Marcelle Danelon

EFEITO DE DENTIFRÍCIOS FLUORETADOS E SUPLEMENTADOS COM NANOPARTÍCULAS DE TRIMETAFOSFATO DE SÓDIO SOBRE A DESMINERALIZAÇÃO, REMINERALIZAÇÃO E EROSÃO DENTÁRIA

Araçatuba – SP
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Orientador: Profº Drº Alberto Carlos Botazzo Delbem

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"A tarefa essencial do professor é despertar a alegria de trabalhar e de conhecer."

(Albert Eisntein)

Dedicatória

Marcelle Danelon

Dedico este trabalho,

Aos meus pais *ALZIRA e JOSÉ ANTONIO (In Memorian),*

Pela confiança que depositaram em mim... Exemplos de dedicação, honestidade, simplicidade, felicidade e amor. Agradeço por todos os momentos em que estivemos juntos e pelas palavras de conforto que sempre trouxeram segurança e tranquilidade.

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"Ser profundamente amado por alguém nos dá força; Amar profundamente alguém nos dá coragem". Lao-Tseu

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(Erasmo)

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(Vinícius de Moares)

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Escudo e Proteção

Achei o amor que não me deixa só
Achei a alegria que é maior
Achei descanso para o meu coração
Achei alívio e libertação

Escudo e proteção acima da razão
Em Ti eu posso confiar

És liberdade pra recomeçar
És esperança pra continuar
És meu abrigo quando a chuva vem
Eu deixo tudo e corro para Ti

Escudo e proteção acima da razão
Em Ti eu posso confiar
Tua força me faz crer
Além do que eu posso ver
Em Ti eu posso confiar

Eu confio, eu confio em Ti!

(Diante do Trono)

Epígrafe

Resumo Geral

Danelon M. Efeito de dentifrícios fluoretados e suplementados com nanopartículas de trimetafosfato de sódio sobre a desmineralização, remineralização e erosão dentária [tese]. Araçatuba: Universidade Estadual Paulista; 2014.

Resumo Geral

O objetivo deste estudo foi avaliar a capacidade de dentifrícios convencionais (1100 ppm F) suplementados com trimetafosfato de sódio micrométrico ou nanoparticulado (TMP; TMPnano), em reduzir a desmineralização (*in vitro*), promover a remineralização (*in situ*) e reduzir a erosão dentária (*in vitro*). No estudo de desmineralização, blocos de esmalte bovino (*n* = 96) selecionados pela dureza de superfície inicial (SHi) foram divididos em oito grupos de dentifrícios (*n* = 12): sem fluoreto e sem TMP (Placebo); 1100 ppm F (1100 ppm F); 1100 ppm F associado 1% TMP micrométrico e nanoparticulado (1100 1%TMP; 1100 1%TMPnano), 1100 ppm F associado a 3% TMP micrométrico e nanoparticulado (1100 3%TMP; 1100 3%TMPnano), 1110 ppm F associado a 6% TMP micrométrico e nanoparticulado (1100 6%TMP; 1100 6%TMPnano). Os blocos foram submetidos a ciclagem de pH durante cinco dias, sendo o tratamento com os respectivos dentifrícios realizados 2x/dia. A seguir, determinou-se a dureza de superfície final (SHf), perda mineral integrada (IML), diferencial da perda mineral integrada (ΔIML) e fluoreto (F) no esmalte. Os resultados foram submetidos à análise de variância seguida pelo teste Student-Newman-Keuls ($p < 0,001$). O grupo 1100 3%TMPnano apresentou a menor perda mineral (SHf, IML e ΔIML) seguido pelo grupo 1100 3%TMP ($p < 0,001$). O grupo 1100 3%TMPnano apresentou a maior concentração de F no esmalte, seguido pelo 1100 6%TMPnano ($p < 0,001$). Para o estudo de remineralização, blocos de esmalte bovinos (*n* = 192) foram selecionados pela dureza de superfície

pós-desmineralização (SH_1), e divididos em quatro grupos experimentais: Placebo, 1100 ppm F, 1100 3% TMP micrométrico (1100 TMP), 1100 3% TMP nanoparticulado (1100 TMPnano). Doze voluntários utilizaram dispositivos palatinos, com quatro blocos de esmalte desmineralizados, durante três dias, sendo a escovação realizada 3x/dia. Após o período de remineralização determinou-se a porcentagem de recuperação de dureza de superfície ($\%SH_R$), recuperação da perda mineral integrada (IML_R), ΔIML e F no esmalte. Os resultados de $\%SH_R$, ΔIML e F foram submetidos à análise de variância seguida pelo teste Student-Newman-Keuls ($p < 0,001$), já os resultados de IML_R foram submetidos ao teste Kruskal-Wallis seguido pelo teste Student-Newman-Keuls ($p < 0,001$). A adição de TMP aos dentifrícios fluoretados aumentou a capacidade remineralizadora ($\%SH_R$) dos mesmos quando comparado ao grupo 1100 ppm F ($p < 0,001$). Maiores valores de IML_R, ΔIML e F foram encontrados no grupo 1100 TMPnano ($p < 0,001$). No estudo de erosão, blocos de esmalte bovinos ($n = 120$) foram selecionados pela dureza de superfície (SHi) e divididos em cinco grupos experimentais: Placebo, 1100 ppm F (1100 ppm F), 1100 3% TMP (1100 TMP), 1100 3% TMPnano (1100 TMPnano) e 5000 ppm F (5000 ppm F). Os grupos foram divididos sob duas condições de erosão (ERO): erosão na presença de saliva humana (ERO-SH) e erosão na presença de saliva artificial (ERO-SA). O desafio erosivo foi produzido pelo ácido cítrico 4x/dia. O fator estudado foi o tipo de dentifrício (5 tipos) e condição (2 tipos: ERO-SH, ERO-SA). Os dados de SHf, desgaste e dureza em secção longitudinal (ΔKHN) foram analisados como variáveis de resposta, e a seguir submetidos à análise de variância seguido pelo teste Student-Newman-Keuls ($p < 0,001$). Os valores de SHf foram maiores nos grupos tratados com 1100 TMPnano e 5000 ppm de F, diferindo no grupo placebo e 1100 ppm de F ($p < 0,001$). Os grupos 1100

TMPnano e 5000 ppm de F apresentaram maior efeito protetor quando comparado com o grupo 1100 ppm F, tanto para o desgaste como ΔKHN sob as duas condições SH e SA ($p < 0,001$). Conclui-se que é possível melhorar a eficácia de um dentifrício contendo 1100 ppm de F através da adição de TMPnano, mostrando uma eficácia superior ao dentifrício 1100 na cérie e similar ao 5000 ppm de F na erosão, e que a presença da película adquirida não interfere na ação do TMP.

Palavras-chave: Esmalte dentário, Flúor, Fosfato, Nanopartícula Desmineralização, Remineralização, Erosão Dentária, Dentifrício.

General Abstract

Danelon M. Effect of fluoride toothpastes and supplemented with nano-sized sodium trimetaphosphate on enamel demineralization and remineralization and on dental erosion [tese]. Araçatuba: Universidade Estadual Paulista; 2014.

General Abstract

The aim of this study was to evaluate the ability of conventional toothpaste (1100 ppm F) supplemented with micrometric or nano-sized sodium trimetaphosphate (TMP; TMPnano), in reducing demineralization (*in vitro*), promote remineralization (*in situ*) and reduce erosion tooth (*in vitro*). In the study of demineralization of bovine enamel blocks (*n* = 96) selected by the initial surface hardness (SHi) were divided into eight groups of toothpaste (*n* = 12) without fluoride and without TMP (Placebo), 1100 ppm F (1100 ppm F) 1100 ppm F associated 1% TMP micrometric and nano-sized (1100 1%TMP; 1100 1%TMPnano), 1100 ppm F associated with 3% TMP micrometric and nano-sized (1100 3%TMP; 1100 3%TMPnano), 1110 ppm F associated with 6% TMP micrometric and nano-sized TMP (1100 6%TMP; 1100 6%TMPnano). The blocks were subjected to pH cycling for five days, and treatment with their toothpastes made 2x/day. Next, we determined the final surface hardness (SHf), integrated mineral loss (IML), differential integrated mineral loss (ΔIML) and fluoride (F) in the enamel. The results were subjected to analysis of variance followed by Student-Newman-Keuls test ($p < 0.001$). The group 1100 3 %TMPnano had the lowest mineral loss (SHF, IML and ΔIML) followed by 1100 3%TMP group ($p < 0.001$). The group 100 3%TMPnano showed a higher concentration of F in the enamel, followed by 1100 6%TMPnano ($p < 0.001$). For the study of remineralization of bovine enamel blocks (*n* = 192) were selected by the hardness of the surface after demineralization (SH_1), and divided into four groups: Placebo, 1100 ppm F, 1100 3 % TMP micrometric (1100 TMP) and 1100 3% TMP nano-sized (1100 TMPnano) .

Twelve volunteers wore palatal appliances with four blocks of demineralized enamel, for three days, and brushing held 3x/day. After the period of remineralization determined the percentage of recovery of surface hardness (%SH_R), recovery of integrated mineral loss (IMLR), and F ΔIML enamel. The %SH_R, ΔIML and F were subjected to analysis of variance followed by Student-Newman-Keuls test ($p < 0.001$), since test results IMLR were subjected to Kruskal-Wallis test followed by Student-Newman-Keuls ($p < 0.001$). The addition of TMP to fluoridated toothpaste increased remineralizing capacity (%SH_R) when compared to the same 1100 ppm F group ($p < 0.001$). Higher values of IML_R, ΔIML and F were found in 1100 TMPnano group ($p < 0.001$). In the study of erosion of bovine enamel blocks (*n* = 120) were selected for surface hardness (SHi) and divided into five groups: Placebo, 1100 ppm F (1100 ppm F), 1100 3% TMP micrometric (1100 TMP), 1100 3% TMP nano-sized (1100 TMPnano) and 5000 ppm F (5000 ppm F). The groups were divided under two conditions of erosion (ERO): erosion in the presence of human saliva (ERO-HS) and erosion in the presence of artificial saliva (ERO-AS). The erosive challenge was produced by citric acid 4x/day. The factor studied was the kind of toothpaste (5 types) and condition (2 types: ERO-HS; ERO AS). The data SHF wear and toughness in longitudinal section (ΔKHN) were analyzed as response variables, and then subjected to analysis of variance followed by Student-Newman-Keuls test ($p < 0.001$). SHF values were greater in the groups treated with 1100 TMPnano and 5000 ppm F, differing in the placebo and 1100 ppm F ($p < 0.001$). 1100 TMPnano and 5000 ppm F groups had higher protective effect when compared to the 1100 ppm F group, both as to wear ΔKHN under both conditions AS and HS ($p<0.001$). It follows that it is possible to improve the effectiveness of a toothpaste containing 1100 ppm F by adding TMPnano showing a superior efficacy in toothpaste caries and 1100

similar to the 5000 ppm F erosion, and the presence of the acquired pellicle not interferes with the action of TMP.

Keywords: Dental enamel, Fluoride, Phosphate, Nano-sized, Demineralization, Remineralization, Tooth Erosion, Toothpaste.

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
IML	Perda mineral integrada
IML_R	Recuperação da perda mineral integrada
KCl	Cloreto de potássio
kgf/mm^2	Quilograma-força por milímetro quadrado
KHN	Unidade de dureza Knoop
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 de Hidrogênio
s	Segundo
SH	Dureza de superfície inicial

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
FI	Fluoreto iônico
FT	Fluoreto total
$\%SH_R$	Porcentagem de recuperação de dureza de superfície
TISAB	Tampão ajustador de força iônica total
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	nanomicrométrico
PO_4^-	Íon fosfato
SD	Desvio padrão
vol% min	Porcentagem de volume mineral
µg	Micrograma
$µg/mm^3$	Micrograma por milímetro cúbico
µg F/mL	Micrograma de fluoreto por mililitro
µm	Micrômetro
∆KHN	Perda integrada de dureza de subsuperfície
ΔIML	Diferencial de perda mineral integrada

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Sumário

SUMÁRIO

Introdução Geral

1 Introdução Geral

A utilização do fluoreto (F) como forma de controle da cárie dentária teve início na década de 50, o qual passou a ser adicionado à água de abastecimento público [Narvai, 2000]. A partir disso, várias formas foram empregadas e oferecidas à população de maneiras diversas e em diferentes concentrações: prescrição de suplementos, aplicação tópica de géis e soluções, vernizes fluoretados e ainda os dentifrícios fluoretados, veículo este, mais utilizado pela população [Van Rijkom et al., 1998; Marinho et al., 2003a; Marinho et al., 2003b; Marinho et al.,2004]. Devido à introdução no mercado de vários métodos alternativos, o F alcançou comunidades onde não havia água de abastecimento fluoretada, o que é conhecido como —efeito halo" [Lima e Cury, 2001].

O principal efeito preventivo de produtos de alta concentração de F, relaciona-se à maior formação de reservatórios de F na superfície do dente na forma de fluoreto de cálcio (CaF_2), o qual denomina-se de flúor fracamente ligado [Saxegaard e Rölla, 1988]. Este fica adsorvido sobre a superfície do dente, agindo como um reservatório de F disponível para atuar nos momentos de queda de pH, intervindo diretamente na dinâmica do processo des-remineralização e, dessa forma, interferindo com a progressão da lesão de cárie [Featherstone, 2000; Jardim et al., 2008].

Embora se tenha observado na maioria dos países em desenvolvimento e desenvolvidos um declínio da cárie dentária, ainda, no interior desses países existem diferenças importantes em termos da prevalência da cárie entre regiões, cidades e diferentes grupos populacionais [Antunes et al., 2004]. Apesar da existência de inúmeras fontes de F disponíveis para a população, observa-se atualmente uma tendência de polarização da doença, englobando indivíduos com

alto risco de cárie, incluindo crianças [Ministério da Saúde, 2004; Dye et al., 2007], adolescentes e pessoas que não possuem acesso à água de abastecimento fluoretada e aos serviços odontológicos [Dtmyer et al., 2011]. Narvia et al. [2000], relatam em um estudo que 25% dos indivíduos concentram aproximadamente 75% dos dentes com prevalência de cárie dentária. Outros estudos correlacionam a polarização da doença principalmente com a privação econômica [Baldani et al., 2004]. Por isso, muitos autores consideram os fatores socioeconômicos como indicadores de risco para o desenvolvimento da cárie dentária [Gillcrist et al., 2001; Sogi e Bhaskar, 2002; Baldani et al., 2004].

Outro problema, embora não seja caracterizado como uma questão de ordem pública vem acometendo principalmente crianças e adolescentes nos últimos anos, é o aparecimento da erosão dentária, que é uma alteração com uma forte correlação com o estilo e qualidade de vida [Grippo et al., 2004]. Devido ao aumento no consumo de alimentos e bebidas ácidas [Lussi et al., 2004; Gambon et al., 2012], ocorre a dissolução química dos tecidos dentais mineralizados, causando perda progressiva e irreversível da estrutura mineralizada do dente, tornando-o mais susceptível à ação de distúrbios mecânicos [Jaeggi e Lussi, 1999; Attin et al, 2000; Attin et al., 2001]. Estudos relatam um aumento na prevalência, variando de 30% a 68%, especialmente entre crianças e adolescentes [Van Rijkom H, 2002; Kazoullis et al., 2007; El Aidi H et al., 2008]. No Brasil, estudos sobre a prevalência de erosão dentária em escolares com idade entre 6-12 anos relatam 19,9% de prevalência [Mangueira et al., 2009]. Já no estudo de Rios et al. [2007], o desgaste foi maior em escolares com 6 anos de idade. Moimaz et al. [2013], avaliaram o índice de erosão dentária em 1.993 pré-escolares brasileiros, com idade entre 4-6 anos. A

idade, dentes e superfícies foram utilizados como fatores de avaliação, sendo a maior prevalência observada em crianças com idade de 6 anos (58,3%), e as superfícies oclusais as mais envolvidas.

Dentre as medidas eficazes para o controle e prevenção deste tipo de alteração dentária, além da mudança dos hábitos alimentares [Millward et al., 1994; Truin et al., 2005; Sales-Peres et al., 2008], encontram-se também os agentes terapêuticos, como o F [Zero et al., 2006; Ganss et al., 2007]. A capacidade do F em inibir a desmineralização e melhorar a remineralização foi relatado por Ganss et al. [2004]. Os dentifrícios são os principais veículos de liberação de F devido à sua disponibilidade e utilização generalizada pela população [Kanapka, 1990]. Assim, sabendo-se que os mesmos se destacam dentre as formas de administração tópicas mais utilizadas pela população, e que contribuem para a redução da prevalência/incidência da cárie [Stookey et al., 2004; Newby et al., 2013; Wright et al., 2014] e da erosão dentária [Faller et al., 2011; Ganss et al., 2013], seria importante aumentar a eficácia dos mesmos contra os problemas descritos acima.

Para minimizar os efeitos da cárie e erosão dentária, além do F, estudos demonstram que a suplementação de dentifrícios com sais de polifosfatos possuem a capacidade de diminuir a desmineralização dentária [Takeshita et al., 2009; Moretto et al., 2010; Delbem et al., 2012].

