Eliana Mitsue Takeshita

AVALIAÇÃO DA AÇÃO DO TRIMETAFOSFATO SOBRE O BIOFILME DENTÁRIO, DESMINERALIZAÇÃO E REMINERALIZAÇÃO DO ESMALTE: ESTUDOS *IN VITRO* E *IN SITU.*

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Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para obtenção de título de Doutorado em Odontopediatria.

Orientador: Alberto Carlos Botazzo Delbem
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...Isso é ter tido sucesso".

Ralph Waldo Emerson

Epígrafe

Resumo

Takeshita EM. Avaliação da ação do trimetafosfato sobre o biolfime dentário, desmineralização e remineralização do esmalte: estudos in vitro e in situ [tese]. Araçatuba: Universidade Estadual Paulista; 2010.

O uso de dentifrícios fluoretados por crianças menores de seis anos de idade é um fator de risco para a fluorose dentária. Uma maneira de diminuir a ingestão de fluoreto (F) seria reduzir a concentração desse íon; entretanto, é necessário manter a mesma efetividade de um dentifrício padrão (1100 µgF/g). Alguns estudos têm mostrado que dentifrícios suplementados com fosfato e cálcio são mais efetivos em prevenir a desmineralização e melhor a remineralização. O objetivo do presente estudo foi avaliar o efeito do trimetafosfato de sódio (TMP) associado à baixa concentração de F sobre o biofilme, a desmineralização e remineralização do esmalte através de modelos de estudo *in vitro* e *in situ*. Para avaliação *in vitro* sobre des-remineralização do esmalte foi utilizado o modelo de ciclagem de pH. Blocos de esmalte (n=35) com lesão de cárie artificial foram submetidos a 15 dias de ciclagem de pH com 6 ciclos diários de 0,5 h em solução desmineralizadora e 2,5 h em solução remineralizadora. O tratamento foi realizado durante 5 min com soluções diluídas de 0, 500, 1500 ou 3000 µg F/g com ou sem a adição de 3% TMP. Após a ciclagem de pH os blocos de esmalte foram analisados através da microrradiografia (TMR) que mostrou a formação de lesão secundária nos grupos com baixa concentração de F. Essa lesão, entretanto, não foi observada nos grupos associados ao TMP. Para avaliação *in vitro* sobre o biofilme foi utilizado um novo modelo que possibilita a formação de biofilme de multi e simples espécies. Com essa finalidade foram testados diferentes substratos, vidro e esmalte de dente bovino e os tratamentos realizados com soluções de TMP associado ou não a antimicrobianos. Os resultados mostraram que em ambos os biofilmes de multi- e simples espécie, o TMP não altera a produção de ácido lático nem a contagem de unidades formadoras de colônia. Para avaliação *in situ* sobre a des-remineralização do esmate foram utilizados dois modelos de estudo. Para a avaliação sobre a desmineralização do esmalte *in situ*, 10 voluntários, utilizando dispositivo palatino com 4 blocos de esmalte bovinos selecionados através da dureza de superfície, foram submetidos a 4 fases experimentais de 14 dias cada. Os blocos de esmalte (n=160) foram alocados em 4 grupos de tratamento: placebo (controle negativo), dentifrício com 500 µg F/g,

dentifrício com 500 µg F/g e 1% TMP e um dentifrício com 1100 µg F/g (controle positivo). Durante o período experimental foram aplicados sobre os blocos: tratamento com dentifrício 2x/dia e uma solução de sacarose 20% 6x/dia. Após cada etapa, foi realizada a coleta do biofilme para análise da concentração de F, cálcio (Ca), fósforo (P) e polissacarídeos insolúveis extracelulares (EPS). Em cada bloco de esmalte, foram determinadas a alteração de dureza de superfície (%SH), perda integrada de dureza de subsuperfície (ΔKHN), e concentração de F, Ca e P. Os resultados mostraram que o dentifrício com F suplementado com 1% TMP apresentou a menor %SH ($p < 0,05$). Quanto à F e Ca no esmalte e biofilme não houve diferença entre dentifrício com 500 µg F/g e 1% TMP e controle positivo ($p > 0,05$), mas foram diferentes quando comparados ao dentifrício com 500 µg F/g e ao placebo ($p < 0,05$). Para avaliação sobre a remineralização do esmalte *in situ*, 11 voluntários, utilizando dispositivos palatinos com 4 blocos submetidos à lesão de cárie artificial, foram submetidos a 4 fases experimentais com duração de 3 dias cada. Os tratamentos foram realizados *in vivo* com os dentifrícios: placebo (controle negativo), 500 µg F/g, 500 µg F/g e 1% de TMP, e 1100 µg F/g (controle positivo); 3x ao dia por 1 minuto. Após cada período experimental, foi determinada a porcentagem de recuperação de dureza de superfície ($\%SH_R$), ΔKHN e as concentrações de F, Ca e P no esmalte. Os resultados mostraram que os grupos controle positivo e 500 µg F/g 1% de TMP apresentaram similar %SHR e ΔKHN ($p>0,05$), porém diferentes dos grupos placebo e 500 µg F/g ($p<0,05$). A concentração de F e P no esmalte foi semelhante nos grupos controle positivo e 500 µg F/g e 1% de TMP ($p<0,05$). A concentração de Ca foi maior no grupo controle positivo ($p<0,05$) seguido do grupo 500 µg F e 1% TMP. Com base nos resultados obtidos e considerando-se as limitações dos modelos utilizados, pode-se concluir que o TMP quando adicionado a um dentifrício de 500 µg F/g produz um efeito anticariogênico semelhante ao de um dentifrício de 1100 µg F/ g, impede a formação da lesão secundária e que o TMP não apresenta atividade antimicrobiana.

