
240
200
160
120
80
40
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340
Depth (µm)
Placebo
5% TMP
2,5% NaF
5% NaF
2,5% NaF + 5% TMP
5% NaF + 5% TMP
Duraphat

Figure 1.

Capítulo 3

4 In vitro effect of fluoride varnishes containing sodium trimetaphosphate on enamel demineralization

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Short title: Effect of varnishes containing F and TMP on enamel caries

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*** De acordo com as instruções aos autores do periódico International Journal of Pediatric Dentistry (Anexo C)**

4.1 In vitro effect of fluoride varnishes containing sodium trimetaphosphate on enamel demineralization

Abstract:

Objective: This study evaluated the effects of fluoride varnishes containing sodium trimetaphosphate (TMP) on bovine enamel demineralization *in vitro*.

Methods: Enamel bovine discs were randomly assigned into six groups (n=20/group): placebo, 2.5% NaF, 5% NaF, 2.5% NaF/5% TMP, 5% NaF/5% TMP, and a commercial formulation (Duraphat). Varnishes were applied on all enamel discs and kept for 6 h. Loosely and firmly bound fluoride formed on/in enamel after treatment were analyzed in 10 discs from each group. The other 10 discs were subjected to a pH-cycling regimen for 7 days, and analyzed for surface (SH) and cross-sectional hardness (ΔKHN), as well as for loosely and firmly bound fluoride in/on enamel. Data were analyzed by ANOVA followed by Student-Newman-Keuls' test ($p<0.05$).

Results: The lowest SH change and ΔKHN were observed for the 5%NaF/5%TMP varnish, which was significantly different from all the other groups. Both fluoridated varnishes containing TMP promoted significantly lower SH change and ΔKHN when compared with their counterparts without TMP. Loosely and firmly bound fluoride was significantly lower in groups treated with varnishes containing TMP and fluoride.

Conclusion: TMP and fluoride added to varnishes have a synergistic effect against enamel demineralization in vitro.

Clinical Significance: The use of TMP-containing fluoride varnishes was shown to be more effective in reducing enamel demineralization when compared with formulations without TMP, suggesting that this formulation could potentially be used in patients at high caries risk and activity, aiming to prevent the development of new caries lesions.

4.2 Introduction

The anticaries properties of fluoride, as well as the mechanisms of action of the fluoridated salts used in vehicles for community, professional and self-application have been well documented for several decades.[1,2] Given that fluoride alone is not able to completely prevent, arrest or reverse the caries process, recent attention has been given to alternatives to increase the effectiveness of topically applied fluoridated products, among which the addition of sodium trimetaphosphate (TMP) has been shown to have a synergistic effect when associated with fluoride in dentifrices, rinses and gels, in studies with different protocols.[3-5]

The evidence for varnishes, however, is scarcer. It was shown that enamel erosive wear is significantly reduced when TMP is added to fluoride varnishes, both *in vitro*[6] and *in situ*[7]. As for dental caries, the only study available showed a synergistic effect between fluoride and TMP on the remineralization of caries-like lesions *in vitro*.[8] Since varnishes are also recommended for preventing the development of new caries lesions[9], it would be interesting to evaluate the effects of fluoride varnishes containing TMP using a demineralization *in vitro* protocol. This would also contribute for a better understanding of the mechanism of action of TMP when used in association with fluoride, which has not yet been fully elucidated.

Thus, this *in vitro* study assessed the effects of fluoride varnishes containing TMP on enamel demineralization. Based on previous studies, the null hypothesis was that no additional protective effect would be attained by the addition of TMP to varnishes with different fluoride concentrations.

4.3 Materials and Methods

Experimental Groups

All test varnishes contained the same basic formulation, differing regarding fluoride and sodium trimetaphosphate concentrations, as follows: placebo (no fluoride or TMP), 2.5% NaF, 5% NaF, 2.5% NaF plus 5% TMP, 5% NaF plus 5% TMP, and a commercial formulation (Duraphat™, batch 02, expiration date February 2015), hereafter abbreviated as PLA, 2.5%NaF, 5%NaF, 2.5%NaF/5%TMP, 5%NaF/5%TMP and Duraphat, respectively.

Varnish formulation and determination of fluoride in products

All varnishes were manufactured by SS White Dental Products (Rio de Janeiro, RJ, 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 of TMP at concentration 5% (Sigma-Aldrich Co., St. Louis, MO, USA). A varnish without F and TMP was also prepared (PLA).

The varnishes were weighed (0.015 to 0.020 g) from each sample separately in a 500 mL Erlenmeyer, in triplicate. Chloroform (15 mL) was added to dissolve the resin, followed by the addition of 285 mL of distilled water to recover fluoride. The vials were then vigorously stirred for 15 seconds (3 times) to allow water and chloroform to separate. Fluoride concentrations in the varnishes were determined using a fluoride ion specific electrode (9609 BN – Orion Research Inc., Beverly, MA, USA), coupled to an ion analyzer (Orion 720 A+, Orion Research Inc., Beverly, MA, USA), calibrated with standards containing 2.0 to 32.0 µg F/mL. Two samples of 0.5 mL were collected from the water phase and buffered with 0.5 mL of TISAB II prior to analysis.[10]

Treatment with F varnishes and pH-cycling[30]

Enamel discs (120; 20 discs/ group; 16 mm^2) were obtained from bovine incisors, previously stored in 2% formaldehyde solution pH 7.0 for 30 days at room temperature.[11] The enamel surface of the discs was then serially polished and selected on the basis of their surface hardness (320.0 to 380.0 KHN). Half the discs were subjected to seven days of a pH-cycling model, based on Vieira et al.[12]. Enamel discs were treated with the varnishes and immersed in a demineralizing solution ("DE": 2.0 mmol L^{-1} calcium and phosphate in 75 mmol L^{-1} acetate buffer, pH 4.7; 0.04 µg F/mL, 2.2 mL/mm^2). After 6 h, the varnishes were removed with a blade and acetone[13] and half the discs were transferred to a remineralizing solution ("RE": 1.5 mmol L^{-1} calcium, 0.9 mmol L^{-1} phosphate, 150 mmol L^{-1} KCl in 0.1 mol L^{-1} cacodylic buffer, pH 7.0; 0.05 mg F/mL, 1.1 mL/mm^2) for 18 h. On the following day, both solutions were changed due to F

[30] Anexo H

release from the varnishes. These solutions were kept until the 5th day. On the 6th and the 7th day, the enamel discs were kept in the remineralizing solution.

Analysis of enamel hardness[31]

Enamel surface hardness (SH) was determined using a microhardness tester (Buehler, Lake Bluff, IL, USA and Mitutoyo Corporation, Kanagawa, Japan) and a Knoop diamond under a 25 g load for 10 seconds.[12] Five indentations spaced 100 µm apart were made at the center of the enamel surface (SH). After pH-cycling, five indentations were made (SH_1) spaced 100 µm apart from each other and from SH.[12] For cross-sectional hardness measurements, enamel discs were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and polished. A sequence of 14 indentations at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel were created in the central region. This was achieved using a hardness tester (Micromet 5114) and the Buehler OmniMet software, with a Knoop diamond indenter under a 5 g load for 10 seconds (Buehler, Lake Bluff, IL, USA and Mitutoyo Corporation, Kanagawa, Japan).[14] The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated using the trapezoidal rule (GraphPad Prism, GraphPad Software Incorporation, La Jolla, CA, USA, version 3.02) in each depth (µm) from the lesion up to sound enamel. This value was subtracted from the integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN × µm).[15]

Calcium fluoride analysis on enamel[32]

The amount of loosely bound fluoride (CaF_2) on enamel was measured 6 h after varnish application and 7 days after pH-cycling, in two different sets of specimens (*n*=10/group). A digital caliper (Mitutoyo CD-15B) was used to measure the surface area of the enamel. Assessment of CaF_2 uptake by enamel was performed according to Caslavska et al.[16]. Briefly, the surfaces of

[31] Anexo F e G
[32] Anexo I

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 hours under constant agitation. The solution was neutralized and buffered with 0.5 mL of TISAB II + 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 F/mL were used. The data were obtained in mV and converted to µg F/cm^2 using Microsoft Excel.

Firmly bound fluoride analysis in enamel[32]

After extraction of CaF_2, enamel biopsy was performed according to the method of Weatherell et al.[17], as modified by Alves et al.[18]. Blocks measuring were obtained from a half of the longitudinally sectioned discs. The discs were fixed to a mandrel and attached to a handpiece, which was fixed to the top of a modified microscope with a micrometer to measure depth. Self-adhesive polishing discs (13 mm diameter) and 400 grit silicon carbide were fixed to the bottom of polystyrene crystal tubes. A 100 µm layer was removed from each enamel disc. The discs were stored in tubes, and their surfaces were washed with 0.5 mL of 0.5 mol/L HCl. The tubes were agitated for 1 hour, and 0.5 mL of 0.5 mol/L NaOH was then added.[3] For F analysis, a specific electrode (Orion 9609) was connected to an ion analyzer (Orion 720+) and TISAB II. A 1:1 ratio (TISAB: sample) was used.

Statistical analysis

Analyses were performed using the SigmaPlot software (version 12.0) (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA), and the level of statistical significance was established at 5%. The statistical power was calculated considering all the differences among groups of each primary outcome (%SH and ΔKHN). The variables %SH, ΔKHN showed normal (Shapiro-Wilk) and homogeneous (Cochran test) distributions. One-way ANOVA was then performed, followed by the Student-Newman-Keuls test. For CaF_2 and F data, after verifying homogeneity (cubic root transformation), were subjected to two-way ANOVA followed by Student-Newman-Keuls test.

4.4 Results

Mean (SD) fluoride concentrations (µg F/g) in the varnishes were 302.2 (62.8), 22,187.5 (1,053.1), 12,470.2 (712.2), 12,645.5 (603.5), 22,061.6 (1,101.5) and 21,107.6 (138.3), respectively for PLA, 5%NaF, 2.5%NaF, 2.5%NaF/5%TMP, 5%NaF/5%TMP and Duraphat.