Dentre os sais de polifosfatos com atividade anticariogênica, o trimetafosfato de sódio (TMP) micrométrico tem-se destacado na literatura [Harris et al., 1967; Gonzalez, 1971; Larson et al., 1972; Gonzalez et al., 1973; Ständtler et al., 1996; Takeshita et al., 2009; Danelon et al., 2013a; Danelon et al., 2013b, Moretto et al., 2010; Manarelli et al., 2011, Favretto et al., 2013; Pancote et al.,

2014]. De acordo com Gonzalez et al. [1973] o TMP e o F não competem para os mesmos sítios de ligação na superfície do esmalte. Entretanto, estudo recente mostra que o TMP micrométrico pode competir com o F nos sítios de ligação na hidroxiapatita se não forem associados em uma proporção molar adequada (TMP:F) [Souza et al., 2013]. Como a adsorção dos polifosfatos ocorre rapidamente [Anbar et al., 1979] e compete com a adsorção do F, o TMP e F devem ser combinados em uma proporção adequada para que não haja competição. A adição do TMP micrométrico em dentifrícios com reduzida concentração de F, exaguatórios, géis e vernizes fluoretados, mostram um efeito contra a desmineralização, remineralização do esmalte e erosão dentária [Takeshita et al., 2009; Moretto et al., 2010; Manarelli et al., 2011; Danelon et al., 2013a; Danelon et al., 2013b; Manarelli et al., 2013; Pancote et al., 2014], cujo mecanismo da ação está relacionado com uma ação local, como descrito acima, sendo adsorvido à superfície do esmalte, causando uma redução na solubilidade da hidroxiapatita e reduzindo as trocas minerais entre o meio e o esmalte [McGaughey e Stowell, 1977, Van Dijk et al., 1980; Takeshita et al., 2011]. Takeshita et al. [2009] demostraram *in vitro*, que a suplementação com 1% de TMP micrométrico em dentifrício de reduzida concentração de F (500 ppm F) proporcionou um efeito semelhante à um dentifrício convencional (1100 ppm F) no processo de desmineralização do esmalte. Já a associação de 3% de TMP micrométrico em soluções dentifrícias com 1500 ppm F, mostrou semelhante remineralização quando comparado à solução dentifrícia contendo 3000 ppm F suplementados ou não com 3% de TMP. Já no estudo de Moretto et al. [2010] a associação de 3% de TMP micrométrico em um dentifrício de 500 ppm F, demonstrou que o mesmo possuiu um efeito protetor semelhante ao de 5000

ppm F e maior efeito contra a erosão e erosão/abrasão quando comparado ao 1100 ppm F.

Recentemente, um estudo *in vitro* mostrou que a adição de 3% de TMP micrométrico em um dentifrício com 1100 ppm F melhorou seu efeito anticárie [dados não publicados]. Entretanto, estudos clínicos mostraram que esta associação (F/TMP) não apresentou benefício contra a cárie dentária [Stephen et al., 1994; O'Mullane et al., 2007], mas segundo os próprios autores este resultado por ser devido ao erro de calibração. Apesar dos estudos *in vitro* [Buzalaf et al., 2010] e *in situ* [Roberts, 1995] serem utilizados para identificar agentes anticárie, os modelos pré-clínicos não são necessariamente preditivos da atuação clínica destes agentes [Roberts, 1995]. Assim, para melhorar a efetividade do dentifrício fluoretado (1100 ppm F) associado ao TMP e aumentar a probabilidade de se obter bons resultados clínicos, seria de grande valor na odontologia avaliar se a redução das partículas do TMP a uma escala nanométrica pode aumentar o efeito do dentifrício fluoretado quando comparado ao TMP micrométrico.

Hoje em dia têm-se observado um grande crescimento nos estudos sobre nanotecnologia, através da criação de nanomateriais, os quais possuem uma escala métrica de 1-100 nm. As propriedades especiais das nanopartículas derivam de sua elevada proporção entre área superficial e seu volume. Elas também têm uma porcentagem consideravelmente mais alta de átomos em sua superfície quando comparadas com partículas maiores, o que pode torná-las mais reativas. Atualmente, a nanotecnologia passa por um rápido crescimento, com grande potencial de aplicações em odontologia, como por exemplo, em dentifrícios fluoretados [Karlinsey e Zero, 2006]. A nanotecnologia

torna-se responsável pelo desenvolvimento de estratégias bio-inspiradas na remineralização do esmalte e da erosão dentária, respectivamente, dentre outras funções, como na medicina [Hannig e Hannih, 2012]. Dessa forma podemos melhorar a ação dos dentifrícios suplementando-os com nanopartículas de sais de fosfatos.

Assim, com o objetivo de otimizar o efeito dos mesmos sobre o processo de des/remineralização dentária, estudos têm analisado o impacto de fosfatos nanoparticulados no processo de remineralização do esmalte [Karlinsey e Zero, 2006]. A adição de nanopartículas de fosfato tri-cálcio em dentifrícios fluoretados reduziram o processo de desmineralização em esmalte em comparação a um dentifrício convencional (1100 ppm F) [Karlinsey et al., 2007]. Nanocompósitos contendo fosfato de cálcio amorfo (ACP), CaF_2 e clorexidina têm mostrado ação na atividade metabólica do biofilme e, consequentemente, uma redução da produção de ácidos [Cheng et al., 2012]. Ainda, Segundo Xu et al. [2010], compósitos contendo nanopartículas possuem a vantagem em impedir a desmineralização dentária, por apresentarem melhores propriedades físicas e mecânicas quando comparadas a compósitos tradicionais.

Para a proteção do esmalte contra a cárie e erosão dentária, um fator muito importante que deve ser mencionado é a ação da saliva humana, uma vez que a mesma apresenta a capacidade de proteger o esmalte contra os desafios erosivos, através da presença de tampões e da formação da película adquirida sobre o esmalte [Lendenmann et al., 2000; Sreebny, 2000]. Esta, por sua vez, funciona como uma barreira, que impede a difusão de ácidos a partir do biofilme bacteriano para a superfície do esmalte, fornecendo proteção contra a desmineralização [Buzalaf et al., 2010]. É importante ressaltar que a maioria dos

estudos *in vitro* sobre erosão dentária é realizada com a presença de saliva artificial, entretanto, a mesma não possui proteínas salivares, não permitindo assim, a adequada formação da película adquirida do esmalte, a qual tem um grande impacto sobre o processo erosivo [Buzalaf et al., 2010].

Considerando-se uma situação clínica, na qual o dentifrício é aplicado sobre a estrutura dentária que contém a película formada, o efeito do mesmo poderá ser influenciado, pois esta agirá como uma barreira impedindo a interação do F com a superfície dentária, restringindo o acesso do mesmo para a hidroxiapatita e/ou funcionará como um reservatório de F [White et al., 2012].

Sabendo-se de todas as propriedades do TMP micrométrico, bem como a ação de nanopartículas de fosfatos, seria interessante a realização de estudos que avaliem novas formulações dentifrícias contendo 1100 ppm F, o qual é classificado como um dentifrício padrão de mercado, suplementadas com TMP micrométrico ou nanoparticulado, sobre a redução da desmineralização e erosão, assim como na remineralização em lesões de cárie, principalmente quando se trata de populações com alto índice de cárie.

*** As referências estão no anexo Q.**

Capítulo 1

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

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*** De acordo com as instruções aos autores do periódico Acta Biomaterialia (Anexo A)**

2.1 ABSTRACT

The aim of this study was to evaluate conventional toothpastes containing 1100 ppm F associated or not with different concentrations of micrometric or nano-sized TMP on enamel demineralization, using a pH cycling model. Bovine enamel blocks (4 mm x 4 mm, *n* = 96) selected by initial surface hardness (SHi) were allocated into eight groups (*n* = 12), according to the test toothpastes: without fluoride and TMP (Placebo), 1100 ppm F (1100 ppm F), 1100 ppm F plus micrometric or nano-sized TMP at concentrations of 1% (1100 1%TMP; 1100 1%TMPnano), 3% (1100 3%TMP; 1100 3%TMPnano), and 6% (1100 6%TMP; 1100 6%TMPnano). Blocks were treated 2x/day with slurries of toothpastes and submitted to pH cycling for five days. Next, final surface hardness (SHf), integrated mineral loss (IML), differential profile of integrated mineral loss (ΔIML) and enamel fluoride (F) concentrations were determined. The results were subjected to ANOVA followed by Student-Newman-Keuls test ($p < 0.001$). Blocks treated with 1100 3%TMPnano showed significantly lower mineral loss (SHf, IML and ΔIML), followed by 1100 3%TMP group ($p < 0.001$). The 1100 3%TMPnano group showed significantly higher enamel F concentration followed by 1100 6%TMPnano ($p < 0.001$). It was concluded that supplementation of conventional toothpaste with 3%TMPnano produce a synergistic effect on the inhibition of enamel demineralization when compared to its counterpart without TMP and micrometric TMP.

Keywords: Demineralization, Phosphates, Toothpastes, Nano-sized.

2.2 Introduction

The prevention and treatment of early caries lesions, especially in patients at high risk are constant challenges in dentistry. In recent years, several studies with nano particles have been conducted attempting to reduce mineral loss, which have been shown to have unique remineralization properties [1-4]. Conversely, studies have been conducted to enhance the effects of fluoride (F) products against dental caries, among which the use of inorganic phosphates have been proven to produce a synergistic effect [5-8]. This has later prompted to studies assessing the impact of nano-sized phosphates addition to F toothpastes on the process of enamel remineralization [9-10].

The addition of micrometric sodium trimetaphosphate (TMP) to low-fluoride toothpastes (500 ppm F) has been studied in recent years, significantly enhancing the effects of the product against demineralization [5,11]. The addition of 3% TMP to a toothpaste with 1100 ppm F improves its anticarie effect [unpublished data], which is in line with a previous *in situ* study [12]. Surprisingly, however, a clinical trial showed that the association between TMP and F did not produce any additional effect against dental caries [13-14]. Clinical models and methodologies have certain limitations, which may cause the non-observation of positive results [13]. Therefore given the scarcity of studies assessing the effects of TMP added to F toothpastes on the dynamics of dental caries and considering the promising effects of nanoparticles, it would be interesting to assess whether the addition of nano-sized TMP to a conventional F toothpaste would further enhance the effects of micrometric TMP.

Therefore, the purpose of the present study was to evaluate conventional toothpastes containing 1100 ppm F associated or not with different concentrations

of micrometric or nano-sized TMP on enamel demineralization, using a pH cycling model. The null hypothesis was that fluoride toothpaste associated to nano-sized TMP would present the same ability to reduce enamel demineralization when compared to its counterpart without TMP and micrometric TMP.

2.3 Materials and Methods

Experimental design

Enamel blocks (4 mm × 4 mm, *n* = 96) were obtained from bovine incisors; enamel surfaces were polished (outer enamel removed ~120 μm) and the blocks selected by initial surface hardness test (SHi; 320.0 to 380.0 kgf/mm^2). Blocks were then randomly distributed into 8 groups (*n* = 12). The experimental toothpastes contained either concentrations of 1, 3, and 6% micrometric TMP (TMP) or nano-sized TMP (TMPnano). NaF (Merck, CAS 7681-49-4, Germany) was also added at 1100 ppm F. In addition, toothpastes without TMP and F (Placebo), as well as with 1100 ppm F (without TMP) were prepared. Blocks were subjected to pH cycling and treatment with toothpaste slurries. Next, final surface hardness (SHf), integrated mineral loss (IML), differential profile of integrated mineral loss (ΔIML) and enamel fluoride (F) concentrations were determined.

Synthesis and characterization of nano-sized (TMP) particles

To prepare the TMP nano-sized, 70 g of pure (micrometric) sodium trimetaphosphate ($Na_3O_9P_3$, Aldrich, purity ≥ 95% CAS 7785-84-4) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol. After 48 h, the powder was separated from the alcoholic media and ground in a mortar. The powder crystallinity was characterized by X-ray diffraction (XRD) using a

Rigaku Dmax 2500 PC difractometer in the 2θ range from 10 to 80° with a scanning rate of 2°/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation:

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

where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9.

Toothpaste formulation and fluoride and pH assessment

The toothpastes were produced with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate and deionized water. Toothpastes containing micrometric or nano-sized TMP were prepared (Aldrich Chemistry, CAS 7785-84-4, China) at concentrations of 1, 3, and 6% micrometric TMP (TMP) or nano-sized TMP (TMPnano). To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpastes without TMP and F (Placebo), as well as with 1100 ppm F (without TMP) were prepared. The F concentrations [15] and pH of the all toothpastes were checked [11] prior to the beginning of the study.

Toothpastes treatments and pH cycling

The blocks were subjected to five pH cycles during 7 days, at constant temperature of 37 °C [16]. The blocks were kept in a demineralizing solution (DE) (6 h; 2.0 mmol/L calcium and phosphate in 75 mmol/L acetate buffer, pH 4.7; 0.04 µg F/mL, 2.2 mL/mm^2) followed by their immersion in a remineralizing solution (RE) (18 h; 1.5 mmol/L calcium; 0.9 mmol/L phosphate; 150 mmol/L KCl in 0.02 mol/L cacodylic buffer, pH 7.0; 0.05 µg F/mL, 1.1 mL/mm^2). The treatment consisted of a 1-min soak under agitation in 2 mL/block of toothpaste:deionized water slurries (1:3 w/w), between immersion in the DE and RE solutions (twice a day). Deionized water rinses were performed between each step. The blocks were kept in fresh remineralizing solution during the last 2 days.

Hardness measurements

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

For cross-sectional hardness measurements (KHN), enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed. The samples were then gradually polished until the total exposition of the enamel. A sequence of 14 indentations were created at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the surface of the enamel in the central region using a Micromet 5114 hardness

tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s [17]. The averages were calculated for each distance and the values converted into mineral content (vol% min. = 4.3*(√KHN) + 11.3) [18]. The integrated mineral loss (IML; % vol min. × μm) of the lesion and sound enamel was calculated using the trapezoidal rule (Graph Pad Prism, version 3.02) and subtracted from the integrated area of the hardness of the sound enamel resulting in the integrated mineral loss (IML).

To analyze the patterns of demineralization, differential mineral content profiles for F and F + TMP (i.e. value of 1100 TMPs groups minus 1100 group), at each of the micrometric TMP concentrations, were determined. Also, the differential profiles were calculated for the F + nano-sized TMP and F + micrometric TMP at each TMP concentrations. These differential profiles were then integrated over three depth zones in the lesion (zone A, 5–15 μm; zone B, 15–50 μm; zone C, 50–130 μm) and underlying sound enamel to yield ΔIML values [7-8].

Analysis of the F concentration present in enamel

Blocks measuring 2 mm × 2 mm (*n* = 96) were obtained from half of the longitudinally sectioned blocks, and were fixed to a mandrel. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50.0 ± 0.05 μm was removed from each enamel block [5,19]. A total of 0.5 mL of 0.5 mol/L HCl was added to the enamel powder retained on the polishing disc fixed to the polystyrene crystal tube. This mixture was agitated

for 1 h, and 0.5 mL of 0.5 mol/L NaOH solution was added [5, 20]. For the F analysis, samples were buffered with TISAB II and analyzed with an ion-specific electrode (Orion 9609) connected to an ion analyzer (Orion 720^{+}). A 1:1 ratio (TISAB:sample) was used. The electrodes were previously calibrated with standards containing from 0.125 to 2.00 mg F/mL under the same conditions of the samples. The results were expressed as $\mu g/mm^3$.

Statistical analysis

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

2.4 Results

The milling processing reduced the particle size of the TMP powders without affecting the crystalline structure of the material. The X-ray diffraction (XRD) pattern of the nano-sized TMP after 48 h of milling (Figure 1) shows broader peaks due the smaller crystallites, which could be used to estimate an average particle size of 22.7 nm.

The concentration of fluoride referred to as total F (TF) and ionic F (IF) of the placebo toothpaste were 9.5 (1.1) and 9.7 (0.4) ppm F, respectively. For the toothpastes with 1100 ppm F, mean values (SD) from the groups were 1,162.0 (44.1) and 1,157.2 (16.8), ranging from 1,111.0 and 1,183.9 ppm F. Mean pH of

toothpastes was 7.3 (0.3) ranging from 6.8 to 7.7. Mean (SD) SHi considering all blocks was 372.8 (0.2) kgf/mm^2. No significant differences were observed among the groups after random allocation (p = 0.610).

The toothpaste with 1100 ppm F (Table 1) promoted SHf 3 times harder when compared with the placebo group (p < 0.001). The addition of TMP to fluoride toothpastes lead to enamel surface with lower softening when compared to fluoride toothpaste without TMP (p < 0.001). Blocks treated with 1100 3%TMPnano showed SHf 30% higher than those treated with 1100 (p < 0.001), while this effect was around 15% with after treatment with 1100 3%TMP (p < 0.001). TMP concentrations over 3% did not promote significant reduction of surface enamel softening, regardless of the particle size.

The integrated mineral loss (IML, vol%min·μm) was 2 times lower in the presence of F (placebo × 1100 ppm F toothpastes). The association TMP/F reduced the IML when compared with fluoride toothpaste without TMP (p < 0.001). The 1100 3%TMPnano lead to the lowest mineral loss, which was ~ 80% smaller when compared to 1100 (p < 0.001). The 1100 3%TMP toothpaste resulted in a ~ 64% reduction of mineral loss when compared to the 1100 toothpaste.

The results of F present in the enamel (Table 1) showed that the addition of 1%TMP and 3%TMP increased the F concentration in 20% and 50%, respectively, when compared to 1100 ppm F group. With TMPnano toothpastes, the increase of fluoride uptake was 90%, 160% and 100%, respectively for the concentrations of 1%, 3% and 6% compared to 1100 ppm F group. Positive and significant correlations were observed between F and SHf (Pearson's r = 0.689; p < 0.001) as well as between F and IML (Pearson's r = -0.658; p < 0.001).