Abstract

Takeshita EM. Evaluation of sodium trimataphosphate (TMP) effect on biolfim and enamel des- and remineralization: *in vitro* and *in situ* study. [thesis]. Araçatuba: São Paulo State University; 2010.

The use of fluoridated dentifrices by children under 6 years old is a risk factor to dental fluorosis development. One way to reduce fluoride (F) ingestion would be the reduction of this ion concentration in dentifrices; however, it is necessary to maintain similar effectiveness to that of a standard dentifrice (1100 µgF/g). Some studies have demonstrated that dentifrices supplemented with phosphate and/or calcium are more effective in reduce the demineralization and increase the remineralization. The aim of this study was to evaluate the effect of TMP combined to low F concentration on enamel de- and remineralization and biofilm using *in vitro* and *in situ* models. To evaluate *in vitro* de- and remineralization, a pH cycling model was used. Bovine enamel specimens were submitted to 15 days of pH-cycling with a daily schedule of 6 cycles, each 0.5 h demineralization and 2.5 h remineralization. The treatment was performed for 5 min in aliquots of 30wt% dilutions of 0, 500, 1500 or 3000 µg F/g with or without the addition of 3% TMP. After the pH-cycling period the enamel specimens were analyzed by microradiography (TMR). The results showed that the TMR data showed that the formation of a second lesion in the low F groups. This phenomenon was not found in the TMP groups. To evaluate *in vitro* TMP effect on microcosm and single species biofilm, a new *in vitro* model was used. The experiments were carried out using different substratum, glass and enamel, and treatment was performed with antimicrobials with or without TMP. The results showed that in both microcosm and single species biofilm TMP did not alter lactic acid production and CFU counts. To evaluate *in situ* enamel de- and remineralization two different models were used. In the first model, 10 volunteers wearing acrylic palatal appliance containing four enamel bovine blocks selected through surface hardness, were submitted to 4 phases of 14 days each. The specimens (n=160) were allocated to four treatment groups: placebo, dentifrice with 500 µg F/g, dentifrice with 500 µg F/g TMP 1% and dentifrice with 1100 µg F/g. Dentifrices treatment was performed 2x/day, and 20% sucrose solution was applied 6x/day. After each experimental period, the biofilm was collected for F, calcium (Ca), phosphorus (P) and insoluble extracellular polysaccharides (EPS) analysis. In each enamel block, the percentage change of

surface hardness (%SH), integrated loss of subsurface area (ΔKHN), Ca, F and P content were determined. The results showed that the dentifrice with 500 F 1% TMP showed the lowest percentage change of surface hardness ($p<0.05$). Regarding F and Ca in enamel and biofilm there were no differences between dentifrice with 500 TMP 1% and positive control ($p>0.05$), but they were different when compared to dentifrice with 500F ($p<0.05$). In the second model, eleven volunteers, wearing palatal appliance containing four bovine enamel blocks with artificial caries lesion, were subjected to 4 experimental phases during 3 days each. The treatment was carried out, *in vivo*, 3x/day during 1 min with dentifrices: placebo (negative control), 500F, 500F and TMP 1%, and 1100µgF/g (positive control). After each phase, the percentage of surface hardness recovery ($\%SH_R$), ΔKHN, F, Ca and P content in enamel was determined. The results showed that the positive control and dentifrice with 500F and TMP 1% showed similar $\%SH_R$ and ΔKHN ($p>0.05$), but they were different when compared to placebo and dentifrice with low-F ($p<0.05$). Regarding F and P in enamel there were no differences between dentifrice with low-F and TMP 1% and positive control ($p>0.05$). Ca content in enamel was higher in positive control ($p<0.05$), followed by dentifrice 500F and TMP 1%. Based on this results and considering the limitations of the models used, we concluded that the addition of TMP to dentifrice with 500 µg F/ g allowed similar anticariogenic effect to that of a standard dentifrice (1100 µg F/g), and prevent the second lesion formation and TMP showed no antibacterial effect on microcosm and single species biofilm.