A dose-response relationship was observed between fluoride concentration in the varnishes and %SH. When TMP was used in association with fluoride, however, significantly lower %SH were observed in comparison to their counterparts without TMP. The lowest %SH was obtained for the 5%NaF/5%TMP, which was significantly different from all the other groups.

A similar pattern was observed for ΔKHN, with the highest values found for the PLA, followed by the 2.5% NaF (Table 1). No significant differences were observed between the 2.5%NaF/5%TMP, 5% NaF and Duraphat. Moreover, ΔKHN obtained for the 5%NaF/5%TMP was significantly lower among all groups tested.

The highest concentration of CaF_2 formed after varnish application was observed for the 5% NaF group when compared to other varnishes (Table 2). After 7 days of pH-cycling, the CaF_2 concentration was reduced for all groups (Table 2). The 5% NaF varnish led to the highest concentration of F present in enamel among all groups, both after varnish application (6 h) and after pH-cycling (7 days). Unlike CaF_2, the F concentration present in enamel increased after pH-cycling in all groups.

4.5 Discussion

The use of fluoridated varnishes is regarded as a safe, effective and well accepted measure in the clinical practice, especially by young children due to the prolonged contact time between fluoride and the tooth surfaces, and the possibility of using very small amounts of the product.[2] The addition of TMP to fluoridated varnishes has been shown to significantly increase the remineralizing effect of caries-like lesions *in vitro*.[8] Confirming the study hypothesis, the present study showed that the association between fluoride and TMP was also able to significantly reduce demineralization of sound enamel using an *in vitro* protocol. This is paramount from a clinical perspective, given that fluoride varnishes are not only used for reversing or arresting existing caries lesions, but also for preventing the development of new lesions.[9]

A similar pattern was observed for surface and cross-sectional hardness, in which the varnishes containing fluoride and TMP significantly reduced enamel demineralization when compared with varnishes containing the same amount of fluoride, without TMP. Such trend had already been described for varnishes in dental caries and erosion using different research protocols[6-8], as well as for other topically applied fluoridated vehicles.[3-5,19,20] Although the mechanism of action of TMP used in association with fluoride has not already been fully described, this synergistic effect has been attributed to the high affinity of TMP to enamel.[21,22]

Unlike traditional fluoride formulations, in which the anticaries effect has been attributed to the formation of high amounts of CaF_2-like material on enamel, as well as to the incorporation of fluoride into deeper layers of enamel (firmly bound fluoride), the addition of TMP to the varnishes significantly reduced the amount of both firmly and loosely bound fluoride when compared with their counterparts without TMP, confirming previous findings on enamel remineralization.[8] These studies together provide new insights into the mechanisms of action of TMP when associated with fluoride.

It has been demonstrated that the effects of TMP used alone in topically applied formulations are minimum or negligible[8,19], so that the mechanism of action of TMP cannot be attributed to the phosphate release to enamel. At an appropriate molar ratio with fluoride, however, TMP can significantly increase the protective effects against dental caries and erosion. Such trend is in line with a mechanism of action recently proposed for TMP used in association with fluoride[8], which seems to be similar to that for CPP-ACP.[23] According to that rationale, TMP adsorbs to Ca^{++} on enamel surface after becoming negatively charged in the oral environment (due to release of Na^{+} ions). Following, intraoral Ca^{++} and CaF^{+} can be retained by other negatively charged sites of TMP, which are released to saliva upon acidification of the oral environment. These would then react with salivary $H_2PO_4^{-}$, leading to the formation of $CaHPO_4^{0}$, whose diffusion coefficient into enamel is much higher when compared to ionic calcium.[22]

This theory seems to explain why the association between fluoride and TMP is able to significantly increase enamel remineralization of preexistent carious lesions[8], as well as to reduce demineralization in sound enamel, as in

the present study. Such mechanism is also in line with the reduced amount of firmly and loosely bound fluoride formed in the presence of TMP; due to the high affinity of TMP, it would rapidly adsorb to enamel surfaces, therefore acting as a partial barrier to CaF_2 deposition on enamel and to ionic fluoride penetration into enamel.

The results of the present study should be interpreted within the limitations of an *in vitro* protocol. Therefore, before these formulations can be recommended for clinical use, future studies should be conducted in conditions that better resemble the clinical situation, in which the interactions with salivary pellicle, proteins, buffers and microorganisms are considered. *In situ* studies would be helpful in that regard, besides providing further evidence for a better understanding of the mechanism of action of TMP-supplemented fluoridated vehicles. The use of nanoparticles could also provide new insights for a better understanding of the mechanisms involved, as well as for the determination of the appropriate TMP:F ratio.

To conclude, the addition of TMP to fluoride varnishes significantly reduces demineralization of sound enamel *in vitro* when compared to varnishes with same fluoride concentration without TMP. Considering the chemical properties of this cyclic polyphosphate and the reduced amount of loosely and firmly fluoride resulting from the use of TMP in fluoride varnishes, a possible mechanism of action is proposed, which seems to be related to the ability of TMP to retain positively charged species, which further take part in the formation of more reactive remineralizing compounds.

Why this study is important to paediatric dentists

This study is important to paediatric dentists given that:

(i) The use of TMP-containing fluoride varnishes was shown to be more effective in reducing enamel demineralization when compared with formulations without TMP, including a formulation commercial available;

(ii) TMP-supplemented varnishes could potentially be used in patients at high caries risk and activity, aiming to prevent the development of new caries lesions.

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Table 1. Mean (SD) surface hardness percentage (%SH) and integrated of subsurface hardness (∆KHN) of enamel after treatment with the varnishes and pH-cycling

Groups	Hardness	
	%SH	∆KHN
Placebo	-73.3[a] (0.5)	5,747.5[a] (351,8)
2.5% NaF	-51.2[b] (2.0)	3,931.5[b] (253.6)
5% NaF	-42.5[c] (0.9)	3,080.3[c] (193.5)
2.5%NaF/5%TMP	-41.7[c] (0.9)	3,003.1[c] (92.3)
5%NaF/5%TMP	-25.0[d] (1.2)	2,040.6[d] (125.1)
Duraphat	-40.4[c] (1.2)	2,992.9[c] (187.1)

%SH: Percentage of surface hardness change. ∆KHN: Integrated of subsurface hardness.

*Distinct superscript lowercase letters indicate statistical significance in each column (Student-Newman-Keuls Method, $p < 0.05$, *n*=10).

Table 2. Mean (SD) concentration of calcium fluoride (CaF_2) and firmly-bound fluoride (F) in the enamel 6 h after varnish application and after pH-cycling

Groups	CaF_2 (µg/cm^2)		F (µg/mm^3)	
	Formed	Retained	Formed	Retained
Placebo	$^{A}2.0^{a}$ (0.3)	$^{B}0.4^{a}$ (0.1)	$^{A}0.20^{a}$ (0.04)	$^{B}0.42^{a}$ (0.04)
2.5% NaF	$^{A}89.4^{c}$ (1.8)	$^{B}1.8^{c}$ (0.3)	$^{A}0.53^{c}$ (0.07)	$^{B}1.16^{b}$ (0.11)
5% NaF	$^{A}166.9^{e}$ (4.0)	$^{B}4.5^{d}$ (0.7)	$^{A}0.77^{d}$ (0.16)	$^{B}2.66^{d}$ (0.18)
2.5%NaF/5%TMP	$^{A}37.9^{b}$ (0.2)	$^{B}1.2^{b}$ (0.3)	$^{A}0.39^{b}$ (0.06)	$^{B}1.09^{b}$ (0.08)
5%NaF/5%TMP	$^{A}95.6^{c}$ (1.9)	$^{B}2.1^{c}$ (0.5)	$^{A}0.70^{d}$ (0.10)	$^{B}1.94^{c}$ (0.13)
Duraphat	$^{A}114.5^{d}$ (1.7)	$^{B}4.7^{d}$ (0.8)	$^{A}0.74^{d}$ (0.11)	$^{B}2.08^{c}$ (0.15)

Bars indicate SD (*n*=10). Different upper case superscript letters indicate differences between CaF_2 formed and retained, as well as between fluoride formed and retained for each group. Different lower case superscript letters indicate significant differences among the groups for each individual column. (Student-Newman-Keuls, $p< 0.05$). CaF_2: Calcium fluoride; F: Firmly-bound fluoride.

Capítulo 4

5 In situ remineralizing effect of fluoride varnishes containing sodium trimetaphosphate

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Short title: Remineralizing effect of varnishes containing F and TMP

Key words: Fluoride varnish. Demineralization. Remineralization.

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*** De acordo com as instruções aos autores do periódico Archives of Oral Biology (Anexo D)**

5.1 Abstract

Objective: This study analyzed the effects of fluoride (F) varnishes supplemented with sodium trimetaphosphate (TMP) on the remineralization of caries-like lesions *in situ*.

Design: Twelve subjects used palatal devices with demineralized enamel discs for 3 days, following a double-blind, crossover protocol. Test groups included Placebo (no F or TMP), 5% NaF and 5% NaF/5% TMP varnishes. The percentage of surface hardness recovery (%SHR) and cross-sectional hardness (ΔKHN) were determined.

Results: The 5% NaF/5% TMP varnish promoted significantly higher %SHR and lower ΔKHN when compared to the other groups.

Conclusion: The remineralizing effect of a 5% NaF varnish is significantly enhanced when associated with TMP.

Key words: Fluoride varnish. Polyphosphates. Remineralization.