Differential profile hardness (Figure 2 and Table 2) show different subsurface lesion patterns. The addition of TMP lead to mineral content values greater in the middle part of the lesion (15–50 μm) which was improved up to 3%TMP ($p < 0.001$). At 6%TMP (Figure 2a), the mineral content was lower in the zone A (5–15 μm) and B (15–50 μm) when compared to 3%TMP ($p < 0.001$). The differential profile hardness from figure 2b, show the additional effect of nano-sized TMP when compared to micrometric TMP. At 1%TMPnano, the mineral content was only higher ($p < 0.001$) in the inner part of the lesion (zone C, 50–130 μm). The addition of 3%TMPnano lead to higher mineral content ($p < 0.001$) being greater at zone B ($p < 0.001$). With 6% of TMPnano, the gain mineral occurred in the zone B and C, despite being lower than 3%TMPnano ($p < 0.001$).

2.5 Discussion

The addition of TMP to topical fluoride products, especially toothpastes, has been shown to improve their anticaries effect [5-6]. The increase of TMP concentration up to 3% improved the anticaries effect and was positively and significantly correlated to F present in the enamel. At concentrations greater than 3% of TMP, enamel fluoride uptake and enamel hardness was significantly reduced. As the effect of TMP is related to its ability to adsorb on the enamel, the TMP:F molar ratio has a strong influence on the resulting anticaries effect [5, 21-22]. Thus, the strength of chemical bonding of TMP to enamel becomes greater than that of F in high TMP concentrations [22]. This reduces the F uptake and increases enamel demineralization (Table 1). Even though, fluoride toothpaste containing 6% TMP showed better anticaries effect than 1100 ppm F toothpaste. With regards to 1100 ppm F toothpaste, the high amount of F in enamel can

explain its anticarie effect in relation to placebo group. An increase in F concentration is correlated with increased Ca concentration, indicating a close association between Ca and F ions [23-25]. To toothpastes containing TMP, probably its adsorption on enamel lead to formation of a barrier causing a reduction of acid diffusion, avoiding enamel demineralization in high degree [6,22].

Nano-sized TMP reduced the integrated mineral loss (IML, vol%·μm) in 20% compared to micrometric TMP, which suggests an increased capacity of adsorption of nano-sized TMP to enamel. TMP has been shown to enhance incorporation of Ca [5,6,26] ions as well as F ions in the enamel [5,6,22,26]. The effect of TMP probably is retain charged ions of CaF^{+} and Ca^{2+} by replacing Na^{+} from cyclic structure [27]. The nano-sized TMP adsorbed on enamel seems to be more reactive and retain a greater amount of Ca^{++} and CaF^{+} in its negatively charged structure. At acidic pH these linkages are broken, releasing Ca^{++} and CaF^{+}, which can further take part in a series of events that ultimately lead to the formation of species ($CaHPO_4^{0}$ and HF^{0}) that have a higher diffusion coefficient into the enamel [28]. These results can be explained due to Properties of nanoparticles, such as their high ratio of surface area to volume, as well as a high percentage of atoms on the surface compared to larger particles, which makes them more reactive. The mechanism above seems to explain why fluoride toothpaste with nano-sized 3% TMP reduced the mineral loss in ~ 44% compared to its micrometric counterpart, mainly in the depth of 15–50 μm (Figure 2 and Table 1). The impact of this effect can be observed in the amount of F present in the enamel when nano-sized TMP is used (Table 1), since there is an increase of ~ 75% on enamel F uptake when compared to micrometric TMP. An important

factor that promotes such effects is the ability of TMP to remain bound to enamel for a longer period than other polyphosphates.

The current study shows that TMP affects the processes of enamel de- and re-mineralization, especially in the deeper regions of the enamel lesion. While in the outer part of the lesion (5 μm) only a small additional effect was produced by the addition of TMP to the toothpastes, a greater effect was observed in the depth of the lesion (mainly at 15–50 μm), which is consistent with previous findings [6-8, 22]. Recently studies have shown that TMP inhibits remineralization in the outer layer [6] as well as reduces the precipitation of CaF_2 and firmly bound fluoride [7-8, 26-27]. Thus, the lower mineral content in the outer enamel (5–15 μm) observed with nano-sized TMP can be explained by: (1) its higher reactivity and retaining of Ca^{++} and CaF^{+} retention in its structure; (2) the reduction of F precipitation in the outer enamel and (3) the reduction of mineral content. Probably, this phenomenon reduces the obstruction of the pores of the enamel surface facilitating neutral species diffusion ($CaHPO_4^0$ and HF^0) into the enamel, mainly during the remineralization phase in the inner part of the lesion.

Since the adsorption of polyphosphates occurs rapidly [29], which is followed by the adsorption of F; TMP and F must be combined in an appropriate molar proportion. Combining 500 ppm F with micrometric TMP observed a better efficacy when compared to toothpaste with 1100 ppm F with a molar proportion of 1.2 to 3.7 [5]. With conventional F concentration in the formulation (1100 ppm F) the equilibrium adsorption of TMP and F reaches a maximum effect with 3%TMP (TMP/F: 1.7), unlike what happens with 500 ppm F. For TMP concentrations above 3% (TMP/F: 2.5, 3.4 and 5.1), the bonding strength of hydroxyapatite with TMP reduces the link sites of F on enamel decreasing the synergistic anti-caries

effect between them. When the fluoride concentration is raised to 3000 ppm F, the bond strength of the F to enamel is greater than TMP at 3% with no improvement in anticarie effect [6].

On the basis of the findings of this *in vitro* study, the addition of nano-sized of TMP at 3% to toothpaste with a concentration of 1100 ppm F has promoted greater inhibition of enamel demineralization. Therefore the null hypothesis is rejected

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Table legend

Table 1. Mean (SD) surface hardness (SHf), integrated mineral loss (IML, vol%) and fluoride in enamel (F) according to groups (*n* = 12).

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

Figure legends

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

Figure 2. Graphic of differential profile hardness as a function of depth according to the groups. : Zone A (5–15µm), Zone B (15–50µm) e Zone C (50–130µm) (*n* = 12).

Table 1. Mean values (SD) of surface hardness (SHf), integrated mineral loss (IML, vol%) and fluoride in enamel (F) according to groups (*n* = 12)

Groups	**SHf (kgf/mm^2)**	**IML, vol%·µm**	**F (µg/mm^3)**
Placebo	73.7^{a} (8.0)	1,637.8^{a} (203.4)	0.4^{a} (0.1)
1100 ppm F	241.0^{b} (8.7)	791.0^{b} (138.7)	1.0^{b} (0.2)
1100 1%TMP	254.9^{c} (7.1)	587.9^{c} (83.4)	1.2^{b,d} (0.2)
1100 1%TMPnano	280.3^{d} (5.5)	604.2^{c} (40.0)	1.9^{c} (0.6)
1100 3%TMP	276.6^{d} (4.4)	287.1^{d} (89.4)	1.5^{d} (0.3)
1100 3%TMPnano	311.5^{e} (4.4)	161.5^{e} (25.6)	2.6^{e} (0.7)
1100 6%TMP	223.2^{f} (4.5)	548.3^{c} (100.3)	1.0^{b} (0.4)
1100 6%TMPnano	261.4^{g} (9.9)	387.8^{f} (41.1)	2.0^{c} (0.4)

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

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

Groups	**ΔIML[a], vol%·μm**		
	Zone A (5–15 μm)	Zone B (15–50 μm)	Zone C (50–130 μm)
1%TMP	31.1^{a,A} (8.9)	138.2^{a,B} (49.2)	46.0^{a,A} (4.9)
3%TMP	84.4^{b,A} (17.5)	323.7^{b,B} (57.5)	79.6^{b,A} (14.8)
6%TMP	20.3^{a,A} (7.6)	195.3^{a,B} (49.7)	73.3^{b,C} (14.6)
1%TMPnano	-92.4^{a,A} (7.9)	-18.2^{a,B} (5.4)	26.2^{a,C} (8.8)
3%TMPnano	21.5^{b,A} (6.5)	107.9^{b,B} (25.2)	25.1^{a,A} (5.4)
6%TMPnano	-11.2^{c,A} (6.1)	49.1^{c,B} (10.3)	2.6^{b,C} (2.4)

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

[a]ΔIML: positive values denote higher integrated mineral content and vice versa.

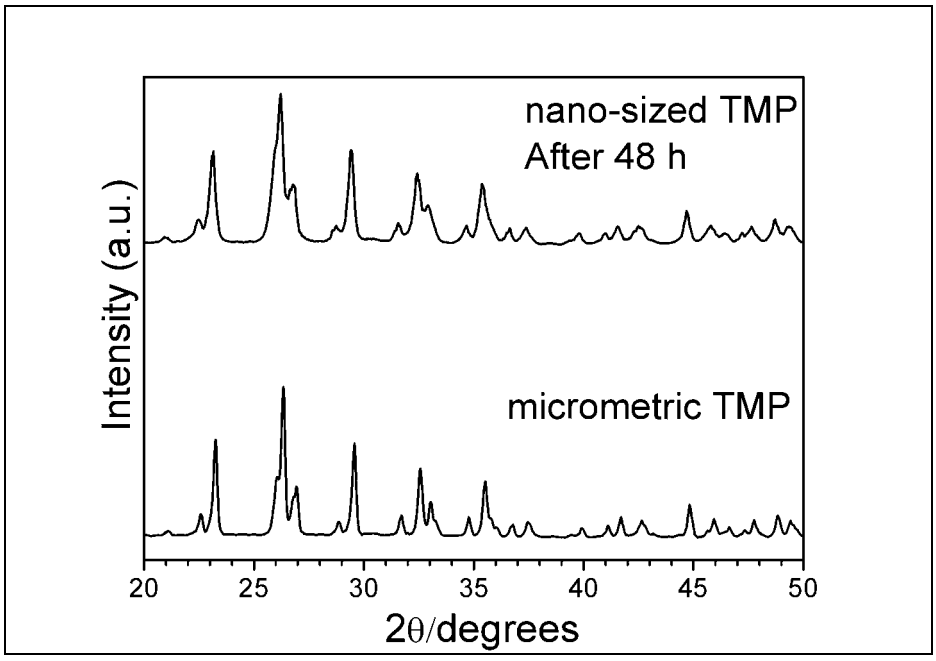

Figure 1. X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

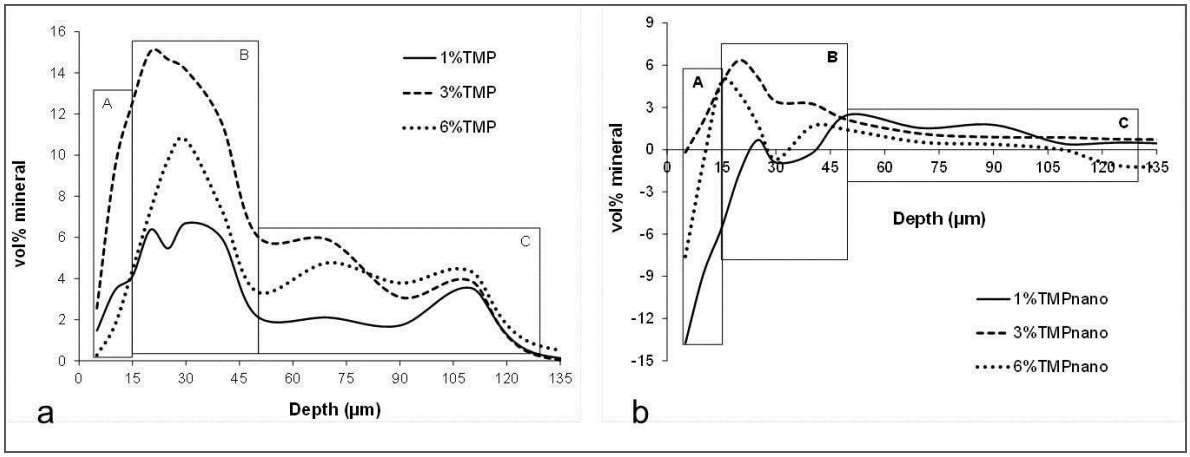

Figure 2. Graphic of differential profile hardness as a function of depth according to the groups. : Zone A (5–15µm), Zone B (15–50µm) e Zone C (50–130µm) (*n* = 12). (ANOVA, Student-Newman Keuls, p < 0.001).

Capítulo 2

3.0 Effect of fluoride toothpaste supplemented with nano-sized trimetaphosphate on enamel remineralization: an *in situ* study

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De acordo com as instruções aos autores do periódico Journal of Dental Research (Anexo B)

3.1 Abstract

The aim of this *in situ* study was to evaluate the effect of fluoride toothpaste supplemented with nano-sized sodium trimetaphosphate (TMP) on enamel remineralization. This blind and cross-over study was performed in 4 phases of 3 days each. Twelve subjects used palatal appliances containing four bovine enamel blocks with artificial caries lesions. Volunteers were randomly assigned into the following treatment groups: Placebo (without F and TMP); 1100 ppm F without TMP (1100), or supplemented with 3% micrometric TMP (1100 TMP) or nano-sized TMP (1100 TMPnano). Volunteers were instructed to brush their natural teeth with the palatal appliances in the mouth during 1 minute (3 times/day), so that blocks were treated with natural slurries of toothpastes. After each phase, the percentage of surface hardness recovery ($\%SH_R$), integrated recovery mineral loss (IML_R) and differential profile of integrated mineral loss (ΔIML) in enamel lesions were calculated. F in enamel was also determined. The results were analyzed by ANOVA and Student-Newman-Keuls tests ($p < 0.05$). Enamel surface became 20% harder when treated with 1100 TMPnano in comparison with 1100 ($p < 0.001$). Also, 1100 TMP nano showed capacity to reduce the lesion body (IML_R; ΔIML) 66% higher when compared with 1100 TMP ($p < 0.001$). Enamel F uptake in the 1100 TMPnano group was 2-fold higher when compared to its counterpart without TMP ($p < 0.001$). It was concluded that the addition of 3% TMPnano to a conventional toothpaste was able to promote an additional remineralizing effect of artificial caries lesions.

Keywords: Toothpastes, Tooth remineralization, Phosphates, Nano-sized.

3.2 Introduction

The regular use of fluoride (F) toothpaste has been associated with a decline in dental caries in both developed and developing countries (Bratthall *et al*., 1996; Marinho *et al*., 2004). The greatest advantage when compared to alternative forms of topical application is the regular delivery of F (Mellberg *et al*., 1985), associated to the removal or disruption of the dental biofilm (Pessan *et al.*, 2011). Given the limited effect of fluoride on the dynamics of dental caries, attempts to enhance the anticaries properties of F dentifrices have been made, among which the use of inorganic phosphates have been the most studied over recent years (Takeshita *et al*., 2009, 2011; Danelon *et al*., 2013a, 2013b). Sodium trimetaphosphate (TMP) seems to be the most effective polyphosphate against dental caries. The addition of micrometric (regular sized) TMP to low-F fluoride toothpastes has been shown to be more effective than its counterpart without TMP on enamel demineralization (Takeshita *et al*., 2009; Moretto *et al*., 2010). It has been suggested that TMP is adsorbed to enamel surface reducing enamel demineralization (Henry and Navia, 1969, Gonzalez, 1971; Gonzalez *et al*., 1973), reducing hydroxyapatite solubility (Mcgaughey and Stowell, 1977; Manarelli *et al*., 2013; Favretto *et al*., 2013) and mineral exchange.

In recent years, several studies with nano-sized compounds other than fluoride have also been conducted, aiming to produce formulations that are effective in reducing mineral loss, as well as in enhancing mineral gain (Roveri *et al*., 2008; Hannig and Hannig, 2012; Comar *et al*., 2013). In this sense, studies have also analyzed the impact of nano-sized phosphates added to fluoride toothpastes in the process of enamel remineralization (Karlinsey *et al*., 2007; Huang *et al*., 2009). The addition of nano-sized tri-calcium phosphate fluoride

toothpastes reduced the enamel demineralization process compared to a conventional toothpaste. Also, nanocomposites ACP, CaF_2 and chlorhexidine have shown action in the metabolic activity of the biofilm and consequently reducing acid production (Cheng *et al*., 2012).

Knowing that conventional toothpastes (1100 ppm F) are used by the population, especially those with high caries development it would be interesting to assess the association between nano-sized TMP with a F-toothpaste in order to verify their effectiveness in remineralizing pre-existing caries lesions when compared to counterparts with micrometric TMP or without TMP.

Thus, the purpose of the study was to evaluate the remineralizing effects of conventional toothpastes (1100 ppm F) associated or not with micrometric or nano-sized 3%TMP, using an *in situ* model and artificially demineralized bovine enamel blocks. The null hypothesis was that fluoride toothpaste associated to nano-sized TMP would present the same ability to remineralization the enamel when compared to fluoride toothpaste (1100 ppm F).

3.3 MATERIAL AND METHODS

Experimental Design

This study was previously approved by the Human Ethical Committee (Protocol: 17888413.1.0000.5420). This was a blind and cross-over *in situ* study performed in four phases of 3 days each (Afonso *et al*., 2013) A sample size of twelve volunteers was calculated considering α-error level of 5%, β-error level of 20% (www.dssresearch.com). Volunteers aged 20-30 years, who were in good general and oral health (Delbem *et al*., 2005), presented normal salivary flow (Rios *et al*.,

2006), 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. All participants read and signed informed consent statements prior to study initiation. Enamel blocks (4 mm × 4 mm, *n* = 192) from bovine incisive teeth were sequentially polished and selected through surface hardness test (SH: range of 370.0 up 377.0 kgf/mm^2; p = 0.080). The blocks were demineralized and submitted to post demineralization surface hardness (SH_1). Based on the percentage of surface hardness loss (post demineralization) the enamel blocks were divided into four treatments groups: Placebo (no F and TMP); 1100 ppm F (1100); 1100 ppm F and 3% micrometric TMP (1100 TMP); and 1100 ppm F and 3% nano-sized TMP sized (1100 TMPnano). After each experimental period, surface hardness (SH_2) was again assessed to calculate the percentage of surface hardness recovery (%SH_R). The blocks were sectioned and to perform cross-sectional hardness test to calculate the integrated recovery of mineral loss (IML_R) and the differential profiles of integrated mineral loss (ΔIML). Fluoride (F) content in enamel was also determined.