5.2 Introduction

The synergistic effects of fluoride (F) and sodium trimetaphosphate (TMP) have been extensively reported over recent years, following different *in vitro* and *in situ* protocols assessing professionally and self-applied fluoride vehicles against dental caries and erosive wear.[1-7] Given the advantages of varnishes regarding their mode of application, safety and patient's acceptability, the association of F and TMP has also been tested in this vehicle. It has been recently shown that a low-F varnish (2.5% NaF) supplemented with TMP promoted significantly lower erosive wear and subsurface mineral loss when compared to a conventional varnish (5% NaF varnish), despite the 2-fold difference in fluoride concentration, following *in vitro* and *in situ* protocols.[5,6]

As for dental caries, the remineralizing effects of low-fluoride (2.5% NaF) and conventional (5% NaF) varnishes supplemented with 5% TMP were assessed *in vitro*.[8] It was demonstrated a significant increase in surface and cross-sectional hardness when TMP was added to the varnishes in comparison with varnishes containing the same amount of NaF, but without TMP. Contradictorily, enamel loosely and firmly bound F was significantly reduced for groups treated with TMP-supplemented varnishes, which led to the conclusion that the mechanism of action of fluoride products containing TMP is related to its high affinity to enamel, what would retain calcium species that are released to saliva under acidic conditions, forming more reactive species thereafter.

Considering the scarcity of studies assessing the effects of TMP on enamel remineralization when added to varnishes, the limitations of *in vitro* protocols, as well as the uncertainty surrounding the mechanisms of action of TMP, aim of the present study was to evaluate the remineralizing effect of a fluoride varnish supplemented with TMP on the remineralization of artificial caries-lesions using an *in situ* protocol in individuals regularly using a fluoridated dentifrice. It was hypothesized that F and TMP would promote a synergistic effect on enamel remineralization, leading to a significantly higher effect in comparison with a fluoride varnish without TMP.

5.3 Materials and Methods

Experimental design

This was a blind and cross-over *in situ* study performed in 3 phases of 3 days each. A 7-day washout period was incorporated after each phase to eliminate possible residual effects from the treatments.[9] On the basis of a previous study and considering an α-error level of 5% and a β-error level of 20%, the required number of volunteers was calculated to be 12.[9] The study was approved by the Institutional Review Board for Human Studies (# 2011-01444), and all participants read and signed informed consent statements prior to the beginning of the study. Volunteers aged 20-30 years, who were in good general and oral health,[10] presented normal salivary flow,[11,12] and did not violate the exclusion criteria (use of any form of medication likely to interfere with salivary secretion, use of fixed or removable orthodontic appliances, pregnancy or breastfeeding, smoker, or systemic illness), were included in the study. Enamel discs were obtained from bovine incisors previously stored in 2% formaldehyde solution pH 7.0 for 30 days at room temperature.[13] The enamel surface of the discs was then serially polished and selected on the basis of their surface hardness (SH, 320.0 to 380.0 KHN). The discs were then demineralized and post-demineralization surface hardness (SH_1) was assessed. Each acrylic palatal appliances had 4 enamel discs. The experimental groups comprised: (a) varnish without F or TMP (placebo), (b) varnish containing 5% NaF (5% NaF) and (c) varnish containing 5% NaF plus 5%TMP (5% NaF/5% TMP). Two enamel discs were removed from the appliances 6h after varnish application for CaF_2 and F analysis. After a 3-day *in situ* period, the remaining 2 discs were removed from the appliances. Surface hardness was assessed (SH_2) for analysis of mineral gain, evaluated in terms of percentage of surface hardness recovery (%SHR). Cross-sectional hardness (ΔKHN) and concentrations of CaF_2 and F in enamel after remineralization were also determined.

Varnish formulation and determination of fluoride in products

All varnishes were manufactured by SS White Dental Products (Rio de Janeiro, RJ, Brazil) and contained the following components: colophony, ethyl cellulose, tolu balsam, beeswax, toluene sulfonamide, vanillin, saccharin and ethanol. Fluoride concentration was 5% of NaF (Merck, Germany), with or without the addition of TMP at concentration 5% (Sigma-Aldrich Co., St. Louis,

MO, USA). A varnish without F and TMP was also prepared (Placebo). Fluoride concentrations in the varnishes were determined as previously reported.[8]

Subsurface enamel demineralization

All surfaces of each specimen, except the enamel surface, were coated with acid-resistant varnish and subsurface enamel demineralization was produced by immersing each enamel disc in 32 mL of a solution with 1.3 mmol/L Ca, and 0.78 mmol/L P in 0.05 mol/L acetate buffer, pH 5.0; 0.03 ppm F, for 16 hours at 37°C.[14,15]

Clinical phase of experimental groups[33]

During the 7-day pre-experimental period, the 3-day experimental period and the 7-day washout period, the subjects brushed their natural teeth and the palatal device with a fluoridated toothpaste (1,100 ppm F as NaF, Sorriso Fresh plus gel, Colgate-Palmolive, São Paulo, Brazil).

The volunteers inserted the appliance in the mouth one hour before study onset, to allow formation of acquired pellicle.[16] Treatment of the bovine enamel discs with the varnishes was performed outside the oral cavity. Palatal devices were immediately inserted in the volunteer's mouth and 6 hours after the varnishes were removed with a blade and acetone;[17] two discs of each device were removed for the determination of CaF_2 on enamel and firmly bound F. After the 3-day experimental period, the discs were removed from the appliance and cleaned using a soft brush, gauze, and 5% NaOCl. Final surface hardness (SH_2) was evaluated and the discs were isolated for determination of CaF_2.[18] Subsequently, the discs were cut in halves for cross-sectional hardness analysis and determination of the firmly bound F.[9,10]

Analysis of enamel hardness [34]

Enamel surface hardness was determined using a microhardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and a Knoop diamond under a 25 g load for 10 seconds.[19] Five indentations spaced 100 µm apart were made at the center of the enamel surface for determining

[33] Anexo K e L
[34] Anexo F e G

initial surface hardness (SH) and also after artificial caries-lesions were induced (SH_1), spaced 100 µm apart from SH. After the experimental periods, five other indentations were made (SH_2) spaced 100 µm apart and from SH_1.[19] The percentage of surface hardness recovery (%SHR) was calculated using the following formula: $\%SHR = [(SH_2 - SH_1)/(SH - SH_1)] \times 100$. For cross-sectional hardness measurements, the enamel discs were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and polished. A sequence of 14 indentations at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel were created in the central region, spaced 100 µm apart, using the above-mentioned microhardness tester, with a Knoop diamond indenter under a 5 g load for 10 seconds (Buehler, Lake Bluff, USA).[9] The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated using the trapezoidal rule (GraphPad Prism, version 3.02) in each depth (µm) from the lesion up to sound enamel. This value was subtracted from the integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN × µm).[15] To analyze the patterns of remineralization, differential hardness profiles were calculated by subtracting the hardness values of the placebo group from those of the F groups (i.e., placebo group values minus the 5% NaF and 5% NaF/5% TMP group values) and by subtracting the hardness values of the 5% NaF group from those of the 5% NaF/5% TMP group at each depth. These differential profiles were then integrated over two depth zones in the lesion (zone A, 5–25 µm; zone B, 25–110 µm) and underlying sound enamel to yield ΔIH values.[2]

Fluoride analysis[35]

Loosely bound fluoride (CaF_2) on enamel was quantified 6 h after varnish application and after the *in situ* phase in two different sets of specimens. A digital caliper (Mitutoyo CD-15B) was used to measure the surface area of the enamel. Assessment of CaF_2 uptake by enamel was performed following the methodology of Caslavska et al.[19] After extraction of CaF_2, enamel biopsy was

[3535] Anexo I

performed according to the method of Weatherell et al.,[20] as modified by Alves et al.[21] For F analysis, a specific electrode (Orion 9609) was connected to an ion analyzer (Orion 720+) and TISAB II. A 1:1 ratio (TISAB: sample) was used.

Statistical analysis

Analyses were performed using the SigmaPlot software (version 12.0) and the level of statistical significance was established at 5%. The statistical power was calculated considering all the differences among groups of each primary outcome (%SHR and ∆KHN). The variables %SHR and ∆KHN showed normal (Shapiro-Wilk) and homogeneous (Cochran test) distributions. One-way ANOVA was then performed, followed by the Student-Newman-Keuls test. CaF_2 and F data, after verifying homogeneity (cubic root transformation), were subjected to two-way ANOVA followed by Student-Newman-Keuls test.

5.4 Results

Mean (SD) fluoride concentrations (µg F/g) in PLA, 5%NaF, and 5% NaF/5% TMP were respectively, 302.2 (62.8), 22,187.5 (1,053.1) and 22,061.6 (1,101.5).

Significantly differences were observed among all groups regarding %SHR and ΔKHN, as shown in Table 1. The highest %SHR was observed for the varnish containing TMP, followed by 5% NaF and PLA. An inverse pattern was seen for ΔKHN, with the lowest values found for the 5% NaF/5% TMP varnish, followed by 5% NaF and PLA, with significant differences among all groups. The differential hardness profiles as a function of depth are shown in Figure 1. While the additional effect of TMP on enamel remineralization (dotted line) at the external part of the subsurface lesion (A) was 9%, a more pronounced effect (around 70%) was seen at deeper regions of the lesion (B).

The highest concentration of CaF_2 formed after varnish application was observed for the 5% NaF group when compared to other varnishes (Table 1). After 3 days, the same trend was observed, although a significant decrease was observed for all groups. Similarly, the highest concentration of firmly bound F was seen for the 5% NaF group, which was significantly higher than the other varnishes. Unlike CaF_2, firmly bound F concentrations significantly increased after 3 days for all groups.

5.5 Discussion

The present study demonstrated that the use of a conventional, colophony-based fluoride varnish (5% NaF) in association with 5% TMP leads to an enhanced effect on enamel remineralization *in situ* when compared to a conventional varnish without TMP, thus confirming the study hypothesis. Although the present results confirm those reported using an *in vitro* protocol,[8] a few points need to be addressed for a better understanding of the present data.