Synthesis and characterization of nano-sized (TMP) particles

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

scanning rate of 2°/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation:

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

where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9.

Toothpaste formulation and fluoride and pH assessment

The toothpastes were produced with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate and deionized water. Toothpastes containing micrometric or nano-sized TMP were prepared (Aldrich Chemistry, CAS 7785-84-4, China) at concentration of 3% micrometric TMP (TMP) or nano-sized TMP (TMPnano). To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpastes without TMP and F (Placebo), as well as with 1100 ppm F (without TMP) were prepared.

The F concentrations (Delbem *et al*., 2009) and pH (Moretto *et al*., 2010) of all the toothpastes were checked. The mean (SD) concentration of total F (TF) and ionic fluoride (IF) (n = 3) were: placebo – 9.5 (1.1) 9.7 and (0.4), 1100 ppm F – 1162.0 (44.1) and 1157.2 (16.8), 1100 TMP – 1162.0 (44.1) and 1157.2 (16.8),

and 1100 TMPnano – 1162.0 (44.1) and 1157.2 (16.8). The pH value from the groups was 7.3 (0.3) ranging from 6.8 to 7.7

Subsurface enamel demineralization

All surfaces of each specimen, except the enamel surface, were coated with acid resistant varnish and subsurface enamel demineralization (Spiguel *et al*., 2010; Danelon *et al*., 2013a) 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). Mean (SD) of surface hardness after demineralization (SH_1) was 63.1 KHN (2.7), and the means varied between 61.4 and 63.7 ($p = 0.093$).

Palatal appliance preparation and treatments

The oral appliance was prepared in acrylic resin (Jet - Articles Classic Odontológico, São Paulo) in accordance with Danelon *et al*. (2013a). Twelve volunteers wearing acrylic palatal appliance with four demineralized enamel bovine blocks were subjected to four phases of 3 days each with 7 days washout period among experimental phases (Afonso *et al*., 2013). The treatments with the toothpastes were performed 3 times per day inside the mouth, during the volunteers' habitual oral hygiene routine. The volunteers were oriented initially brushing their natural teeth and following to conduct three brushing strokes in each row of enamel blocks on the oral appliance, with the natural slurry (saliva/tootphaste) formed. During 7-day pre-experimental period and washout periods, the volunteers brushed their teeth with non-fluoridated toothpaste. The volunteers received all the instructions previously. After the 3-day experimental

period, the blocks were removed from the appliance, cleaned using gauze and deionized water.

Microhardness Analysis

The enamel surface hardness was determined before (SH_1) and after each phase (SH_2) using a a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s. Five indentations, spaced 100 μm from each other, were made in the center of the enamel block. After each phase, five indentations were made spaced 100 μm from the baseline indentations for determination enamel surface hardness (SH_2). The-recovery percentage of surface hardness ($\%SH_R$) was calculated [$\%SH_R = 100\ (SH_2 – SH_1)/SH_1$].

For the cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed. The samples were then gradually polished until the total exposition of the enamel. One 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 using a a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s (Delbem *et al*., 2010). The averages were calculated for each distance and the values converted into mineral content (vol% min. = 4.3*(√KHN) + 11.3) (Kielbassa *et al*., 1999). The integrated mineral loss (IML; vol% min × μm) of the lesion and sound enamel was calculated using the trapezoidal rule (Graph

Pad Prism, version 3.02) and subtracted from the integrated area of the hardness of the sound enamel. These values were subtracted from the integrated area of the post demineralized enamel resulting in the integrated recovery of mineral loss (IML_R).

To analyze the patterns of remineralization, differential mineral content profiles were calculated by subtracting the mineral values of each group at each depth from of the Placebo group (i.e., 1100 ppm F, 1100 TMP and 1100 TMPnano groups values minus the Placebo group). These differential profiles were then integrated over three depth zones in the lesion (zone A, 5–15 μm; zone B, 15–50 μm; zone C, 50–110 μm) and underlying sound enamel to yield ΔIML values (Danelon *et al*., 2013a, Danelon *et al*., 2013b).

Analysis of the F concentration present in enamel

Blocks measuring 2 mm × 2 mm (*n* = 192) were obtained from half of the longitudinally sectioned blocks, and were fixed to a mandrel. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tube (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of 50.0 ± 0.05 μm was removed from each enamel block (Weatherell *et al*., 1985; Takeshita *et al*., 2009). A total of 0.5 mL of 0.5 mol/L HCl was added to the enamel powder retained on the polishing disc fixed to the polystyrene crystal tube. This mixture was then agitated for 1 h, and then, 0.5 mL of 0.5 mol/L NaOH solution was added (Alves *et al*., 2007; Takeshita *et al*., 2009). For the 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. The electrodes were previously calibrated with standards containing from 0.125 to

2.00 mg F/mL under the same conditions of the samples. The results were expressed as μg/mm^3.

Statistical Analysis

Analyses were performed using the Sigma Plotm (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. The variables $\%SH_R$ and F (log transformation) showed normal (Shapiro-Wilk) and homogeneous (Cochran test) distributions. One-way ANOVA was then performed, followed by the Student-Newman-Keuls. The IML_R data showed heterogeneous distribution and it were submitted to Kruskal-Wallis followed by the Student-Newman-Keuls. The ∆IML values were submitted to two-way ANOVA followed by the Student-Newman-Keuls. Pearson's correlation coefficients between F present in enamel with $\%SH_R$ and IML_R were also calculated.

3.4 Results

The milling processing reduced the particle size of the TMP powder without affecting the crystalline structure of the material. The X-ray diffraction (XRD) pattern of the nano-sized TMP after 48 h of milling (Figure 1) shows broader peaks due the smaller crystallites, which could be used to estimate an average particle size of 22.7 nm.

The addition of TMP to fluoride toothpastes increased the $\%SH_R$ in 10% (Table 1) when compared to fluoride toothpaste without TMP ($p < 0.001$). With nano-sized TMP, the enamel surface became 20% harder than 1100 ($p < 0.001$). In addition, the capacity to reduce the lesion body (IML_R) was ~ 20 higher with the addition of micrometric TMP and ~ 43% higher with the TMPnano compared with

the 1100 ppm F group ($p < 0.001$). The statistical power ($\alpha = 0.05$) calculated for $\%SH_R$ and IML_R was 1.00.

The integrated values from differential mineral profile showed different mineral recovery patterns (Figure 2 and Table 2). The addition of micrometric TMP did not improve the remineralization capacity of fluoride toothpaste in the outer enamel (zone A, 5–15 μm, $p = 0.109$) as well as in the inner enamel (zone C, 50–110 μm, $p = 0.679$) when compared to 1100 ppm F group, but an effect was observed in the middle of lesion (zone B, 15–50 μm) with mineral recovery of 36% ($p < 0.001$) for the same comparison. The 1100 3% TMPnano significantly increased mineral recovery (Figure 2 and Table 2) in all zones when compared to all other groups ($p < 0.001$). The additional effect of nano-sized TMP on enamel remineralization (dotted line, Figure 1) was 66% with a more pronounced effect at deeper regions of the lesion (considering all the extension). The statistical power ($\alpha = 0.05$) calculated was 1.00 for ΔIML analysis (considering group vs. zone).

The addition of TMP at both particle sizes significantly increased enamel F concentrations (Table 1) when compared to the 1100 group ($p < 0.001$). With the nano-sized TMP, the increase was ~ 150% higher when compared to 1100 group ($p < 0.001$). The statistical power ($\alpha = 0.05$) calculated was 1.00 for F analysis. Enamel F concentration was positively correlated with $\%SH_R$ (Pearson's $r = 0.894$, $p< 0.001$) and enamel F with IML_R (Pearson's $r = 0.896$, $p < 0.001$).

3.5 Discussion

The present study evaluated the remineralization potential of a toothpaste with 1100 ppm F supplemented with 3% of TMP nano-sized, comparing it to a standard toothpaste (1100 ppm F) using an *in situ* model. Previous studies have shown the effect of TMP in preventing enamel demineralization and promote remineralization when added to fluoridated formulations for topical application (Takeshita *et al*., 2009, 2011; Danelon *et al*., 2013a,b). Recently, in an *in vitro* study showed that the addition of 3% of micrometric TMP to a 1100 ppm F toothpaste improves its anticaries effect [data not published]. However, the clinical trials showed that improvement was not enough to bring some benefice against dental caries (Stephen *et al*., 1994; O'Mullane *et al*., 2007). Clinical models and methodologies have certain limitations, which may cause the non-observation of positive results [Stephen *et al*., 1994].The present study showed nano-sized TMP increases the probability of obtaining good clinical outcomes.

As the effect of TMP depends on its ability to adsorb to enamel (McGaughey and Stowell, 1977; van Dijk *et al*., 1980) as well as retaining fluoride and calcium in its structure (Danelon *et al*., 2013b; Manarelli *et al*., 2014), the option to reduce the size of particles appears to be more effective than increasing the concentration of phosphate in the formulation. The nano-sized TMP adsorbed on enamel seems to be more reactive and retain a greater amount of Ca^{++} and CaF^{+} in its negatively charged structure. At acidic pH these linkages are broken, releasing Ca^{++} and CaF^{+}, which can further take part in a series of events that ultimately lead to the formation of species ($CaHPO_4^0$ and HF^0) that have a higher diffusion coefficient into the enamel [28]. These results can be explained due to Properties of nanoparticles, such as their high ratio of surface area to volume, as

well as a high percentage of atoms on the surface compared to larger particles, which makes them more reactive. The mechanism above seems to explain why fluoride toothpaste with nano-sized 3% TMP reduced the mineral loss in ~ 44% compared to its micrometric counterpart, mainly in the depth of 15–50 μm (Figure 2 and Table 1). The impact of this effect can be observed in the amount of F present in the enamel when nano-sized TMP is used (Table 1), since there is an increase of ~ 75% on enamel F uptake when compared to micrometric TMP. An important factor that promotes such effects is the ability of TMP to remain bound to enamel for a longer period than other polyphosphates.

The present data showed that the higher remineralization rate was associated with higher enamel fluoride uptake. This means reducing the deposition of calcium fluoride (CaF_2) in the enamel and increase retention of calcium (Ca^{++}) and F on the TMP molecule adsorbed to enamel (Danelon *et al*., 2013b; Manarelli *et al*., 2014). A greater availability of Ca^{++} and CaF^{+} may lead to the formation of $CaHPO_4^{0}$ and HF^{0}, which are known to have a much higher diffusion coefficient into the enamel when compared to charged species (Cochrane *et al*., 2008). The impact of this can be observed in the mineral content (IML_R) of the enamel when nano-sized TMP is used (Table 1).

ΔIML values observed in this study confirm previous findings that TMP reduces mineral loss deep in the enamel (Table 2, Figure 2) (Takeshita *et al*., 2011; Danelon *et al*., 2013a, Danelon *et al*., 2013b). Recently studies have shown that TMP inhibits remineralization in the outer layers of tooth enamel (Takeshita *et al*., 2011) as well as reduces the precipitation of CaF_2 and firmly bound fluoride (Danelon *et al*., 2013b; Souza *et al*., 2013; Manarelli *et al*., 2014). It could be speculated that this phenomenon reduces the obstruction of the pores of the

enamel surface facilitating the neutral species diffusion ($CaHPO_4^0$ and HF^0) into the enamel, increasing the remineralization in the zone B (15–50 μm) of the lesion, but not in the deeper of the lesion (zone C, 50–110 μm). This area could further reduce the mineral ion diffusion to the region deeper lesion (zone C) not allowing a mineral recovery throughout the body of the lesion. In the presence of nano-sized TMP, there is a higher gain of mineral in the deeper part of the lesion (zone C). This can be explained by its higher reactivity and retaining of Ca^{++} and CaF^+ in its structure that leads to reducing the precipitation of F in the outer enamel and consequent lower obstruction of the pores of the enamel surface. This phenomenon may facilitate the diffusion of ions carrying the process of remineralization occurs throughout the body of the lesion and in a greater degree at the deepest part of the lesion. It is likely that this effect can produce better clinical outcomes to those observed previously (Stephen *et al*., 1994; O'Mullane *et al*., 2007). Hence, the toothpaste with 1100 ppm F associated with nano-sized TMP should be a matter of clinical trials.

Based on the above, it was concluded that the addition of nano-sized TMP to a conventional toothpaste promoted a significantly higher remineralizing effect when compared to a toothpaste of same F concentration, without TMP, so that the null hypothesis could be rejected. The effects of nano-sized TMP should also be assessed on enamel demineralization, in order to confirm the existence of additional or synergistic effects, prior to clinical trials.

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Table legend

Table 1. Mean (SD) percentage surface hardness recovery (%SH_R), integrated recovery of mineral loss (IML_R) and enamel fluoride concentrations (F) according to groups (*n* = 12).

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

Figure legends

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

Figure 2. Differential profile hardness as a function of depth according to the groups: Zone A (5–15μm), Zone B (15–50μm) e Zone C (50–110μm) (*n* = 12).

Table 1: Mean (SD) percentage of surface hardness recovery ($\%SH_R$), integrated recovery of subsurface mineral (IML_R) and enamel fluoride concentrations (F) according to groups (*n* = 12)

Groups	$\%SH_R$ (kgf/mm^2)	IML_R (vol%·µm)	F (µg/mm^3)
Placebo	18.1^a (2.3)	360.9^a (74.8)	0.36^a (0.06)
1100 ppm F	31.7^b (1.4)	751.5^b (27.0)	0.85^b (0.06)
1100 TMP	41.8^c (4.9)	896.3^c (13.6)	1.05^c (0.10)
1100 TMPnano	51.4^d (3.8)	1,073.5^d (46.6)	2.12^d (0.17)

Distinct superscript lowercase letters indicate statistical significance in each column (Student–Newman–Keuls test, $p < 0.001$).

Table 2: Mean (SD) integrated differential mineral area profile (∆IML) calculated for three zones in the enamel lesions according to groups (*n* = 12)

Groups	∆IML*, vol%·µm		
	zone A (5-15 µm)	zone B (15-50 µm)	zone C (50-110 µm)
1100 ppm F	133.2^{a,A} (7.5)	396.0^{a,B} (39.4)	92.1^{a,C} (29.7)
1100 TMP	154.5^{a,A} (5.9)	539.0^{b,B} (20.9)	74.9^{a,C} (33.8)
1100 TMPnano	201.6^{b,A} (14.1)	781.1^{c,B} (120.0)	280.3^{b,C} (103.1)

Distinct superscript capital letters indicate the differences between zones A, B and C in each line (Student–Newman–Keuls test, $p < 0.001$). Distinct superscript lowercase letters indicate statistical significance in each column (Student–Newman–Keuls test, $p < 0.001$).

* ∆IML: positive values denote higher integrated mineral and vice versa.

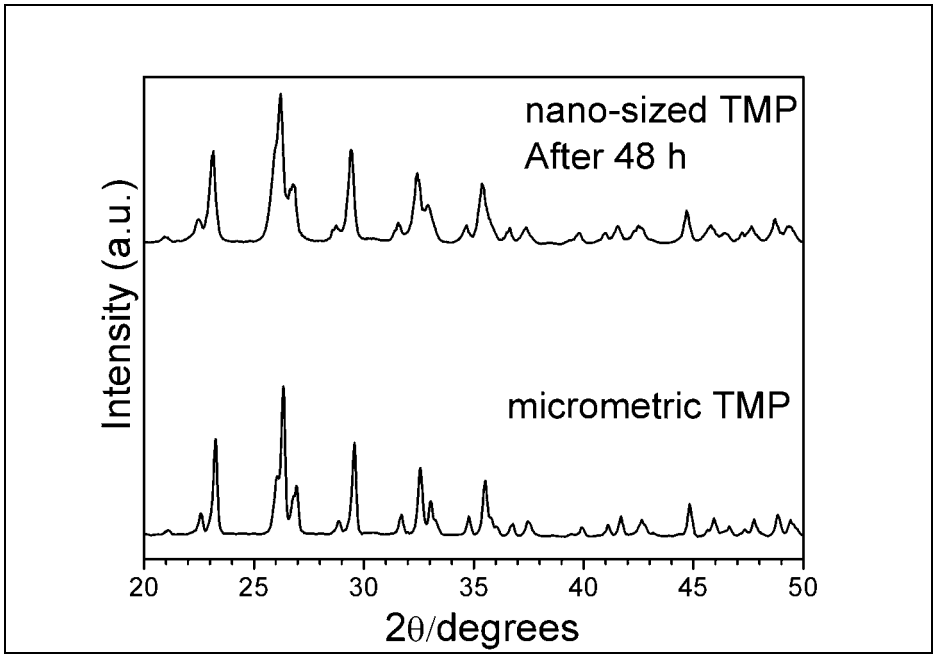

Figure 1. X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

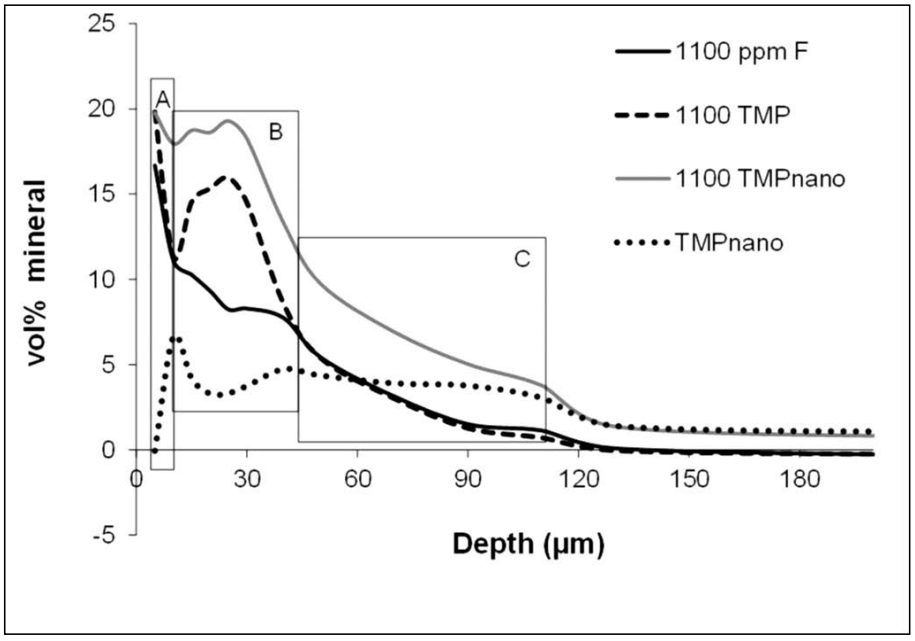

Figure 2: Graphic of differential profile hardness as a function of depth according to the groups: Zone A (5–15µm), Zone B (15–50µm) e Zone C (50–110µm) (*n* = 12).

Capítulo 3

4.0 Effect of fluoride toothpaste containing nano-sized sodium trimetaphosphate on enamel erosive wear *in vitro*

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Running title: Toothpaste with Nano-sized and enamel erosive wear *in vitro*

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

4.1 Abstract

Objective: The aim of this study was to investigate the effectiveness of nano-sized sodium trimetaphosphate (TMPnano) added to a fluoride (F) toothpaste on enamel erosive wear in the presence or not of the acquired pellicle. Design: Bovine enamel blocks (4 mm x 4 mm, *n* = 120) were randomly assigned into the following experimental toothpastes: Placebo (no F or TMP); 1100 ppm F (1100); 1100 ppm F containing 3% micrometric TMP (1100 TMP); 1100 ppm F containing 3% nano-sized TMP (1100 TMPnano) and 5000 ppm F (5000). The groups of blocks were further divided into two conditions: erosion in the presence of human saliva (ERO-HS, *n* = 60) and artificial saliva (ERO-AS, *n* = 60). The erosive challenge was be produced by immersion in citric acid 4x/day. The hardness surface (SHf) and cross-sectional hardness were analyzed as response variables as well as surface wear. The data were submitted to two-way ANOVA followed by the Student–Newman–Keuls test ($p < 0.001$). Results: SHf values were significantly higher in groups treated with TMP-supplemented and 5000 toothpastes, when compared to Placebo and 1100 ($p < 0.001$); no significant difference was observed between 1100 TMPnano and 5000 ($p = 0.202$). 1100 TMPnano and 5000 toothpastes had a greater protective effect when compared with the positive control (1100) for all variables studied regardless the use in the presence or not of the acquired pellicle ($p < 0.001$). Conclusion: the effectiveness of a toothpaste with 1100 ppm F on enamel erosive wear was significantly improved by addition of TMPnano, reaching levels similar to those obtained after the use of 5000, regardless the presence of acquired pellicle.

Keywords: Toothpastes, Dental erosion, Nano-size, Phosphates, Fluoride.

4.2 Introduction

Dental erosion is an irreversible process, in which the etiological factors must be identified and controlled in order to avoid further progression of tooth wear. Preventive measures may be adopted with the aim of reducing mineral loss due to exposure to acids, which are responsible for enamel demineralization [Hove et al., 2007]. The acidic attack leads to an irreversible loss of dental hard tissue, which is accompanied by a progressive softening of the surface [Amaechi and Higham, 2001].

Although fluoride-containing dental care products have been used to prevent erosive wear [Lussi, 2008], it has been shown that moderate or low fluoride (F) concentrations are unable to provide a significant preventive effect against erosion [Larsen and Richards, 2002]. Nevertheless, F may have a protective effect under conditions in which the erosive factors are not excessive [Lussi and Jaeggi, 2006]. On the other hand, some studies have shown a limited beneficial effect of 1000 ppm F toothpaste compared to nonfluoridated toothpastes on the abrasion of eroded dentin and enamel [Ponduri et al., 2005; Magalhães et al., 2007a].

Several studies have been conducted, attempting to enhance the effects of topically applied F products against dental caries and erosion, among which the use of inorganic phosphates has been showing to produce additional protective effects [Takeshita et al., 2009; Takeshita et al, 2011; Moretto et al., 2010; Manarelli et al., 2011; Danelon et al., 2013a; Danelon et al., 2013b; Manarelli et al., 2013; Pancote et al., 2014]. The addition of sodium trimetaphosphate (TMP) to fluoride toothpastes have been studied in the last years showing to be effective against demineralization [Takeshita et al., 2009; Moretto et al., 2010].

The impact of nano-sized phosphate added to F toothpastes on the enamel remineralization has also been studied [Karlinsey and Zero, 2006]. The addition of nano-sized tri-calcium phosphate to fluoride toothpastes was shown to be effective in reducing enamel demineralization when compared to conventional toothpaste [Karlinsey et al., 2007]. The above-mentioned benefits have not yet been assessed against enamel erosive wear.

Besides therapeutic agents, human saliva has the ability to protect enamel against erosive challenges through its clearing and buffering effects, but also due to the formation of the acquired pellicle on the enamel surface [Lendenmann et al., 2000; Sreebny, 2000]. The pellicle layer, in turn, also possesses buffering capacity and functions as a diffusion barrier that prevents the direct contact between acids and the tooth surface. It is an organic film formed by the continuous selective adsorption of salivary proteins and glycoproteins onto the enamel surface. Moreover, constant exchange of the proteins occurs during the formation of the layer [Buzalaf et al., 2012].

Based on the information above, the aim of this study was to evaluate the effect of fluoride toothpastes supplemented or not with micrometric or nano-sized sodium trimetaphosphate (TMPnano) on enamel erosive wear and whether the presence of acquired pellicle could interfere on the effects of TMP. It was hypothesized that the toothpaste supplemented with nano-sized TMP would lead to additional protective effect against enamel erosive wear, and that this effect would be decreased by the presence of acquired pellicle.

4.3 Materials and Methods

Experimental Design

Bovine enamel blocks (4 mm x 4 mm, *n* = 120) were assigned to the following experimental toothpastes: Placebo (no fluoride or TMP); 1100 ppm F (1100 ppm F); 1100 ppm F with 3% micrometric TMP (1100 TMP), 1100 ppm F with 3% nano-sized TMP (1100 TMPnano) and 5000 ppm F (5000 ppm F). Blocks were further divided into two conditions of erosion: in the presence of human saliva (ERO-HS, *n* = 60) and artificial saliva (ERO-AS, *n* = 60). The erosive challenge was be produced by immersion in citric acid 4 times per day. The sample size of 12 enamel blocks was calculated considering an α-error level of 5% and a β-error level of 20%. The factors studied were type of toothpastes (5 types) and conditions (2 types: ERO-AS and ERO-HS). Enamel wear, as well as surface and cross-sectional hardness were analyzed as response variables.

Synthesis and characterization of nano-sized (TMP) particles

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

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

where L is the linear dimension of a monocrystalline nanoparticle, λ is the wavelength of the incident X-ray, B is the diffraction line width of the diffraction peak, θ_B is the Bragg angle obtained from the XRD pattern, and K is a numerical constant which value is 0.9.

Toothpaste formulation and fluoride and pH assessment

The toothpastes were prepared with the following components: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate and deionized water. TMP (Aldrich Chemistry, CAS 7785-84-4, China) was added at concentration of 3%, for both micrometric and nano-sized particles. To these toothpastes, NaF (Merck, CAS 7681-49-4, Germany) was added to reach a concentration of 1100 ppm F. In addition, toothpastes without TMP and F (Placebo), as well as with 1100 and 5000 ppm F (without TMP) were also prepared. F concentrations [Delbem et al., 2009] and pH of the toothpastes [Moretto et al., 2010] were checked prior to the beginning of the experiment.

The total and ionic F concentrations (mean [SD]; ppm F; *n* = 3) in the toothpastes were, respectively 9.5 [1.1] and 9.7 [0.4] for the Placebo; 1162.0 [44.1] and 1157.2 [16.8] for the 1100 ppm F toothpastes (ranging from 1111.0 and 1183.9 ppm F); and 5387.0 [26.3] and 5028.8 [24.8] for the 5000 ppm F toothpaste. Mean pH of the toothpastes was 7.3 [0.3], ranging from 6.8 to 7.7

Enamel Sample Preparation

Enamel blocks (4 mm x 4 mm, *n* = 120) were prepared from extracted bovine incisors, which were stored in 2% formaldehyde solution (pH 7.0) for 30 days at room temperature. The enamel surface of the blocks was ground flat with water-cooled Carborundum disks (600, 800 and 1,200 grades of Al_2O_3 papers; Buehler, Lake Bluff, Ill., USA) for the removal of ~ 200 µm of enamel surface. After polishing, enamel surface knoop hardness (SHi) was determined using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 25 g load for 10 s and 120 blocks with initial hardness (SHi) numbers ranging from 372.0 up to 377.0 kgf/mm^2 (p = 0.610). Enamel blocks were selected and randomly allocated to ten groups of 12 blocks each. In order to maintain reference surfaces for analysis of enamel loss by profilometry, two layers of nail varnish were applied in the middle of each block.

Saliva Collection

Human subject recruitment, signed informed consent and saliva collection were performed in accordance with the protocol approved by the Human Ethical Committee (Protocol: 15018213.8.0000.5420). Paraffin-stimulated saliva samples from healthy donors of both sexes (aged 22–35 years, *n* = 10) were collected into ice-chilled vials and pooled. Immediately after collection, whole saliva samples were centrifuged for 20 min at 4 °C and 2,000 g. The supernatants were divided into 13-mL aliquots and stored at –80 ° C [Schipper et al., 2007].

Erosive Challenge and Treatment

Enamel blocks were kept immersed in 3 mL of human or artificial saliva, at 37 ºC, during 24 hours [Buzalaf et al., 2012]. Following, blocks were removed from artificial or human saliva and subjected to erosive challenges (0.05 mol/L citric acid, pH = 3.2 [Magalhães et al., 2012]; Labsynth Diadema , SP, Brazil) during 5 days, 4x/day (5 minutes each, 2 mL/block) [Moretto et al., 2010]. A remineralizing period of 2 h (immersion in unstirred artificial saliva, pH 7.0, 3 mL/block) [West et al., 1998; Attin et al., 2001; Hughes et al., 2002; Hunter et al., 2003] was allowed between the erosive challenges and treatments, at room temperature in small containers. The composition of the artificial saliva was: 1.5 mmol/L $Ca(NO_3)_2.4H_2O$, 0.9 mmol/L $NaH_2PO_4.2H_2O$, 150 mmol/L KCl, 0.1 mol/L Tris buffer, 0.03 ppm F, pH 7.0 [Vieira et al., 2005]. Immediately following each erosive challenge, the enamel blocks were exposed to slurries (1 g of toothpastes: 3 mL of water) of the toothpastes for 1 minute (under agitation, 6 mL/block,), at room temperature. The enamel blocks were stored in human saliva and artificial saliva after the last erosive/treatment process.

Hardness and Profilometric Analysis

The nail varnish on the reference surfaces was carefully removed with acetone-soaked cotton wool [Magalhães et al., 2007b]. The mean SHf was calculated based on the unit knoop hardness (KHN) determined by performing 5 indentations in different regions of the blocks surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a

Knoop diamond indenter under a 25 g load for 10 s, before (SHi) and after (SHf) the experiment. The enamel loss was determined in relation to the reference surfaces by profilometry (Hommel Tester T1000, Hommelwerke, VS-Schwenningen, Germany). Profilometric traces were taken from the reference surfaces (baseline) across the exposed surfaces (length = 2 mm). Five readings were performed on each of the blocks, and the average wear depth was calculated.

Analysis of cross-sectional hardness

Blocks were sectioned at the center, and half of each block was embedded in acrylic resin and subsequently polished. Cross-sectional hardness was determined using a hardness tester (Micromet 5114f) and the Buehler OmniMetf software, with a Knoop diamond indenter under a 5 g-load for 10 seconds [Delbem et al., 2010]. A sequence of eight prints at distances of 10, 15, 20, 25, 30, 40, 50 and 70 μm from the external surface of the enamel was performed in the center of blocks, for both the control and the test areas. The integrated area of hardness (KHN x μm) of the demineralized and sound enamel was calculated using the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated area of the hardness of sound enamel loss resulting integrated hardness (ΔKHN) [Spiguel et al., 2009].

Statistical Analysis

For statistical analysis, SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) was used, and the significance limit

was set at 5%. The values of SHf, wear (μm) and ΔKHN were considered as outcome measures and the toothpastes and saliva as the variation factor. The data presented normal (Shapiro-Wilk test) and homogenous (Cochran test) distribution and were submitted to two-way ANOVA followed by the Student–Newman–Keuls test.

4.4 Results

The milling processing reduced the particle size of the TMP powders without affecting the crystalline structure of the material. For instance, the X-ray diffraction (XRD) patterns of the nano-sized TMP after 48 h of milling (Figure 1) show broader peaks due the smaller crystallites, which could be used to estimate an average particle size of 22.7 nm.

The addition of TMP in the toothpaste reduced the enamel wear around ~ 48% when compared to the 1100 ($p < 0.001$). Nano-sized TMP increased the effect against erosion in ~ 30% when compared to 1100 TMP ($p = 0.002$). Moreover, the 1100 TMPnano and 5000 ppm F toothpastes presented similar enamel loss rates ($p = 0.955$) (Table 1). The type of saliva (artificial or human) did not influence enamel erosive wear ($p = 0.332$).

After the erosive challenges, the remaining enamel (SHf) was significantly harder for groups treated with fluoride toothpastes in comparison with the Placebo group ($p < 0.001$). The addition of TMP to the 1100 ppm F toothpaste resulted in enamel softening 84% lower when compared to the counterpart without TMP ($p < 0.001$). The nano-sized TMP improved this effect in 50% ($p < 0.001$), reaching levels similar to those obtained after the use of the 5000 toothpaste ($p = 0.202$).

As for enamel erosive wear, the type of saliva (artificial or human) did not influence the enamel surface hardness ($p = 0.113$).

The demineralization area (ΔKHN) was significantly lower than the Placebo group in around 27%, 58% and 67%, respectively for 1000, 1000 TMP and 1000 TMPnano ($p < 0.001$). The 1100 TMPnano and 5000 ppm F toothpastes showed similar ΔKHN values ($p = 0.212$). When blocks treated with Placebo were kept in artificial saliva, ΔKHN was significantly higher than those kept in human saliva ($p < 0.001$). The cross-sectional hardness profiles (Figure 2) showed that this demineralization was greater in the inner part of the lesion (15–40 µm) utilizing artificial saliva.

4.5 DISCUSSION

The present *in vitro* study confirms previous data that increasing F concentration in the toothpaste leads to improved protective effects against enamel erosive wear [Moretto et al., 2010]. The remaining, less softened enamel (Table 1; SHf and ΔKHN) observed for the 5000 group is related to the greater calcium fluoride formation [Magalhães et al., 2009; Moretto et al., 2010; Danelon et al., 2013a, Danelon et al., 2013b; Pancote et al., 2014]. As the enamel blocks were stored in human saliva and artificial saliva after the last erosive/treatment process, it would be more correct to state that the hardness testing (superficial and cross-sectional) analyzed the remineralizing ability of the formulations on the remaining enamel. This explains the greatest remineralizing capacity of the 5000 ppm F toothpaste observed in the present study, as well as in the in vitro study of Moretto et al. [2010]. However, this is a chemical model and the data obtained using this model (or any other *in vitro* protocol) should be considered carefully

due to limitations in reproducing the oral environment with all of the biological variations known to influence erosion [West et al., 2011]. When carried out in the oral medium, *in situ* studies [Magalhães et al., 2008; Rios et al., 2008] showed that increasing the F concentration has no benefits against dental erosion.

The presence of the acquired salivary pellicle and saliva might play an important role during the erosive challenge and can influence the interaction between the fluoride and mineral dental content [Rios et al., 2006; Rios et al., 2008; Young and Tenuta, 2011; Buzalaf et al., 2012]. This was confirmed for placebo group when analysed by cross-sectional hardness (ΔKHN). However, in the presence of F and TMP no influence was observed utilizing human saliva during the *in vitro* experiment. The differences observed for F when compared *in vitro* and *in situ* studies could be related to salivary clearance of F in the oral medium [Buzalaf et al., 2012], since the dilution of the toothpaste was the same.

In addition to the factor previously mentioned, the molar ratio F/TMP has a strong influence on enamel demineralization [Takeshita et al., 2009; Manarelli et al., 2011; Takeshita et al., 2011; Danelon et al., 2013a; Danelon et al., 2013b; Favretto et al., 2013; Manarelli et al., 2013]. An *in situ* study (data no published) showed that the addition of 3% TMP to a 1100 ppm F toothpaste did not improve its effect against erosive wear, while an additional effect was seen when TMP at the same concentration was added to a 500 ppm F toothpaste. It is possible that a higher F concentration associated to 3% TMP could reduce the adsorption of TMP on enamel owing to strength of chemical bonding to enamel higher for fluoride. This would further reduce Ca^{2+} and CaF^{+} retention [Danelon et al., 2013a; Danelon et al., 2013b; Manarelli et al., 2013; Pancote et al., 2014], what in turn would minimize the formation of $CaHPO_4^0$ and HF^0 species, which are known

to have much higher diffusion coefficient into the enamel when compared to neutrally charged species [Cochrane et al., 2008]. Also decrease the ability of reduction the acid diffusion into the enamel under acidic pH [Danelon et al., 2013a; Danelon et al., 2013b; Manarelli et al., 2013; Pancote et al., 2014].