The TMP-supplemented varnish was 40% more effective in increasing %SHR and resulted in a lesion area (ΔKHN) 23% smaller when compared to the conventional formulation. The results of the *in vitro* study, however, showed a more pronounced effect on the lesion body (63% reduction in the lesion area) than in the surface of the lesion (25% reduction in SHR).[8] The different patterns observed may be due to a number of reasons, which include the complex interactions not only between F and TMP, but also among these and tooth enamel itself, the acquired pellicle, salivary ions and buffers, variables which were not included in the *in vitro* study. Also, the lower duration of the *in situ* study in comparison with the *in vitro* protocol (3 vs. 7 days) might have influenced the results, given that longer remineralizing periods lead to a higher degree of enamel remineralization.[22] Finally, volunteers brushed their teeth with a fluoridated dentifrice during the experimental periods, what contributed to the maintenance of high and more constant intraoral fluoride levels, in contrast to the *in vitro* study that only included a single application of the varnishes. All the above taken together highlights the importance of conducting *in situ* studies after initial (screening) *in vitro* assessments are conducted, since they resemble the clinical situation much more closely than *in vitro* conditions.

Despite the above-mentioned differences, the synergistic effects of F and TMP when used in varnishes are unquestionable, which do not only apply to varnishes, but also to mouthrinses.[1-4] Most importantly, while conventional fluoride formulations promote a more pronounced mineralization of the surface layers of caries lesions, the use of a TMP-supplemented fluoride varnish leads to a higher degree of remineralization of the subsurface, especially at deeper regions of the lesion (Figure 1). This could be regarded as an actual “healing” of

the lesion, instead of a standstill of the caries process, which ultimately leaves a "scar" of the disease (inactive enamel caries).[23]

Loosely and firmly bound fluoride were significantly reduced in specimens treated with the TMP-supplemented varnish, also confirming the results of the *in vitro* study conducted with the same varnishes on enamel remineralization and demineralization (data not shown).[8] These results taken together with %SHR and ΔKHN data suggest that TMP acts like a partial barrier to CaF_2 deposition and to enamel fluoride uptake, but this contradictorily does not imply in a reduction in the remineralizing capacity of the varnish. In this sense, the present study adds to the body of evidence that TMP seems to bind both enamel and calcium species, which further react with salivary phosphates leading to the formation of neutrally charged calcium phosphates, in a similar way as that described for CPP-ACP.[24] Future laboratory studies would be useful in determining the saturation of free fluoride, calcium and phosphate ions when TMP is associated with F, so that the present theory could be tested.

In summary, the present study demonstrated that the addition of TMP to a fluoridated varnish leads to enhanced remineralization of artificial caries lesions *in situ* and that its effects are more pronounced in depth when compared to a conventional formulation. Such effect might have important clinical implications, given that the reduction in the subsurface lesion area observed implies that cavities would take longer to develop, or might not develop at all depending on individual factors, resulting in lower net caries increments at individual and population levels.

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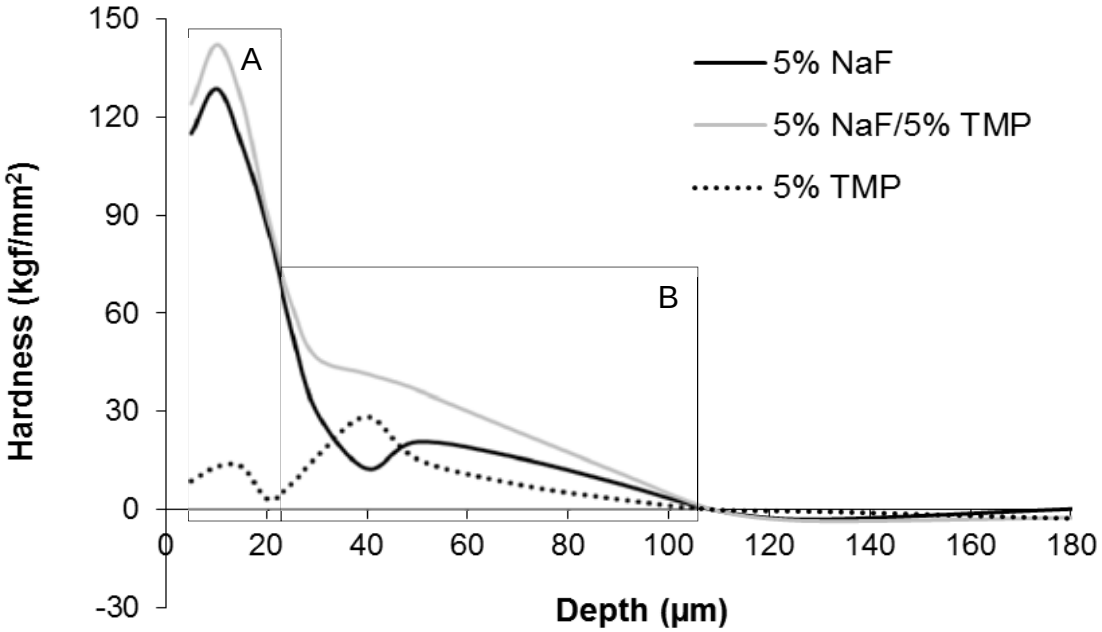

Figure 1. Differential hardness profile as a function of depth according to the varnishes. (A) area calculated between 5 and 25 µm. (B) area calculated between 25 and 110 µm. The values were described in the Table 1. 5% TMP: means differential profile of the 5% NaF minus the 5% NaF/5% TMP.

Table 1. Percentage of surface hardness recovery (%SHR), integrated area of subsurface (ΔKHN), integrated area of differential hardness (ΔIH), enamel calcium fluoride (CaF_2) and firmly bound fluoride, according to the varnishes

Groups	Hardness		ΔIH		CaF_2 (µg/cm^2)		FA (µg/mm^3)	
	%SHR	ΔKHN	zone A	zone B	Formed	Retained	Formed	Retained
Placebo	26.9^{a} (2.0)	6.386.1^{a} (254.9)	–	–	^{A}1.0^{a} (0.3)	^{B}1.5^{a} (0.2)	^{A}0.27^{a} (0.03)	^{B}0.56^{a} (0.04)
5% NaF	50.1^{b} (1.2)	3,671.0^{b} (272.2)	2,052.1^{a,A} (223.3)	1,253.7^{a,B} (515.3)	^{A}49.4^{c} (7.1)	^{B}18.2^{c} (5.5)	^{A}0.93^{c} (0.12)	^{B}2.41^{c} (0.26)
5%NaF/5%TMP	70.0^{c} (2.6)	2,836.2^{c} (158.0)	2,245.0^{a,A} (107.5)	2,134.9^{b,A} (274.0)	^{A}27.9^{b} (3.4)	^{B}9.6^{b} (2.1)	^{A}0.69^{b} (0.04)	^{B}1.23^{b} (0.19)

Mean (SD), *n*=12. Different lower case superscript letters indicate significant differences among the varnishes in each column. Distinct superscript capital letters indicate the differences between zones A and B in each line, as well as between CaF_2 formed and retained, and between fluoride formed and retained for each varnish (Student-Newman-Keuls, $p < 0.05$).

Anexos

ANEXO A

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Metodologia: devem ser fornecidas todas as características do material pertinente ao assunto da pesquisa (ex.: amostras de tecido, pacientes ou população). Os métodos experimentais, analíticos e estatísticos devem ser descritos de forma concisa, porém suficientemente detalhada para permitir que outros possam repetir o trabalho. Os dados de fabricantes ou fornecedores de produtos, equipamentos, ou softwares devem ser explicitados na primeira menção feita no texto, como segue: nome do fabricante, cidade e país. Os programas de computador e métodos estatísticos também devem ser

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Figuras

Fotografias, micrografias e radiografias devem ter uma largura mínima de 10 cm, resolução mínima de 500 dpi, e devem ser fornecidas em formato TIFF. Gráficos, desenhos, esquemas e demais ilustrações vetoriais devem ser fornecidos em formato PDF. Todas as figuras devem ser submetidas, individualmente, em arquivos separados (não inseridas no arquivo de texto). As figuras devem ser numeradas consecutivamente em algarismos arábicos, e todas devem ser citadas no corpo do texto. As legendas das figuras devem ser inseridas todas juntas no final do texto, após as referências.

CARACTERÍSTICAS E FORMATAÇÃO DOS TIPOS DE MANUSCRITOS

Pesquisa Original

Artigos de pesquisa original devem ser limitados a 23.000 caracteres incluindo espaços (considerando-se introdução, metodologia, resultados, discussão, conclusão, agradecimentos, tabelas, referências e legendas de figuras). Será aceito um máximo de 6 (seis) figuras e 30 (trinta) referências. O resumo deve conter, no máximo, 250 palavras.

Formatação - Arquivos de Texto

- Folha de rosto - conforme descrito acima
- Texto principal (23.000 caracteres incluindo espaços)
- Resumo - máximo de 250 palavras
- Descritores - de 3 (três) a 5 (cinco) descritores principais
- Introdução - conforme descrito acima
- Metodologia - conforme descrito acima
- Resultados - conforme descrito acima
- Discussão - conforme descrito acima
- Conclusão - conforme descrito acima
- Agradecimentos - conforme descrito acima
- Tabelas - conforme descrito acima
- Referências - máximo de 30 referências, conforme descrito acima
- Legendas de figuras - conforme descrito acima

Formatação - Arquivos de figuras

- Figuras - máximo de 6 (seis) figuras, conforme descrito acima

"Short Communication"

"Short Communications" devem ser limitados a 10.000 caracteres incluindo espaços (considerando-se, introdução, metodologia, resultados, discussão, conclusão, agradecimentos, tabelas, referências e legendas de figuras). É permitido um máximo de 2 (duas) figuras e 12 (doze) referências. O resumo deve conter, no máximo, 100 palavras.