According to an *in vitro* study (data no published), the increase of TMP concentration did not improve the effect against demineralization in a caries model. Nevertheless, the present study showed that the addition of nano-sized TMP leads to similar outcome with 5000 ppm F, in a trend similar to that previously reported *in vitro* for 500 ppm F toothpaste supplemented with 3% TMP [Moretto et al., 2010]. When a toothpaste with 500 ppm F and 3% TMP was tested in an *in situ* study (data no published), the effect against erosion was superior compared to a 1100 ppm F and 1100 ppm F plus 3% TMP toothpaste. Furthermore, the nano-sized TMP increased enamel fluoride in around 90% (data no published). This can indicate a higher capacity of remineralization and reduction the acid diffusion into the enamel from nano-sized TMP.

These results can be explained due to Properties of nanoparticles, such as their high ratio of surface area to volume, as well as a high percentage of atoms on the surface compared to larger particles, which makes them more reactive. However, as these data are based in *in vitro* protocols, additional *in situ* and clinical studies must be conducted to confirm these results.

To conclude, the addition of 3% TMPnano in toothpastes (1100 ppm F) promoted a synergistic protective effect against enamel erosive wear when compared with its counterparts with micrometric TMP or without TMP, reaching protective levels similar to those seen for the 5000 toothpaste. This effect was not influenced by the presence of acquired enamel pellicle.

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Table legend

Table 1. Mean (SD) enamel wear (µm), final hardness (SHf), integrated subsurface hardness (∆KHN) after erosive challenge, according to the toothpastes and type of saliva (*n* = 12).

Figure legends

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

Figure 2. Cross-sectional hardness profile according to the toothpastes and type of saliva (*n* = 12).

Table 1. Mean (SD) enamel wear (μm), final hardness (SHf), integrated subsurface hardness (ΔKHN) after erosive challenge, according to the toothpastes and type of saliva (*n* = 12)

Groups	Wear (μm)		SHf (kgf/mm^2)		ΔKHN (kgf/mm^2 × μm)	
	Artificial	Human	Artificial	Human	Artificial	Human
Placebo	4.42^{a,A} (0.09)	4.25^{a,A} (0.33)	41.7^{a,A} (5.1)	39.7^{a,A} (4.4)	3,563.7^{a,A} (694.3)	2,858.9^{a,B} (427.1)
1100 ppm F	1.23^{b,A} (0.25)	1.19^{b,A} (0.27)	77.7^{b,A} (8.0)	76.9^{b,A} (8.3)	2,357.0^{b,A} (337.2)	2,337.8^{b,A} (259.4)
1100 TMP	0.65^{c,A} (0.10)	0.67^{c,A} (0.20)	142.4^{c,A} (3.4)	141.0^{c,A} (7.4)	1,466.1^{c,A} (387.9)	1,247.1^{c,A} (396.5)
1100 TMPnano	0.46^{d,A} (0.11)	0.49^{d,A} (0.08)	214.8^{d,A} (6.8)	213.9^{d,A} (4.1)	1,063.4^{d,A} (127.4)	1,075.5^{d,A} (218.4)
5000 ppm F	0.48^{d,A} (0.12)	0.47^{d,A} (0.10)	214.0^{d,A} (3.6)	210.3^{d,A} (6.5)	978.0^{d,A} (170.2)	836.0^{d,A} (166.2)

Distinct lowercase letters show significant difference among the toothpastes in each column. Distinct capital letters show significant difference between the two conditions of saliva (ANOVA, Student–Newman–Keuls, $p < 0.001$).

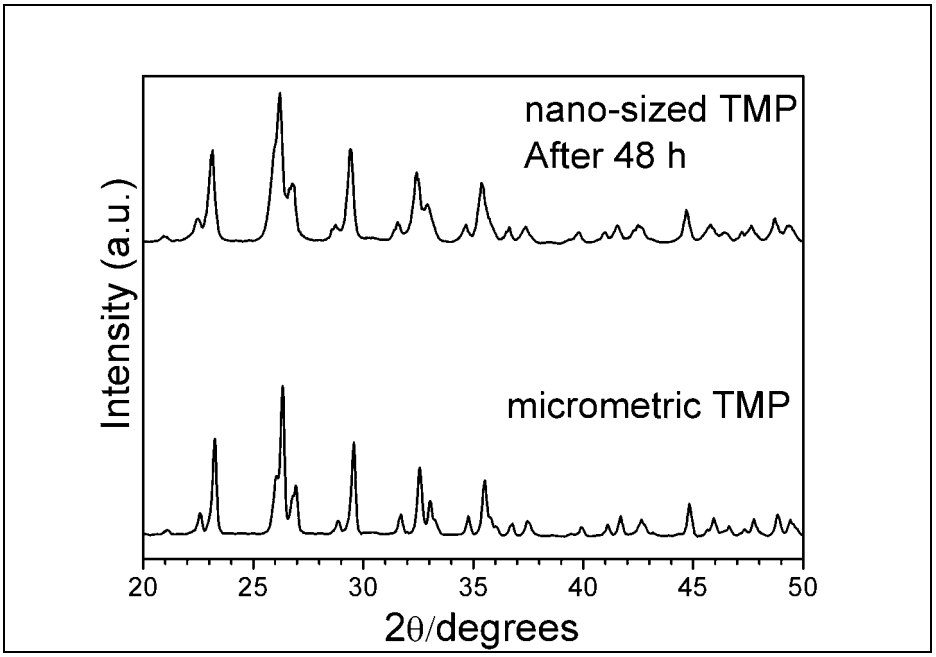

Figure 1. X-ray patterns of the micrometric TMP and of the nano-sized TMP after milling for 48 h.

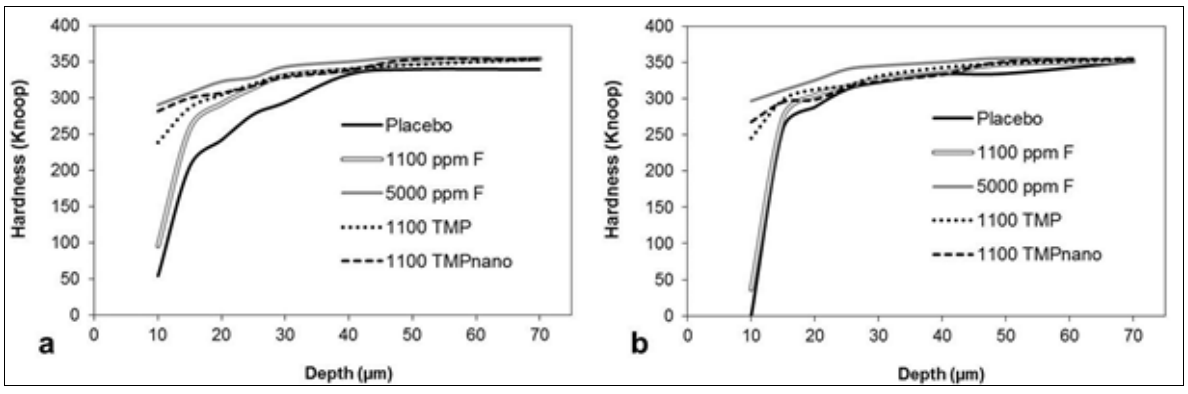

Figure 2. Graphic of cross-sectional hardness profile according to the groups under two conditions of saliva: (a) artificial and (b) human (*n* = 12).

Anexos

ANEXO A

Acta Biomaterialia

Article structure

Papers submitted to **Acta Biomaterialia**, to be acceptable, must normally be fewer than 10 printed pages in length; as a rule of thumb, a paper of 20 double-spaced typescript pages, plus a typical number of figures (8 or so), reduces to 10 printed pages. Papers that are longer than 25 double-spaced typescript pages will likely be returned to the authors with a request that they be shortened before they are considered further. Shortening, almost always, is in the author's best interest: readers read short papers.

Page numbering
Please ensure that your manuscript is paginated, as this will help both editors and reviewers to process it promptly.

Subdivision - numbered sections
Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to "the text". Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction
State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Materials and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Studies Involving Animals or Humans When data from animal subjects are reported, institutional approval of the protocol is required and a statement should be included in the "Methods" section of the text that indicates compliance with the NIH Guide for Care and Use of Laboratory Animals or other appropriate guidelines.

For human subject data, a statement must be added to the "Methods" section indicating that an institutional review committee approved the study (with the date of approval) and that the subjects provided informed consent.

Theory/calculation
A Theory section should extend, not repeat, the background to the article already dealt with in the Introduction and lay the foundation for further work. In contrast, a Calculation section represents a practical development from a theoretical basis.

Results
Results should be clear and concise, in a section separate from the Discussion.

Statistics
Careful statistical analysis must be performed and reported to support any statements regarding the existence of differences in study groups. Statistical support should underlie hypothesis testing. Error bars

are required on all experimental and calculated data points with an explanation in the text as to how the errors were determined.

Discussion

This should explore the significance of the results of the work, not repeat them. Discussion should be reported independently from Results. 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 at the end of the Discussion section.

Appendices

If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

Essential title page information

Title Page

Provide the following data on the title page (in the order given).

• **Title.** Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations, formulae, and new trademarked product names where possible.

• **Author names and affiliations.** Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a lower-case superscript letter immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name, and, if available, the e-mail address of each author.

• **Corresponding author.** Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. **Ensure that telephone and fax numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address.**

• **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a "Present address" (or "Permanent address") may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Abstract

An abstract is required for all papers. The abstract should indicate the content of the paper, and should describe the main conclusions. An effective abstract is brief and normally less than 200 words. Abstracts must not exceed 250 words. References should be avoided, but if essential, they must be cited in full, without reference to the reference list.

Graphical abstract

A Graphical abstract is optional and should summarize the contents of the article in a concise, pictorial

form designed to capture the attention of a wide readership online. Authors must provide images that clearly represent the work described in the article. Graphical abstracts should be submitted as a separate file in the online submission system. Image size: Please provide an image with a minimum of 531 × 1328 pixels (h × w) or proportionally more. The image should be readable at a size of 5 × 13 cm using a regular screen resolution of 96 dpi. Preferred file types: TIFF, EPS, PDF or MS Office files. See http://www.elsevier.com/graphicalabstracts for examples.
Authors can make use of Elsevier's Illustration and Enhancement service to ensure the best presentation of their images also in accordance with all technical requirements: Illustration Service.

Keywords

Immediately after the abstract, authors should list four to five keywords that appropriately represent the contents of their manuscripts.

Abbreviations

Define abbreviations and acronyms when they first appear in the article. Ensure consistency of abbreviations throughout the article.

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,

Math formulae

Present simple formulae in the line of normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article, using superscript Arabic numbers. Many wordprocessors build footnotes into the text, and this feature may be used. Should this not be the case, indicate the position of footnotes in the text and present the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

Table footnotes

Indicate each footnote in a table with a superscript lowercase letter.

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Electronic artwork
General points
• Make sure you use uniform lettering and sizing of your original artwork.
• Embed the used fonts if the application provides that option.
• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
• Number the illustrations according to their sequence in the text.
• Use a logical naming convention for your artwork files.
• Provide captions to illustrations separately.
• Please assure scale bars are present and legible. In SEM images, please remove small automated scale bars and replace with new clear bars.

• Size the illustrations close to the desired dimensions of the printed version.
• Submit each illustration as a separate file.

• Error bars are required on all experimental and calculated data points with an explanation in the text as to how the errors were determined. (See Statistics)

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EPS (or PDF): Vector drawings, embed all used fonts.
TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black & white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of 500 dpi.

Please do not:

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• Supply files that are too low in resolution;

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(for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

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Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

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Number tables consecutively in accordance with their appearance in the text. Place footnotes to tables below the table body and indicate them with superscript lowercase letters. Avoid vertical rules. Be sparing in the use of tables and ensure that the data presented in tables do not duplicate results described elsewhere in the article.

References
All references to other papers, books, etc. must be given at the end of the paper. They should be numbered in sequence starting at the beginning of the paper. The numbers (in brackets) should appear in the text at the appropriate places.

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either "Unpublished results" or "Personal communication". Citation of a reference as "in press" implies that the item has been accepted for publication.

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Archival references are preferred, but if web references must be used, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given.

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Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference management software

This journal has standard templates available in key reference management packages EndNote (http://www.endnote.com/support/enstyles.asp) and Reference Manager (http://refman.com/support/rmstyles.asp). Using plug-ins to word processing packages, authors only need to select the appropriate journal template when preparing their article and the list of references and citations to these will be formatted according to the journal style which is described below.

Reference style

Text
Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

Example: "..... as demonstrated [3,6]. Barnaby and Jones [8] obtained a different result ...."

List

Number the references in the list in the order in which they appear in the text. Please include all authors for citations including seven authors or fewer. For citations with greater than seven authors, cite the first author's name followed by et al. Please include titles of all cited articles as in the following examples.

Examples:

Reference to a journal publication:

1. Jeon SI, Lee JH, Andrade JD, De Gennes PG. Protein-surface interactions in the presence of polyethylene oxide. J Colloid Interface Sci 1991;142:149-158.

Reference to a book:

2. Tjia JS, Moghe PV. "Cell-internalizable" ligand microinterfaces on biomaterials: design of regulatory determinants of cell migration. In: Dillow AK, Lowman AM, Hudgins KA, editors. Biomimetic Materials and Design. New York: Marcel Dekker; 2002. p 335-374.

Journal abbreviations source
Journal names should be abbreviated according to the List of title word abbreviations:

http://www.issn.org/2-22661-LTWA-online.php.

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One author has been designated as the corresponding author with contact details:

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• Full postal address

• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

• Manuscript has been 'spell-checked' and 'grammar-checked'

• References are in the correct format for this journal

• All references mentioned in the Reference list are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Web)

• Color figures are clearly marked as being intended for color reproduction on the Web (free of charge) and in print, or to be reproduced in color on the Web (free of charge) and in black-and-white in print

• If only color on the Web is required, black-and-white versions of the figures are also supplied for printing purposes

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ANEXO B

Journal Dental Research

Instruction to Authors

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Manuscript Submission

All manuscripts must be submitted electronically **through Website:** http://www.journalonweb.com/drj

To submit online please follow on screen instructions and steps to upload different part of your article at the website. If the references have been prepared using Reference Manager software or similar programs, please ensure to remove the reference manager links from the file before submission. Otherwise these references may not be visible under certain platforms. All tables must be present in the main submission file and they may not embedded as graphics. All figures, pictures, graphics or images must be submitted as supplementary files in a format of JPEG, GIF or TIFF which will produce high quality images in the online edition of the journal. Manuscript must be accompanied by a covering letter to the Editor-in-Chief, including title and author(s) name and undertaking that it has not been published or submitted elsewhere.

Manuscript Preparation

Manuscripts must be prepared in accordance with ";Uniform requirements for Manuscripts submitted to Biomedical Journal"; developed by International Committee of Medical Journal Editors (http://www.icmje.com). Authors are advised to write in clear and simple English. Manuscripts must be prepared in two Microsoft Word document file format (*.doc). and a 12 point font:

1- Title page and cover letter file

2- Main file including the Abstract page with Keywords, Text (Introduction, Materials and Methodes, Results, Discussion, Conclusion), Acknowledgements,References, Tables, and Figure legends.

Please do not use sophisticated formatting and page styles, as these lead to some problem in file processing. It should be typed in A4 size (212 × 297 mm) paper, with margins of 25 mm (1 inch) from all the four sides. Use 1.5 spacing throughout. Number pages consecutively, beginning with the title page. Each of the following sections of manuscript should start on a separate page: Title page, Abstract, Text, Acknowledgements, References, Tables, and Figures legends. Indent the first line of each paragraph. Abbreviation, symbols and acronyms must be given in full when first mentioned. Use only standard abbreviations. Avoid using them in the title and abstract. Avoid footnotes in the text.

1. Title page: The title page should contain the following information in the order given: 1) full title of manuscript. 2) Authors' full names. 3) Authors' scientific degree, institutional affiliations including city and country. 4) The name and address of the author responsible for correspondence about the manuscript [including email address and telephone]. 5) The name and address of the author to whom requests for off prints should be sent. 6) Short title that is 45 characters or less as a running title.

2. Abstract page: The abstract should be limited to 250 words. Do not use abbreviation, footnotes, references and authors' names. The abstract page should contain the following: 1) Title of manuscript. 2) A summary of manuscript that is structural for Original Research Article [Background, Materials and Methods, Results, Conclusion].

3) Three to five keywords. Keywords should be placed alphabetical order and adjusted to Medical Subject Headings used in Index Medicus (http://www.nlm.nih.gov/mesh/MBrowser.html)

3. Text Pages: Organize the manuscript into following four main headings:

o Introduction: This should summarize the purpose and the rationale for the study. It should neither review the subject extensively nor should it have data or conclusions of the study.

o Materials and Methods: This should include exact method or observation or experiment. If an apparatus is used, its manufacturer's name and address should be given in parenthesis. If the method is established, give reference but if the method is new, give enough information so that another author is able to perform it. If a drug is used, its generic name, dose and route of administration must be given. For patients, age, sex with mean age ± standard deviation must be given. Statistical method must be mentioned and specify any general computer program used. The Info system used should be clearly mentioned.

o Results: It must be presented in the form of text, tables and illustrations. The contents of the tables should not be all repeated in the text. Instead, a reference to the table number may be given. Long articles may need sub-headings within some sections (especially the Results and Discussion parts) to clarify their contents.

o Discussion: This should emphasize the present findings and the variations or similarities with other work done in the field by other workers. The detailed data should not be repeated in the discussion again. Emphasize the new and important aspects of the study and the conclusions that follow from them. It must be mentioned whether the hypothesis mentioned in the article is true, false or no conclusions can be derived.

o Conclusion

4. Acknowledgement: All contributors who do not meet the criteria for authorship should be covered in the acknowledgement section. It should include persons who provided technical help, writing assistance and departmental head who only provided general support. Financial and material support should also be acknowledged.