Formatação - Arquivos de texto

- Folha de rosto: conforme descrito acima
- Texto principal (10.000 caracteres incluindo espaços)
- Resumo - máximo de 100 palavras
- Descritores - de 3 (três) a 5 (cinco) descritores principais
- Introdução - conforme descrito acima
- Metodologia - conforme descrito acima
- Resultados - conforme descrito acima
- Discussão - conforme descrito acima
- Conclusão - conforme descrito acima
- Agradecimentos - conforme descrito acima
- Tabelas - conforme descrito acima
- Referências - máximo de 12 referências, conforme descrito acima
- Legendas de figuras - conforme descrito acima

Formatação - Arquivos de figuras

- Figuras - máximo de 2 (duas) figuras, conforme descrito acima

Revisão Crítica de Literatura

Em geral, a submissão desse tipo de manuscrito será realizada a convite da Comissão de Publicação da BOR. Autores com expertise em assuntos específicos poderão submeter revisões

críticas, mas o aceite do manuscrito para a avaliação no processo de revisão por pares da BOR ficará condicionado à aprovação da Comissão de Publicação. Todos os manuscritos, convidados ou submetidos espontaneamente, serão submetidos à revisão por pares. Esse tipo de manuscrito deve ter um conteúdo descritivo-discursivo, com foco numa apresentação e discussão abrangente de questões científicas importantes e inovadoras, e ser limitado a 23.000 caracteres incluindo espaços (considerando-se, introdução, metodologia, resultados, discussão, conclusão, agradecimentos, tabelas, referências e legendas de figuras). Incluir uma apresentação clara do objeto científico de interesse, argumentação lógica, uma análise crítica metodológica e teórica dos estudos e uma conclusão resumida. É permitido um máximo de 50 referências. O resumo deve conter, no máximo, 250 palavras. É permitido um máximo de 6 (seis) figuras.

Formatação - Arquivos de texto

- Folha de rosto - conforme descrito acima
- Texto principal (23.000 caracteres incluindo espaços)
- Resumo - máximo de 250 palavras
- Descritores - de 3 (três) a 5 (cinco) descritores principais
- Introdução - conforme descrito acima
- Metodologia - conforme descrito acima
- Resultados - conforme descrito acima
- Discussão - conforme descrito acima
- Conclusão - conforme descrito acima
- Agradecimentos - conforme descrito acima
- Tabelas - conforme descrito acima
- Referências - máximo de 50 referências, conforme descrito acima
- Legendas de figuras - conforme descrito acima

Formatação - Arquivos de figuras

- Figuras - máximo de 6 (seis) figuras, conforme descrito acima

Revisão Sistemática e Meta-Análise

Ao resumir os resultados de estudos originais, sejam eles quantitativos ou qualitativos, esse tipo de manuscrito deve responder a uma questão específica, ser limitado a 23.000 caracteres, incluindo espaços, e seguir o estilo e formato Cochrane (www.cochrane.org). O manuscrito deve informar detalhadamente como se deu o processo de busca e recuperação dos trabalhos originais, o critério de seleção dos estudos incluídos na revisão e fornecer um resumo dos resultados obtidos nos estudos revisados (com ou sem uma abordagem de meta-análise). Não há limite para a quantidade de referências. Tabelas e figuras, caso sejam incluídas, devem apresentar as características dos estudos revisados, as intervenções que foram comparadas e respectivos resultados, além dos estudos excluídos da revisão. Demais tabelas e figuras pertinentes à revisão devem ser apresentadas como descrito anteriormente. O resumo deve conter, no máximo, 400 palavras.

Formatação - Arquivos de texto

- Folha de rosto - conforme descrito acima
- Texto principal (23.000 caracteres incluindo espaços)
- Resumo - máximo de 400 palavras
- Formulação da pergunta - deve seguir as diretrizes descritas em www.cochrane.org
- Localização dos estudos - deve seguir as diretrizes descritas em www.cochrane.org
- Avaliação crítica - deve seguir as diretrizes descritas em www.cochrane.org
- Coleta de dados - deve seguir as diretrizes descritas em www.cochrane.org
- Análise e apresentação dos dados - deve seguir as diretrizes descritas em www.cochrane.org
- Aprimoramento - deve seguir as diretrizes descritas em www.cochrane.org
- Atualização da revisão - deve seguir as diretrizes descritas em www.cochrane.org
- Referências - não há limite para a quantidade de referências, conforme descrito acima
- Tabelas - conforme descrito acima

Formatação - Arquivos de figuras

- Figuras - não há limite para a quantidade de figuras, conforme descrito acima

Carta ao Editor

Cartas devem incluir evidências que sustentem a opinião do(s) autor(es) sobre o conteúdo científico
ou editorial da BOR, e ser limitadas a 500 palavras. Figuras ou tabelas não são permitidas.

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À Comissão de Publicação da Brazilian Oral Research (**BOR**)

Os autores [inserir os nomes e sobrenomes completos e sem abreviaturas de todos os autores] (doravante denominados "Autores") submetem o manuscrito original intitulado [inserir o título do manuscrito] à Brazilian Oral Research - **BOR**, representada pela Comissão de Publicação do periódico, e atestam que o manuscrito ora submetido é original e não infringe patente, marca registrada, direito autoral, segredo comercial ou quaisquer outros direitos proprietários de terceiros.

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- Arquivo de folha de rosto (em formato DOC, DOCX ou RTF), contendo os nomes e sobrenomes de todos os autores por extenso, respectivos números de telefone e endereços de Email. O endereço postal completo do autor de correspondência deve ser fornecido.
- Arquivo do texto principal (manuscrito), em formato DOC, DOCX ou RTF.
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- Justificativa para a participação de cada um dos autores, se aplicável (mais de 5 autores), fornecida em um documento separado e em formato PDF.
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- Gráficos, desenhos, esquemas e demais ilustrações vetoriais em formato PDF.
- Todas as figuras devem ser submetidas em arquivos separados e individuais (não inseridas no arquivo de texto).

EXEMPLOS DE REFERÊNCIAS

Periódicos

Goracci C, Tavares AU, Fabianelli A, Monticelli F, Raffaelli O, Cardoso PC, et al. The adhesion between fiber posts and root canal walls: comparison between microtensile and push-out bond strength measurements. Eur J Oral Sci. 2004 Aug;112(4):353-61.

Bhutta ZA, Darmstadt GL, Hasan BS, Haws RA. Community-based interventions for improving perinatal and neonatal health outcomes in developing countries: a review of the evidence. Pediatrics. 2005;115(2 Suppl):519-617. DOI:10.1542/peds.2004-1441.

Usunoff KG, Itzev DE, Rolfs A, Schmitt O, Wree A. Nitric oxide synthase-containing neurons in the amygdaloid nuclear complex of the rat. Anat Embryol (Berl). 2006 Oct 27. Epub ahead of print.

Artigos com Título e Texto em Idioma Diferente do Inglês

Li YJ, He X, Liu LN, Lan YY, Wang AM, Wang YL. [Studies on chemical constituents in herb of Polygonum orientale]. Zhongguo Ahong Yao Za Zhi. 2005 Mar;30(6):444-6. Chinese.

Suplementos ou Edições Especiais

Pucca Junior GA, Lucena EHG, Cawahisa PT. Financing national policy on oral health in Brazil in the context of the Unified Health System. Braz Oral Res. 2010 Aug;24 Spec Iss 1:26-32.

Periódicos Online

Barata RB, Ribeiro MCSA, De Sordi M. Desigualdades sociais e homicídios na cidade de São Paulo, 1998. Rev Bras Epidemiol. 2008;11(1):3-13 [cited 2008 Feb 23]. Available from: http://www.scielosp.org/pdf/rbepid/v11n1/01.pdf.

Livros

Stedman TL. Stedman's medical dictionary: a vocabulary of medicine and its allied sciences, with pronunciations and derivations. 20th ed. Baltimore: Williams & Wilkins; 1961. 259 p.

Livros Online

Foley KM, Gelband H, editors. Improving palliative care for cancer [monograph on the Internet]. Washington: National Academy Press; 2001 [cited 2002 Jul 9]. Available from: http://www.nap.edu/books/0309074029/html/.

Websites

Cancer-Pain.org [homepage on the Internet]. New York: Association of Cancer Online Resources, Inc.; c2000 [cited 2002 Jul 9]. Available from: http://www.cancer-pain.org/.

Instituto Brasileiro de Geografia e Estatística [homepage]. Brasília (DF): Instituto Brasileiro de Geografia e Estatística; 2010 [cited 2010 Nov 27]. Available from: http://www.ibge.gov.br/home/default.php.

World Health Organization [homepage]. Geneva: World Health Organization; 2011 [cited 2011 Jan 17]. Available from:http://www.who.int/en/.

ANEXO B

Caries Research

Guidelines for Authors www.karger.com/cre_guidelines

Aims and Scope

'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

Submission

Manuscripts written in English should be submitted online:

Online Manuscript Submission

Should you experience problems with your submission, please contact:

Prof. David Beighton
(Editor-in-Chef, 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)
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david.beighton@kcl.ac.uk

During the online submission you will be asked to list complete mailing addresses, including e-mail addresses of three potential reviewers for your manuscript.

Copies of any 'in press' papers cited in the manuscript must accompany the submission. Manuscripts reporting on clinical trials must be accompanied by the CONSORT checklist (see

below).

Plagiarism Policy

Whether intentional or not, plagiarism is a serious violation. We define plagiarism as a case in which a paper reproduces another work with at least 25% similarity and without citation.
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Types of Papers

Original papers or Short Communications are reports of original work (including systematic reviews and meta-analyses). Both have the structure outlined below but for Short Communications the abstract should be less than 100 words and the manuscript should not exceed 3 printed pages, equivalent to about 9 manuscript pages (including tables, illustrations and references).

Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope.

Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.

Letters to the Editor, commenting on recent papers in the journal, are published occasionally, together with a response from the authors of the paper concerned.

Preparation of Manuscripts

Text should be one-and-a-half-spaced, with wide margins. All pages and all lines must be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes.
Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Lines in the manuscript must be numbered consecutively from the title page until the last page. Submissions which do not conform to these simple guidelines will be returned to the author.

Title page: The first page of each manuscript should show, in order:

- the title, which should be informative but concise;
- the authors' names and initials, without degrees or professional status, followed by their institutes;
- a short title, maximum length 60 characters and spaces, for use as a running head;
- a list of 3-10 key words;
- the name of the corresponding author and full contact details (postal address, telephone and fax numbers, and e-mail address).

Declaration of Interests:Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or if an author or their employer holds patent(s) on a product used in the study, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares, travel grant). Investigators should disclose potential conflicts to study participants and should state whether they have done so.

The possible existence of a conflict of interest does not preclude consideration of a manuscript for publication, but the Editor might consider it appropriate to publish the disclosed information along with the paper.

Abstract: The abstract should summarise the contents of the paper in a single paragraph of no more than 250 words (to ensure that the abstract is published in full by on-line services such as PubMed). No attempt should be made to give numerical results in detail. References are not allowed in the abstract.

Introduction: This section should provide a concise summary of the background to the relevant field of research, introduce the specific problem addressed by the study and state the hypotheses to be tested.

Materials and Methods (or Subjects and Methods): All relevant attributes of the material (e.g. tissue, patients or population sample) forming the subject of the research should be provided. Experimental, analytical and statistical methods should be described concisely but in enough detail that others can repeat the work. The name and brief address of the manufacturer or supplier of major equipment should be given.
Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures of error or uncertainty (such as confidence intervals) given. Sole reliance on statistical hypothesis testing, such as the use of P values, should be avoided. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical methods used should be specified. See Altman et al.: Statistical guidelines for contributors to medical journals [Br Med J 1983;286:1489–93] for further information.
Manuscripts reporting studies on human subjects should include evidence that the research was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association).In particular, there must be a statement in Materials and Methods that the consent of an appropriate ethical committee was obtained prior to the start of the study, and that subjects were volunteers who had given informed, written consent.
Information detailing the power and sample size calculations must be included in the manuscript.
Randomized clinical trials should be reported according to the standardised protocol of the CONSORT Statement. The CONSORT checklist must be submitted together with papers reporting clinical trials.
Randomized clinical trials must be registered at clinicaltrials.gov or similar national authority and the trial number included in the manuscript.
Trials beginning after 1 July 2012 must be registered before recruitment of the first patient. Caries Research will accept 'retrospective registration' of trials that began before 1 July 2012 (retrospective meaning registration occurs after patient enrolment begins). When submitting a paper on a clinical trial, the trial registration number should be stated at the end of the abstract in the following format: Trial registration: [name of the trial registry, the registry URL and the trial

registration number].
In studies on laboratory animals, the experimental procedures should conform to the principles laid down in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and/or the National Research Council Guide for the Care and Use of Laboratory Animals.
Unless the purpose of a paper is to compare specific systems or products, commercial names of clinical and scientific equipment or techniques should only be cited, as appropriate, in the 'Materials and Methods' or 'Acknowledgements' sections. Elsewhere in the manuscript generic terms should be used.
In any manuscript involving microradiography, the following information must be included: the radiation source and filters used and the kV used (this determines the wavelength of radiation and hence the validity of using Angmar's equation).
Manuscripts on experimental enamel caries should show that the lesions retain a relatively well-preserved surface layer, i.e. are not surfacesoftened lesions. Proof of surface integrity can be provided either as illustrations in the paper or as supplementary material for the reviewers. Transverse microradiography, polarized light microscopy of a section immersed in water or backscattered scanning electron microscopy of a polished cross-section can be used to provide the necessary proof. To allow the nature of experimental changes to be assessed, microradiographs or micrographs should be provided to show part of the experimental lesion and the adjacent control (e.g. figure 2 of Zaura et al.: Caries Res 2007;41:489–492). Again, these images can be provided as part of the paper or as supplementary material for review purposes.

Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns.

Discussion: This section has the functions of describing any limitations of material or methods, of interpreting the data and of drawing inferences about the contribution of the study to the wider field of research. There should be no repetition of preceding sections, e.g. reiteration of results or the aim of the research. The discussion should end with a few sentences summarising the conclusions of the study. However, there should not be a separate 'Conclusions' section.

Acknowledgements: Acknowledge the contribution of colleagues (for technical assistance, statistical advice, critical comment etc.) and provide the position(s) of author(s) employed by commercial firms. This section should describe the source(s) of funding that have supported the work inlcuding relevant grant numbers. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the

manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE).

Legends: The table headings should be listed first, followed by the legends for the illustrations.

Tables: Tables should be numbered in Arabic numerals. Each table should be placed on a separate page. Tables should not be constructed using tabs but by utilising the table facilities of the word-processing software.

Illustrations:

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- Figures with a screen background should not be submitted.
- When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

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Print edition: Up to 6 color illustrations per page can be integrated within the text at CHF 800.00 per page.

References

Reference to other publications should give due acknowledgement to previous work; provide the reader with accurate and up-to-date guidance on the field of research under discussion; and provide evidence to support lines of argument. Authors should select references carefully to fulfil these aims without attempting to be comprehensive.

Cited work should already be published or officially accepted for publication. Material submitted for publication but not yet accepted should be cited as 'unpublished results', while unpublished

observations communicated to the authors by another should be cited as 'personal communication', with credit in both cases being given to the source of the information. Neither unpublished nor personally communicated material should be included in the list of references. Abstracts more than 2 years old and theses should not be cited without a good reason, which should be explained in the covering letter accompanying the paper.

References should be cited by naming the author(s) and year. Where references are cited in parenthesis, both names and date are enclosed in square brackets. Where the author is the subject or object of the sentence, only the year is enclosed in brackets.

One author: [Frostell, 1984] or Frostell [1984].

Two authors: [Dawes and ten Cate, 1990] or Dawes and ten Cate [1990].

More than two authors: [Trahan et al., 1985] or Trahan et al. [1985].

Several references cited in parenthesis should be in date order and separated by semi-colons: [Frostell, 1984; Trahan et al., 1985; Dawes and ten Cate, 1990].

Material published on the World Wide Web should be cited like a reference to a print publication, and the URL included in the reference list (not in the text), together with the year when it was accessed.

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Examples

(a) Papers published in periodicals: Lussi A, Longbottom C, Gygax M, Braig F: Influence of professional cleaning and drying of occlusal surfaces on laser fluorescence in vivo. Caries Res 2005;39:284-286.

(b) Papers published only with DOI numbers: Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol DOI: 10.1159/000063858.

(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: Diggens 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.

Supplementary Material

Supplementary material is restricted to additional information which is directly pertinent to the contentand conclusion of the paper. Please note that all supplementary files will undergo editorial review and should be submitted together with the original manuscript. The editors reserve the right to reject or limit the scope and length of supplementary material. Supplementary material must meet production quality standards for web publication without the need for any modification or editing. In general, supplementary files should not exceed 10 MB in size. Acceptable file formats are word or pdf, excel spreadsheets (only if the data cannot be converted properly to a pdf file), video files (.mov, .avi, .mpeg), and audio files (.wav), either free standing or incorporated into html or ppt files in each case to illustrate the sound. Accepted supplementary material will be published as submitted and no proofs will be provided to the authors.

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ANEXO C

International Journal of Paediatric Dentistry

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Review Articles: may be invited by the Editor.

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3. Al-Mughery AS, Attwood D, Blinkhorn A. Dental health of 5-year-old children in Abu Dhabi, United Arab Emirates. Community Dent Oral Epidemiol 1991; 19: 308-309.
4. Al-Hosani E, Rugg-Gunn A. Combination of low parental educational attainment and high parental income related to high caries experience in preschool children in Abu Dhabi. Community Dent Oral Epidemiol 1998; 26: 31-36.

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ANEXO D

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Guide for authors

Editors-in-Chief:
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This should be a succinct statement of the problem investigated within the context of a brief review of the relevant literature. Literature directly relevant to any inferences or argument presented in the Discussion should in general be reserved for that section. The introduction may conclude with the reason for doing the work but should not state what was done nor the findings.

Materials and Methods

Enough detail must be given here so that another worker can repeat the procedures exactly. Where the materials and methods were exactly as in a previous paper, it is not necessary to repeat all the details but sufficient information must be given for the reader to comprehend what was done without having to consult the earlier work.

Authors are requested to make plain that the conditions of animal and human experimentation are as outlined in the "Ethics" and "Studies on Animals" sections above

Results or Findings

These should be given clearly and concisely. Care should be taken to avoid drawing inferences that belong to the Discussion. Data may be presented in various forms such as histograms or tables but, in view of pressure on space, presentation of the same data in more than one form is unacceptable.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

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As titles frequently stand alone in indexes, bibliographic journals etc., and indexing of papers is, to an increasing extent, becoming computerized from key words in the titles, it is important that titles should be as concise and informative as possible. Thus the animal species to which the observations refer should always be given and it is desirable to indicate the type of method on which the observations are based, e.g. chemical, bacteriological, electron-microscopic, histochemical, etc. A "running title" of not more than 40 letters and spaces must also be supplied. A keyword index must be supplied for each paper.

Structured abstract

The paper should be prefaced by an abstract aimed at giving the entire paper in miniature. Abstracts should be no longer than 250 words and should be structured as per the guidelines published in the Journal of the American Medical Association (JAMA 1995; 273: 27-34). In brief, the abstract should be divided into the following sections: (1) Objective; (2) Design - if clinical, to include setting, selection of patients, details on the intervention, outcome measures, etc.; if laboratory research, to include details on methods; (3) Results; (4) Conclusions.

Keywords

Immediately after the abstract, provide a maximum of 6 keywords, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, 'and', 'of'). Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible. These keywords will be used for indexing purposes.