5. References: All submitted manuscripts should be in the style of Vancouver Reference System. These should be numbered sequentially as superscripts in order of their appearance in the text and listed in a separate section following the text, double-spaced. List all authors when six or fewer; when seven or more, list the first six and add ";et al";. All published materials, including brief communications and letters to the editor must be cited in the References section. Manuscripts accepted but not yet published can also be included in the references; designate the Journal followed by 'in press' (in parentheses). References to unpublished material, such as personal communications and unpublished data, must be placed within the text and not cited in the references section. Personal communication and unpublished data must include the individual's name, location, and month and year of communication as appropriate. Use Index Medicus abbreviations for Journals that are indexed; if a Journal is not indexed, use full name. A maximum of 40 references is allowed for original articles.

- *Journal:* Motamedi MH, Hashemi HM , Shams MG, Nejad AN. Rehabilitation of war-injured patients with implants: analysis of 442 implants placed during a 6-year period. J Oral Maxillofac Surg 1999; 57(8):907-13.
- *Book:* Torabinejad M, Walton RE. Principels and Practice of Endodontics. 3rd ed. Philadelphia: Saunders, 2002. p. 275-8.
- *Chapter From a Book:* Berman LH, Hartwell GR. Diagnosis. in: Cohen S, Hargreaves KM. Pathways of the pulp. 9th ed. St Louis: Mosby Elsevier, 2006. p. 1-39.
- *E-Journal:* Hasheminia SM, Shojai A. Assessment of canal configuration in maxillary first and second molars in the city of Isfahan, Iran. Inter J Dental Anthropol [Serial Online] 2005; 6:26-32. Available from: http://ijda.syllabapress.com/abstractsijda6.shtml [Cited December 10 2006].
- *Site Reference:* Kuraray Co. Clearfil SE Bond, Technical information. Available at: http://www.kuraray dental.com/downloads.php?type=technical [Cited December 11 2006].

6. Tables: Tables should be presented on separate pages after the references, and numbered in the order in which they are cited in the text. Table headers should be fully descriptive of the contents. Tables should supplement, not duplicate, the text. Use only horizontal rules. Do not submit tables as photograph.

7. Figures: Each figure must be prepared and submitted as JPEG, GIF or TIFF with high resolution. Upon acceptance of the paper the authors must prepare and submit the figures in a high resolution format in accordance with the Pubmed Central preferred image file specification (http://www.pubmedcentral.nih.gov/about/preferred.html) For the details of image file specifications please refer to the Pubmed Central documents (http://www.pub-

medcentral.nih.gov/about/image_quality_table.html). Failure to submit the required image format in time may cause delay in the publication of the accepted papers. Keep wording on figures to a minimum, with explanations written in the figure legends. Legends for figures should be placed at the end of the main submission file. Figure legends should not be part of the figure proper. Line drawings and graphs should be professionally drawn and lettered; freehand or typewritten lettering is unacceptable.

The authors must ensure that before submitting the manuscript for publication, they have taken care of the following:

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2. Abstract in structured format up to 250 words.

3. References mentioned as stated in the Instruction to Authors section.

4. Tables should be typed on separate pages.

6. Make sure for Headings of Tables, their numbers and Captions of illustrations. Don't repeat the information in tables if it is covered in the text.

7. Photographs illustrations with high resolution and along with their captions.

8. Letter of Undertaking signed by all the authors.

9. Disclosure regarding source of funding and conflict of interest if any besides approval of the study from respective Ethics Committee/Institution Review Board.

10. Covering Letter

Anexo C

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 a etiology, 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.

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Fax +44 2071887466
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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).

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All manuscripts are subject to editorial review. Manuscripts are received with the explicit understanding that the data they contain have not previously been published (in any language) and that they are not under simultaneous consideration by any other publication.

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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 should 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.

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

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- 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, for indexing purposes;
- 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 payment to an investigator from organizations with an interest in the study (including employment, consultancies, honoraria, ownership of shares). The fact that a study is conducted on behalf of a commercial body using funds supplied to the investigators' institution by the sponsor does not in itself involve a conflict of interest. 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.

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.

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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.

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 also acknowledge the source of funding for the project. The position(s) of author(s) employed by commercial firms should be included.

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:

- Illustrations should be numbered in Arabic numerals in the sequence of citation. Figure numbers must be clearly indicated on the figures themselves, outside the image area.
- Black and white half-tone illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800-1200 dpi.
- 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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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 is was accessed.

The reference list should include all the publications cited in the text, and only those publications. References, formatted as in the examples below, should be arranged in strict alphabetical order. All authors should be listed. For papers by the same authors, references should be listed according to year. Papers published by the same authors in the same year should be distinguished by the letters a, b, c, ... immediately following the year, in both the text citation and the reference list. For abbreviation of journal names, use the Index Medicus system. For journals, provide only the year, volume number and inclusive page numbers.

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(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.

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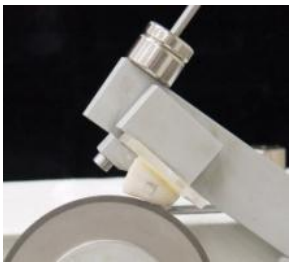
ANEXO D

OBTENÇÃO E PREPARO DOS BLOCOS DE ESMALTE

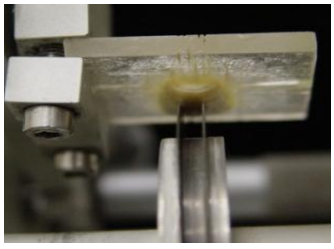
Confecção dos blocos de esmalte bovino (4 mm x 4 mm)

1. Coroa do dente bovino incisivo central inferior, separada da raiz através de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob refrigeração (água destilada/deionizada).

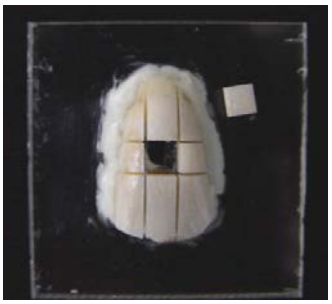


2. Secção da coroa utilizando disco diamantado (série 15 HC Diamond - n. 11-4244 Buehler) separando a superfície vestibular da lingual.

3. Face vestibular fixada na placa de acrílico.

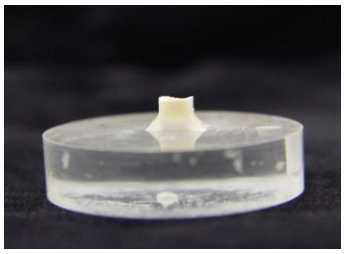


4. Secção da face vestibular no sentido longitudinal, na porção mais plana, utilizando-se 2 discos diamantados (série 15 HC Diamond –n. 11-4243 Buehler), montados em cortadeira sob refrigeração com água destilada/deionizada e separados por um disco espaçador de alumínio com 4 mm de espessura. Em seguida, foi realizado o corte no sentido transversal.



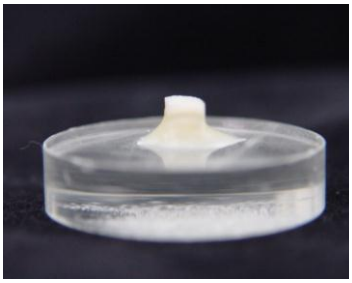
5. Fragmento vestibular do dente bovino, fixado sobre placa de resina. Ao lado, bloco de esmalte dentário.

Planificação da dentina e polimento do esmalte



6. Bloco de esmalte fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 8 mm de espessura), com auxílio de cera pegajosa (Kota Ind. e Com. LTDA), com a superfície dentinária voltada para cima.

7. 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.



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

Seqüência do polimento de esmalte

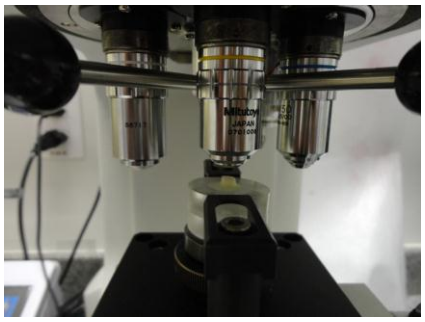
1. Pedra-pomes, água deionizada e taça de borracha montada em contra-ângulo em baixa-rotação.
2. Na Politriz APL-4 AROTEC - lixa de granulação 600, 800 e 1200 (30 segundos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa; Para 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.

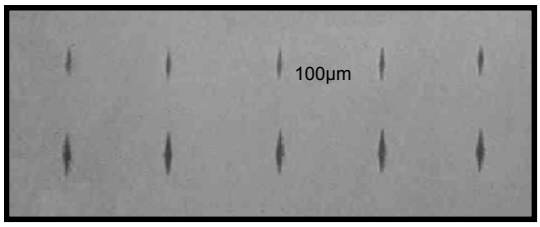
ANEXO E

ANÁLISE DE DUREZA SUPERFICIAL

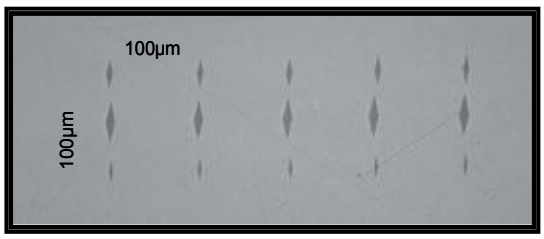
1. Microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, USA e Mitutoyo Corporation, Kanagawa, Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff, USA).

2. Bloco 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.





A



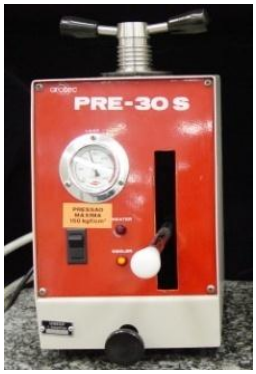
B

3. Fotomicrografia das impressões para análise de dureza de superfície inicial e final (SHi, SHf) (Figura A) (Aumento: 100x) *Capítulo 1 e 3.*

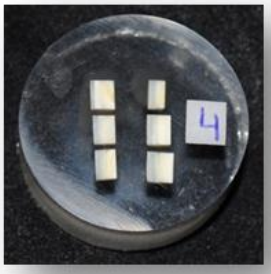
Fotomicrografia das impressões para análise de dureza de superfície inicial (SH), pós-desmineralização (SH_1) e final (SH_2) (Figura B) (Aumento: 100x) *Capítulo 2.*

ANEXO F

ANÁLISE DA DUREZA EM SECÇÃO LONGITUDINAL (CAPÍTULOS 1, 2 e 3)

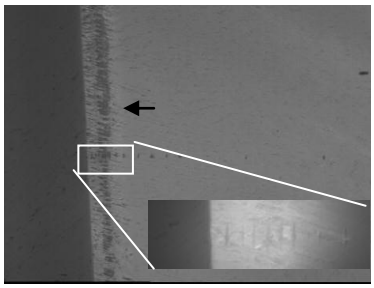


1. Embutidora metalográfica (AROTEC PRE 30S) – utilizada para inclusão dos blocos de esmalte em 5 gramas de resina acrílica (Buehler Transoptic Powder, Lake Bluff, Illinois, USA), pressão de 150 kgf/cm^2, tempo de aquecimento de 7 minutos e mais 7 minutos de resfriamento. Os blocos foram fixados em posição com cola adesiva (Super Bonder – Loctite).



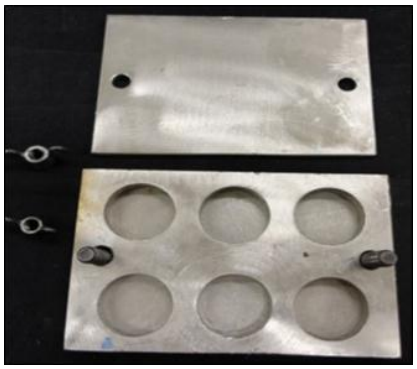
2. Blocos embutidos – plano longitudinal voltado para a superfície da resina acrílica.

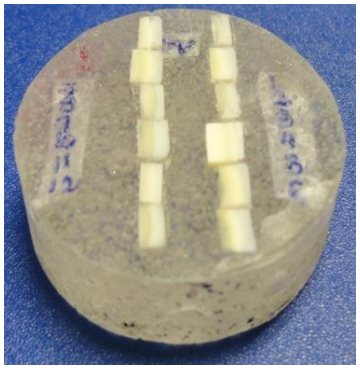
3. Microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, USA e Mitutoyo Corporation, Kanagawa, Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff, USA).



4. Fotomicrografia das impressões. (Aumento: 1000x). Seta: Lesão de subsuperfície.

5. Matriz metálica para embutimento dos blocos submetidos à erosão (Capítulo 3), em resina acrílica e a frio.

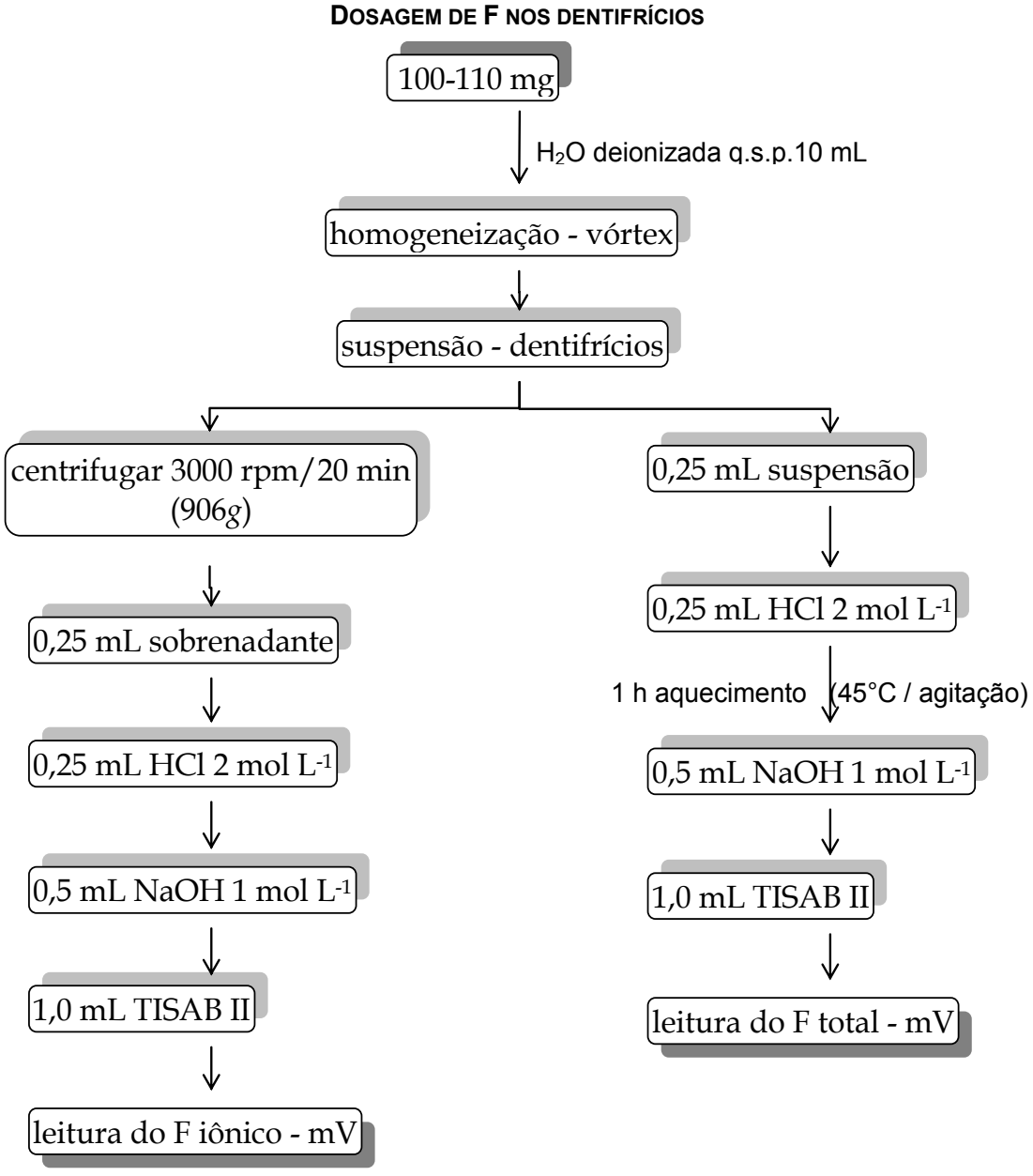




6. Blocos embutidos – plano longitudinal voltado para a superfície da resina acrílica (Capítulo 3), em resina acrílica e a frio.

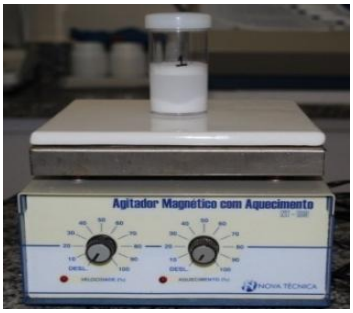
ANEXO G

DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS



ANEXO H

CICLAGEM DE pH (CAPÍTULO 1)



1. Agitador Magnético com Aquecimento TE – 081 (Piracicaba, SP - Brasil) utilizado para agitar o dentifrício com velocidade de 50% durante 15 min.