Abbreviations

As Archives of Oral Biology is a journal with a multidisciplinary readership, abbreviations, except those universally understood such as mm, g, min. u.v., w/v and those listed below, should be avoided if possible. Examples of abbreviations which may be used without definition: ADP, AMP, ATP, DEAE-cellulose, DNA, RNA, EDTA, EMG, tris.
Other abbreviations used to improve legibility should be listed as a footnote on the title page. Chemical symbols may be used for elements, groups and simple compounds, but excessive use should be avoided. Abbreviations other than the above should not be used in titles.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Bacterial nomenclature

Organisms should be referred to by their scientific names according to the binomial system. When first mentioned the name should be spelt in full and in italics. Afterwards the genus should be abbreviated to its initial letter, e.g. 'S. aureus' not 'Staph. aureus'. If abbreviation is likely to cause confusion or render the intended meaning unclear, the names of microbes should be spelt in full. Only those names which were included in the Approved List of Bacterial Names, Int J Syst Bacteriol 1980; 30: 225?420 and those which have been validly published in the Int J Syst Bacteriol since 1 January 1980 have standing in nomenclature. If there is good reason to use a name that does not have standing in nomenclature, the names should be enclosed in quotation marks and an appropriate statement concerning the nomenclatural status of the name should be made in the text (for an example see Int J Syst Bacteriol 1980; 30: 547?556). When the genus alone is used as a noun or adjective, use lower case Roman not italic, e.g.'organisms were staphylococci' and 'streptococcal infection'. If the genus is specifically referred to use italics e.g. 'organisms of the genus Staphylococcus'. For genus in plural, use lower case roman e.g. 'salmonellae'; plurals may be anglicized e.g.'salmonellas'. For trivial names, use lower case Roman e.g. 'meningococcus'

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1. Walsh NP, Montague JC,Callow N and Rowlands AV. Saliva flow rate, total protein concentrationand osmolality as potential markers of whole body hydration statusduring progressive acute dehydration in humans. Arch Oral Biol2004;49(2):149-154.

For book references, the author(s) should be followed by the chapter title (if appropriate), editor(s) (if applicable), book title, place of publication, publisher, year and page numbers. For example:

Nanci A. Ten Cate's Oral Histology: Development, Structure and Function. 6th ed. St. Louis: Mosby; 2003.

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• Use of parametric tests when non-parametric tests are required

• Inconsistencies between summary statistics and statistical tests such as giving means and standard deviations for data which were analysed with non-parametric tests.

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• N values being given in a way which obscures how many independent samples there were (e.g. stating simply n=50 when 10 samples/measurements were obtained from each of 5 animals/human subjects).

• Stating that P=0.000 (a figure which is generated by some computer packages). The correct statement (in this case) is P<0.0005.

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ANEXO E

OBTENÇÃO E PREPARO DOS BLOCOS DE ESMALTE (CAPÍTULOS 2, 3 e 4)

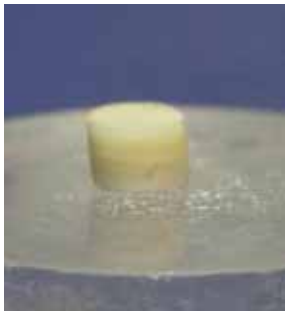
Confecção dos discos de esmalte bovino

1. Separação da coroa do dente bovino utilizando disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob-refrigeração (água destilada/deionizada).

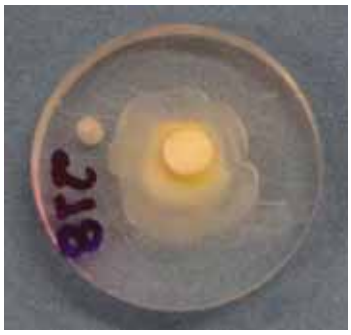
2. Secção da face vestibular no sentido longitudinal, na porção mais plana, utilizando broca diamantada, montada em furadeira de bancada sob-refrigeração com água destilada/deionizada.

Planificação da dentina e polimento do esmalte

3. Ajuste da dentina para obtenção de superfícies paralelas entre esmalte e dentina, utilizando Politriz APL-4 AROTEC e lixas de granulação 320 (CARBIMET Paper Discs, 30-5108-320, BUEHLER), 2 pesos, durante 20 segundos sob baixa rotação e refrigeração.



4. Blocos fixados com a superfície do esmalte voltada para cima, a qual foi polida para análise de dureza.



Seqüência do polimento de esmalte

1. Pedra-pomes, água deionizada e taça de borracha montada em contra-ângulo em baixa-rotação.

2. Na Politriz APL-4 AROTEC - lixa de granulação 600, 800 e 1200 (30 segundos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa; Para o estudo de erosão iniciou-se o polimento com a lixa de granulação 400 e sequencialmente com as outras.

3. Na Politriz APL-4 AROTEC - acabamento final com disco de papel feltro TEXMET 1000 (Buehler Polishing Cloth) (1 minuto – 2 pesos) e suspensão de diamante 1 micron base-água (Buehler);

4. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Solution - Buehler) diluída 20:1 em água destilada/deionizada (2minutos);

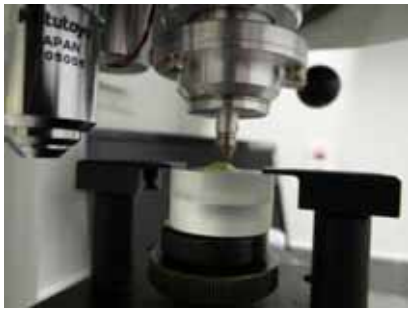
5. Lavagem durante 30 segundos com jato de água destilada/deionizada.

ANEXO F

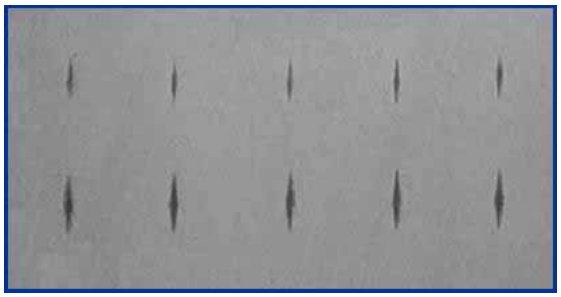
ANÁLISE DE DUREZA SUPERFICIAL (CAPÍTULOS 2, 3 e 4)

1. Microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, USA e Mitutoyo Corporation, Kanagawa, Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff, USA).

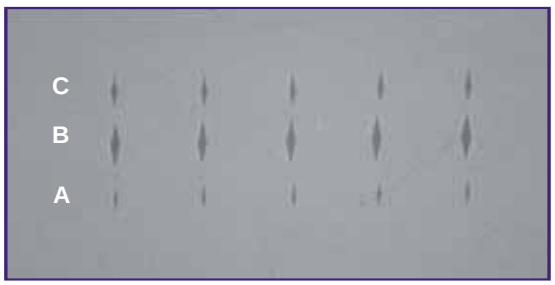
2. Disco de esmalte sendo submetido à determinação de dureza no microdurômetro, carga estática de 25 gramas e tempo de 10 segundos, para análise da dureza de superfície.



3. Fotomicrografia das impressões para análise de dureza de superfície inicial e final (SHi, SHf) (Aumento: 100x) *Capítulo 3.*



Fotomicrografia das impressões para análise de dureza de superfície inicial (SH), pós-desmineralização (SH1) e final (SH2) (Aumento: 100x) *Capítulo 2 e 4.*



ANEXO G

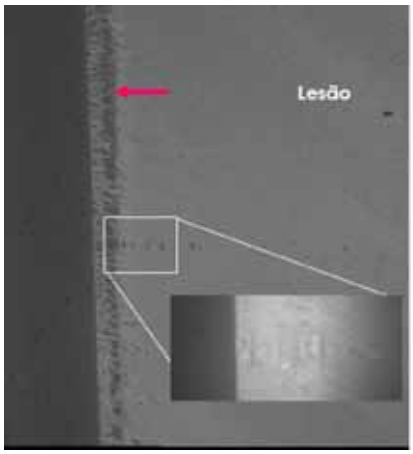
ANÁLISE DA DUREZA EM SECÇÃO LONGITUDINAL (CAPÍTULOS 2, 3 e 4)

1. Embutidora metalográfica (AROTEC PRE 30S) – utilizada para inclusão dos discos de esmalte em 5 gramas de resina acrílica (Buehler Transoptic Powder, Lake Bluff, Illinois, USA), pressão de 150 kgf/cm2, tempo de aquecimento de 7 minutos e mais 7 minutos de resfriamento. Os discos foram fixados em posição com cola adesiva (Super Bonder – Loctite).

2. Discos embutidos – plano longitudinal voltado para a superfície da resina acrílica.

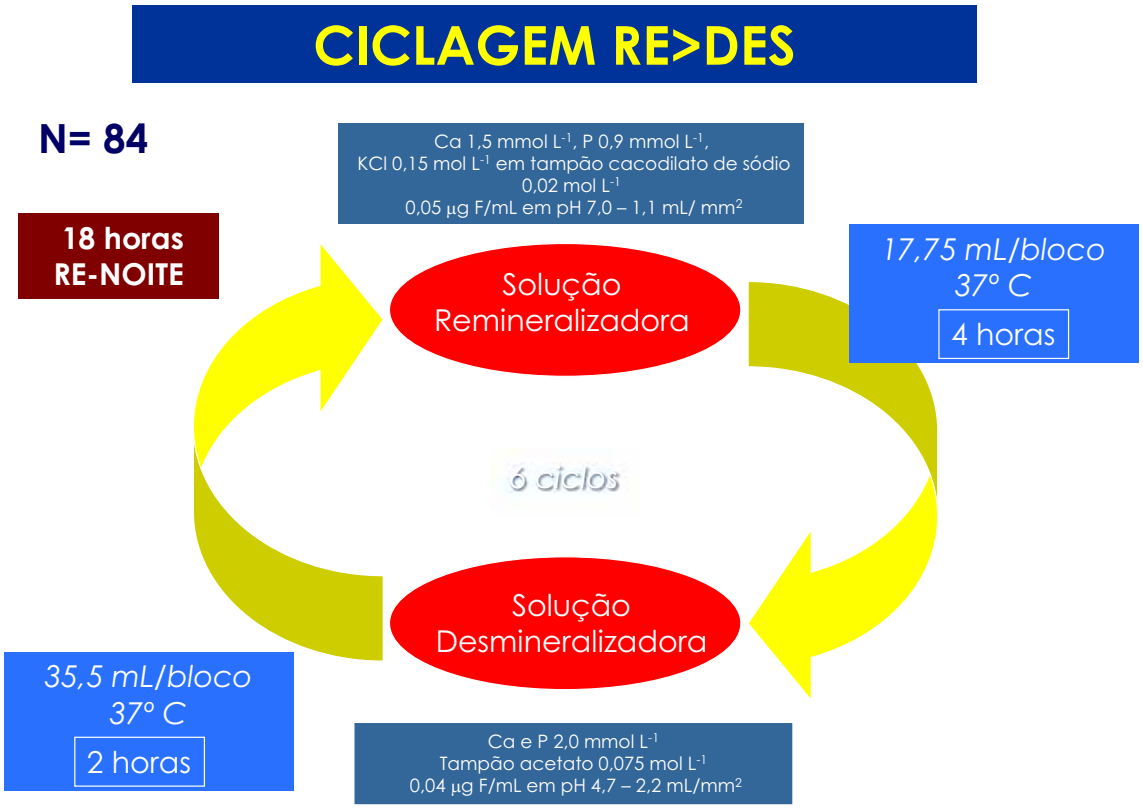
3. Microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, USA e Mitutoyo Corporation, Kanagawa, Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff, USA).

4. Fotomicrografia das impressões. (Aumento: 1000x).

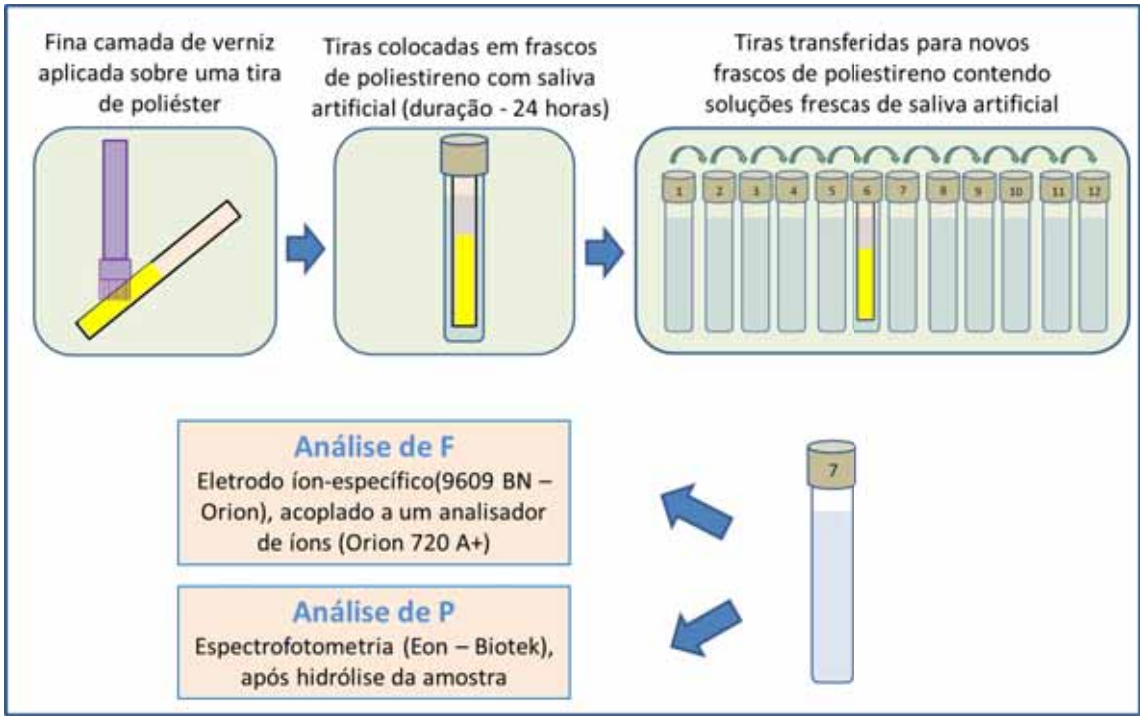


ANEXO H

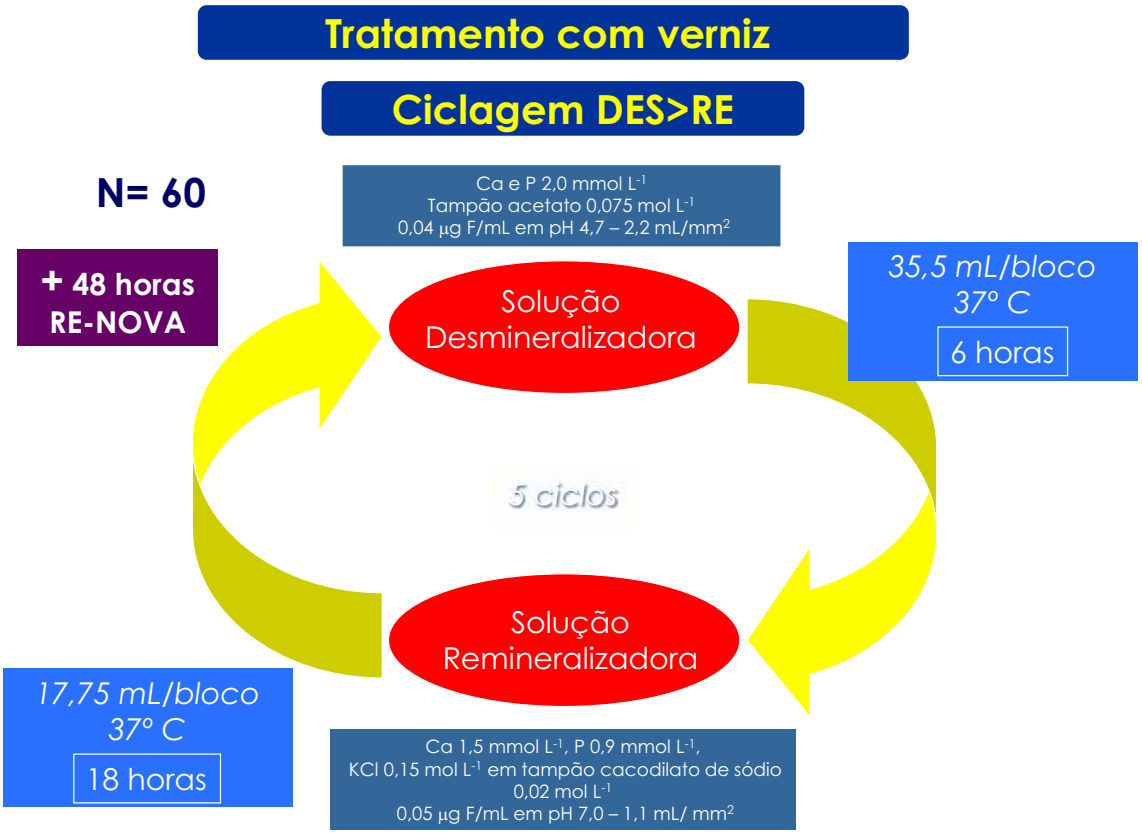
ESQUEMA REPRESENTATIVO DA CICLAGEM DE pH (CAPÍTULO 2)



ESQUEMA REPRESENTATIVO DA CICLAGEM DE pH (CAPÍTULO 1)



ESQUEMA REPRESENTATIVO DA CICLAGEM DE pH (CAPÍTULO 3)

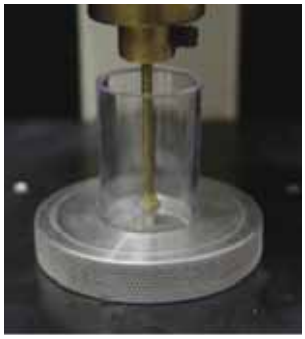


ANEXO I

ANÁLISE DA CONCENTRAÇÃO DE F NO ESMALTE (CAPÍTULOS 2, 3 e 4)

1. Micrômetro eletrônico digital com saída (Starrett, São Paulo – SP) acoplado a uma base de microscópio.

2. Disco de esmalte adaptado ao mandril, sendo submetido à microabrasão, com desgaste de 50 µm, para análise do F, no esmalte.



3. Após desgaste, pó de esmalte presente na lixa adaptada em frascos de poliestireno cristal (J - 10, Injeplast, Brasil).

4. Para análise do conteúdo de F no esmalte utilizou-se:

A- Eletrodo específico Orion 9409-BN (Orion Research, Inc., Beverly, MA, USA.).

B- Microeletrodo de referência (Analyser Comércio e Indústria LTDA, São Paulo, SP.).

C- Analisador de íons Orion 720A (Orion Research, Inc.).

ANEXO J

MICROSCOPIA DE LUZ POLARIZADA (CAPÍTULOS 2)

- Ampliação de 400x (Zeiss, Oberkochen, Alemanha).

- Software AxioVision Rel. 4.3

ANEXO K

LISTA DE INSTRUÇÕES AO VOLUNTÁRIO

1- Todos os materiais utilizados na pesquisa não acarretam em custo ao voluntário.

2- Os voluntários não deverão utilizar qualquer tipo de anti-séptico bucal fluoretado uma semana antes e durante todo o experimento.

3- Uma semana antes do experimento e entre os períodos de washout os voluntários deverão escovar os dentes com dentifrício não fluoretado fornecido pela pesquisadora.

4- A pesquisa será composta por 4 regimes experimentais, com duração de 3 dias cada um e intervalo de uma semana entre eles.

5- Os voluntários deverão utilizar o dispositivo bucal durante todo o dia, inclusive para dormir e **DEVERÁ REMOVÊ-LO SOMENTE PARA AS REFEIÇÕES**, ocasião em que o dispositivo deverá permanecer na caixa própria para armazená-lo.

6- Evite que o dispositivo fique fora da boca por um período prolongado, restringindo-se ao tempo necessário para cada refeição.

7- Realize sua higiene bucal normalmente, utilizando o dentifrício fornecido.

8- Quando qualquer material estiver acabando ou sentir algum desconforto na utilização do dispositivo, entrar em contato com a pesquisadora.

9- Favor verificar todos os dias se os fragmentos estão em suas lojas. Caso não estejam, entrar em contato imediatamente com a pesquisadora.

10- Qualquer dúvida entrar em contato com a pesquisadora (Michele M. Manarelli) pelo telefone : 3636-3235 (Odontopediatria) ou (18) 8116-1623.

ANEXO L

DISPOSITIVO PALATINO

1. Kit fornecido ao voluntário a cada período experimental.