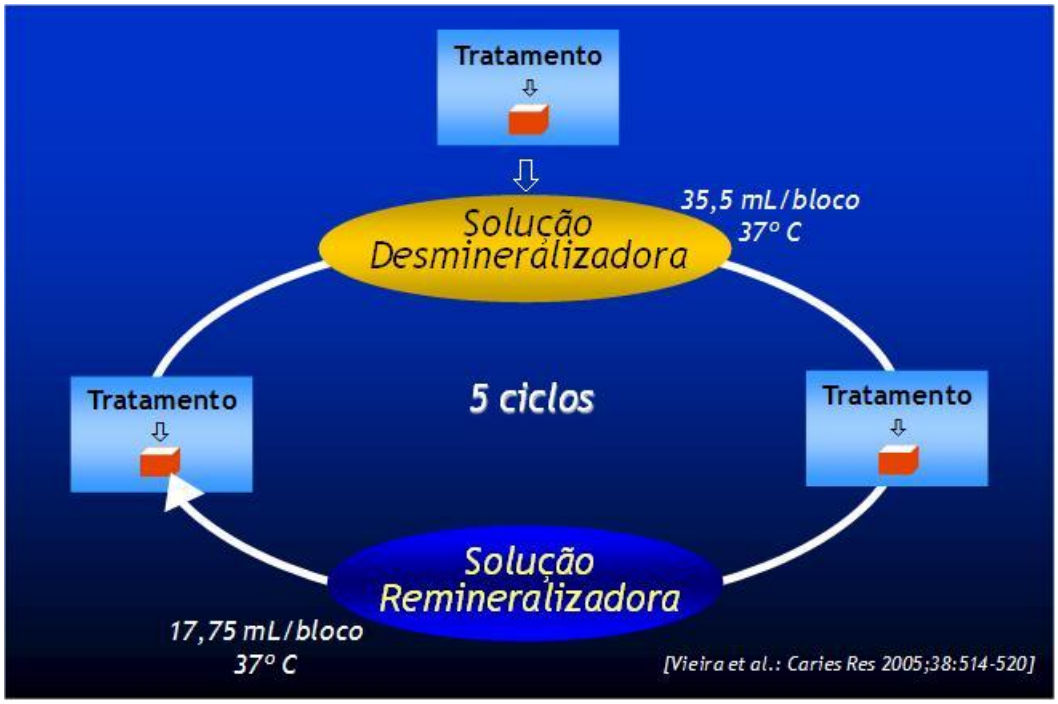
2. Mesa Agitadora TE - 141-Orbital (Tecnal, Piracicaba - SP, Brasil) utilizada para tratamento dos blocos de esmalte, em rotação 7 durante 1 min.

.

3. Lavagem dos blocos de esmalte antes e após os tratamentos, durante 30 s com água deionizada.

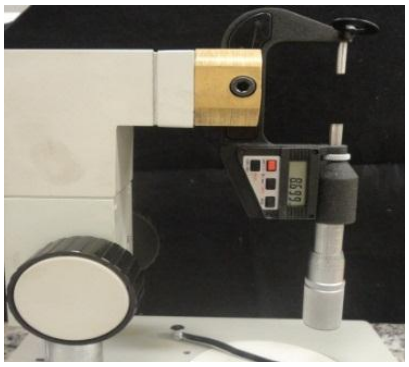
4. Estufa para cultura bacteriológica (Olidef cz Ribeirão Preto – SP, Brasil) utilizada para manter os blocos de esmalte nas soluções de Des e Re em temperatura 37°C, durante o período da ciclagem.

ESQUEMA REPRESENTATIVO DA CICLAGEM DE pH (CAPÍTULO 1)



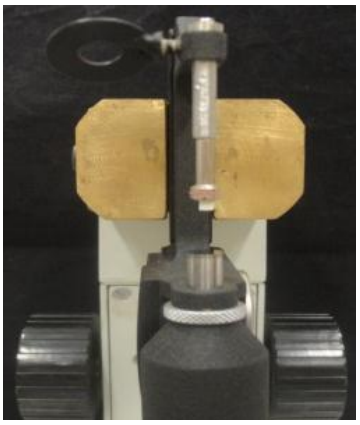
ANEXO I

ANÁLISE DA CONCENTRAÇÃO DE F NO ESMALTE (CAPÍTULOS 1 E 2)



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

2. Bloco 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

COMITÊ DE ÉTICA (Capítulo 2)

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO IN SITU DE DENTIFRÍCIOS FLUORETADOS E SUPLEMENTADOS COM TRIMETAFOSFATO DE SÓDIO NANOPARTICULADO SOBRE A REMINERALIZAÇÃO DENTÁRIA

Pesquisador: Alberto Carlos Botazzo Delbem

Área Temática:

Versão: 2

CAAE: 17888413.1.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Universidade Estadual Paulista Júlio de Mesquita Filho

DADOS DO PARECER

Número do Parecer: 444.027

Data da Relatoria: 23/09/2013

Apresentação do Projeto:

A otimização de dentifrícios fluoretados sobre o processo de remineralização de lesões cariosas é de grande interesse na odontologia, principalmente quando se pensa em populações que apresentam alto risco de desenvolvimento da doença. Para avaliar a capacidade de remineralização de dentifrícios com TMP e diferentes concentrações de fluoreto, blocos de esmalte dental bovino, obtido em frigorífico, submetidos à prévia desmineralização, serão fixados a dispositivos palatinos que serão utilizados por 12 voluntários, previamente selecionados. As formulações testadas serão: 1) Placebo (sem F/TMP), 2) 1100 ppm F (F), 3)1100 ppm F + 3% de TMP (1100 3%TMP) e 4) 1100 ppm F + 3% de TMPn (1100 3%TMPn). Após três dias de uso dos dentifrícios, considerado como período de remineralização, os quatro blocos serão removidos do dispositivo para análise da dureza de superfície final (SH2) e em secção longitudinal para o cálculo da perda integrada de dureza de subsuperfície (delta KHN), e da concentração de F, presente no esmalte. Para

análise estatística, serão considerados como variáveis os valores de SH, SH1,SH2 delta KHN e o conteúdo de F no esmalte e, como fator de variação, os dentifrícios experimentais.O estudo será cego e cruzado consistindo em quatro fases com duração de 3 dias cada e washout de 7 dias entre uma etapa e outra, para eliminar possíveis efeitos residuais dos tratamentos.

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Bairro: VILA MENDONCA **CEP:** 16.015-050
UF: SP **Município:** ARACATUBA
Telefone: (18)3636-3200 **Fax:** (18)3636-3332 **E-mail:** anacmsn@foa.unesp.br

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Continuação do Parecer: 444.027

Objetivo da Pesquisa:

Avaliar in situ o efeito de dentifrícios fluoretados, suplementados ou não com TMP nanoparticulado, sobre a remineralização dentária.

Avaliação dos Riscos e Benefícios:

Durante o experimento 12 voluntários utilizarão dispositivos palatinos na arcada superior contendo 4 blocos desmineralizados por um período de 3 dias e washout de 7 dias entre os tratamentos. Poderá ocorrer desconforto durante o uso do dispositivo, facilmente sanável pela retirada do mesmo pelo voluntário. As concentrações de fluoreto e as formulações utilizadas não apresentam risco. Os riscos são assim considerados mínimos.

Comentários e Considerações sobre a Pesquisa:

O estudo não resultará em benefícios diretos aos participantes, porém é de extrema relevância para a adoção de medidas para a prevenção e tratamento de uma doença vista como um problema de saúde pública, o que justifica sua realização.

Considerações sobre os Termos de apresentação obrigatória:

Os termos de apresentação obrigatória foram apresentados e estão corretamente preenchidos.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O estudo tem por objetivo avaliar in situ o efeito de dentifrícios fluoretados, suplementados ou não com TMP nanoparticulado, sobre a remineralização dentária.Para avaliar a capacidade de remineralização de dentifrícios com TMP e diferentes concentrações de fluoreto, blocos de esmalte dental bovino, obtido em frigorífico, submetidos à prévia desmineralização, serão fixados a dispositivos palatinos que serão utilizados por 12 voluntários, previamente selecionados. As formulações testadas serão: 1) Placebo (sem F/TMP), 2) 1100 ppm F (F), 3)1100 ppm F + 3% de TMP (1100 3%TMP) e 4) 1100 ppm F + 3% de TMPn (1100 3%TMPn). Após três dias de uso dos

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FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Continuação do Parecer: 444.027

dentifrícios, considerado como período de remineralização, os quatro blocos serão removidos do dispositivo para análise da dureza de superfície final (SH2) e em secção longitudinal para o cálculo da perda integrada de dureza de subsuperfície (delta KHN), e da concentração de F, presente no esmalte. O estudo será cego e cruzado consistindo em quatro fases com duração de 3 dias cada e washout de 7 dias entre uma etapa e outra, para eliminar possíveis efeitos residuais dos tratamentos. Os riscos para o voluntários são considerados mínimos e estão restritos a leve e transitório desconforto durante a utilização do dispositivo palatino. As concentrações de fluoreto que serão utilizadas estão embasadas na literatura. Não foram declarados procedimentos que possam ferir a dignidade dos voluntários. O pesquisador responsável apresenta vasta experiência na área de estudo. Sendo de levado interesse público a otimização de dentifrícios fluoretados, principalmente quando se pensa em populações que apresentam alto risco de desenvolvimento da doença e frente a inexistência de procedimentos que não atendam a resolução 466/2012, somos favoráveis a aprovação do projeto. Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/05/2014.

ARACATUBA, 01 de Novembro de 2013

Assinador por:
Ana Claudia de Melo Stevanato Nakamune
(Coordenador)

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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 (Marcelle Danelon) pelo telefone : 3636-3235 (Odontopediatria) ou (18) 8114-7992.

Obrigada pela colaboração.

ANEXO L

DISPOSITIVO PALATINO

1. Kit fornecido ao voluntário a cada período experimental.

ANEXO M

COMITÊ DE ÉTICA (CAPÍTULO 3)

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: ESTUDO IN VITRO DE DENTIFRÍCIOS FLUORETADOS E SUPLEMENTADOS COM TRIMETAFOSFATO DE SÓDIO NANOPARTICULADO SOBRE A EROSÃO

Pesquisador: Alberto Carlos Botazzo Delbem

Área Temática:

Versão: 1

CAAE: 15018213.8.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Faculdade de Odontologia do Campus de Araçatuba - UNESP

DADOS DO PARECER

Número do Parecer: 285.149

Data da Relatoria: 17/05/2013

Apresentação do Projeto:

O projeto foi muito bem elaborado e perfeitamente estruturado para ser realizado adequadamente.

Objetivo da Pesquisa:

Comparar a eficácia do dentifrício 1100 ppm F associado á 3% TMP com o dentifrício 5000 ppm F, na erosão dentária, através da dureza de superfície e desgaste (perfilometria). Visando verificar a melhor eficácia dos dentifrícios suplementados com TMP quando comparado ao dentifrício 5000 ppm F.

Avaliação dos Riscos e Benefícios:

Riscos: Mínimo

Benefícios: Caso comprove-se que o dentifrício 1100 ppm F associado à 3% de TMP nanoparticulado apresente melhores resultados em inibir a erosão dentária, quando comparado ao dentifrício 5000 ppm F, este poderá ser utilizado também por crianças de tenra idade minimizado os riscos de intoxicação crônica.

Comentários e Considerações sobre a Pesquisa:

A realização desta pesquisa será de grande valhia para a odontologia, pois visa a pesquisa de uma produto que contribuirá para a redução da erosão dentária.

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FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Continuação do Parecer: 285.149

Considerações sobre os Termos de apresentação obrigatória:

Os Termos de apresentação obrigatória estão adequadamente apresentados.

Recomendações:

Nada a recomendar.

Conclusões ou Pendências e Lista de Inadequações:

O projeto está muito bem estruturado e perfeitamente exequível.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

A utilização de um produto com maior potencial para reduzir a erosão dentária representará um importante passo para a odontologia.

ARACATUBA, 27 de Maio de 2013

Assinador por:
Ana Claudia de Melo Stevanato Nakamune
(Coordenador)

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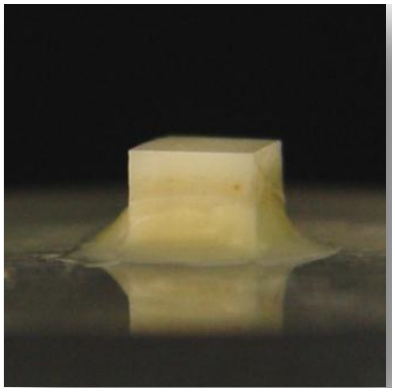
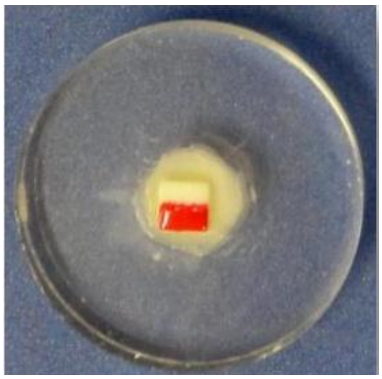
ANEXO N

PROTEÇÃO DOS BLOCOS DE ESMALTE PARA ATAQUE EROSIVO E TRATAMENTO COM OS RESPECTIVOS DENTIFRÍCOS EXPERIMENTAIS (CAPÍTULO 3)



1. Seleção prévia dos blocos realizadas através da dureza e observação a olho nu da superfície de cada bloco.



2. Esmalte cosmético foi utilizado para proteger uma área dos fragmentos necessária para realização dos testes de perfilometria. Uma fita adesiva (Scotch, 3M do Brasil Ltda, Sumaré, SP) foi posicionada ao meio da superfície do esmalte dentário, promovendo a proteção de uma porção e expondo a área a ser pintada.

ANEXO O

DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 1)

Tabela 1. Valores de fluoreto (FI) e (FT) (média ± dp, *n* = 2) nos dentifrícios experimentais

Dentifrícios	FI (ppm F)	FT (ppm F)
Placebo	9,7 ± 0,4 (2)	9,5 ± 1,0 (2)
1100 ppm F	1102,9 ± 4,4 (2)	1106,5 ± 8,8 (2)
1100 1%TMP	1189,0 ± 12,1 (2)	1179,8 ± 22,0 (2)
1100 1%TMPnano	1127,4 ± 18,6 (2)	1112,5 ± 10,8 (2)
1100 3%TMP	1110,3 ± 17,6 (2)	1112,6 ± 13,2 (2)
1100 3%TMPnano	1135,3 ± 4,8 (2)	1103,8 ± 5,1 (2)
1100 6%TMP	1111,0 ± 18,6 (2)	1162,0 ± 6,4 (2)
1100 6%TMPnano	1052,6 ± 14,7 (2)	1107,0 ± 27,1 (2)

DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 2)

Tabela 1. Valores de fluoreto (FI) e (FT) (média ± dp, *n* = 2) nos dentifrícios experimentais

Dentifrícios	FI (ppm F)	FT (ppm F)
Placebo	9,7 ± 0,41 (2)	9,5 ± 1,06 (2)
1100 ppm F	1102,9 ± 4,49 (2)	1106,5 ± 8,82 (2)
1100 TMP	1110,3 ± 17,66 (2)	1112,6 ± 13,22 (2)
1100 TMPnano	1135,3 ± 4,87 (2)	1103,8 ± 5,15 (2)

DOSAGEM DE FLUORETO NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 3)

Tabela 1. Valores de fluoreto (FI) e (FT) (média ± dp, *n* = 2) nos dentifrícios experimentais

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1100 ppm F	1102,9 ± 4,49 (2)	1106,5 ± 8,82 (2)
1100 TMP	1110,3 ± 17,66 (2)	1112,6 ± 13,22 (2)
1100 TMPnano	1135,3 ± 4,87 (2)	1103,8 ± 5,15 (2)
5000 ppm F	5028,8 ± (24,76) (2)	5387,0 ± (26,27) (2)

ANEXO P

DETERMINAÇÃO DO pH NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 1)

Tabela 2. Valores de pH (média ± dp, *n* = 2) nos dentifrícios experimentais

Dentifrícios	pH
Placebo	7,5 ± 0,1 (2)
1100 ppm F	7,7 ± 0,1 (2)
1100 1%TMP	7,6 ± 0,2 (2)
1100 1%TMPnano	7,3 ± 0,3 (2)
1100 3%TMP	7,4 ± 0,1 (2)
1100 3%TMPnano	7,1 ± 0,3 (2)
1100 6%TMP	7,5 ± 0,2 (2)

DETERMINAÇÃO DO pH NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 2)

Tabela 2. Valores de pH (média ± dp, *n* = 2) nos dentifrícios experimentais

Dentifrícios	pH
Placebo	7,5 ± 0,1 (2)
1100 ppm F	7,7 ± 0,1 (2)
1100 TMP	7,4 ± 0,1 (2)
1100 TMPnano	7,1 ± 0,3 (2)

DETERMINAÇÃO DO pH NOS DENTIFRÍCIOS EXPERIMENTAIS

(CAPÍTULO 3)

Tabela 2. Valores de pH (média ± dp, *n* = 2) nos dentifrícios experimentais

Dentifrícios	pH
Placebo	7,5 ± 0,1 (2)
1100 ppm F	7,7 ± 0,1 (2)
1100 TMP	7,4 ± 0,1 (2)
1100 TMPnano	7,1 ± 0,3 (2)
5000 ppm F	7,5 ± 0,2 (2)

ANEXO Q

REFERÊNCIAS INTRODUÇÃO GERAL

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