Listas

LISTA DE ABREVIATURAS

% =	Porcentagem
%SH =	Porcentagem de alteração dureza de superfície
$\%SH_R$ =	Porcentagem de recuperação de dureza de superfície
^oC =	Graus Celsius
µ =	Micro
µg =	Micrograma
µg F/g =	Micrograma de fluoreto por grama
µg /mm^3 =	Micrograma por milímetro cúbico
µL/g =	Microlitro por grama
µm =	Micrômetro
ADA =	American Dental Association
Al_2O_3 =	Óxido de alumínio
BHI =	Brain Heart Infusion
Ca =	Cálcio
CaF_2 =	Fluoreto de cálcio
$CaCl_2$	Cloreto de cálcio
CFU =	Unidades formadoras de colônia
CHX =	Clorexidina
CPC =	Cloreto de cetilpiridínio
CPW =	Cysteine peptone water
CO_2 =	Dióxido de carbono
ΔKHN =	Perda integrada da dureza de subsuperfície
EPS	Polissacarídeos insolúveis extracelulares
F =	Fluoreto
FI =	Flúor iônico
FT =	Flúor total
g =	Aceleração da gravidade
h =	Hora
H_2 =	Hidrogênio
HCl =	Ácido clorídrico
H_2O =	Água

IML = Integrated Mineral Loss
KCl = Cloreto de potássio
KH_2PO_4 = Fosfato dihidrogenado de potássio
KHN = Unidade de dureza Knoop
mL = Mililitros
mL/mm^3 = Mililitros por milímetro cúbico
mm = Milímetros
mm^2 = Milímetros quadrados
Mol/L = Molaridade
n = Número da amostra
N_2 = Nitrogênio
NaCl = Cloreto de sódio
NaF = Fluoreto de Sódio
NaOH = Hidróxido de Sódio
OH = Hidroxila
P = Fósforo
Pi = Fosfato inorgânico
PBW = Buffered peptone water
pH = Potencial hidrogeniônico
pK = Potencial de ionização
ppm = Partes por milhão
s = Segundos
SD = Standard deviation
SH = Dureza de superfície inicial
SH_1 = Dureza de superfície pós-cárie
SH_2 = Dureza de superfície final
TISAB = Tampão de ajustador de força iônica total
TMP = Trimetafosfato de Sódio
TMR = Microrradiografia transversal
TSA = Tryptic (Trypticase) Soy Agar
W = Watts

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Sumário

SUMÁRIO

Introdução Geral

INTRODUÇÃO GERAL

A busca de meios para reduzir a cárie dentária tem sido um dos principais objetivos neste último século. A introdução do fluoreto (F) na década de 50, como principal agente de prevenção, causou uma redução em sua incidência. O sucesso obtido pela utilização do F levou sua adição não só na água de abastecimento público, como também em vários produtos de higiene bucal. O resultado acumulativo do uso excessivo levou ao aumento na incidência de fluorose dentária (Mascarenhas, 2000).

A utilização de dentifrícios fluoretados foi considerada uma das principais causas do aumento da fluorose dentária (Pendrys e Stamm, 1990; Mascarenhas, 2000). Observou-se em um estudo com crianças que viviam em áreas com água não fluoretada, mas que tinham acesso a dentifrícios fluoretados antes de 2 anos de idade, apresentavam fluorose dentária, sugerindo que o uso de dentifrícios antes dessa idade é um fator de significância para o desenvolvimento da fluorose dentária (Lalumandier, 1992). A recomendação do uso de dentifrícios fluoretados por crianças com menos de 6 anos de idade promove alto risco, pois nessa faixa etária o reflexo de expectoração ainda está em desenvolvimento, resultando em maior ingestão de dentifrício durante a escovação. Crianças com idade entre 1-3 anos ingerem entre 30-75% do dentifrício durante a escovação e crianças entre 4-7 anos, 14-48% (Warren e Levy, 1999). Apesar das propostas em diminuir a incidência de fluorose dentária através da escovação supervisionada, uso de quantidades reduzidas de dentifrício e redução do orifício da embalagem, a concentração de F acrescentada à maior parte dos dentifrícios comerciais infantis disponíveis no mercado é de 1100 µg F/g, semelhante aos dentifrícios utilizados por adultos. Embora se saiba que quanto maior a concentração de F, maior a sua efetividade, a utilização de altas concentrações de F não elimina o aparecimento de cárie dentária na população (Roberts, 1995).

Portanto, umas das maneiras para reduzir a ingestão de F pelas crianças seria a utilização de dentifrícios com menores concentrações desse íon (Horowitz, 1992, Tanever et al., 2006), porém dentifrícios com baixa concentração de F têm demonstrado ser menos efetivos que aqueles contendo 1000 µg F/g (Koch et al., 1982, Ammari et al., 2003). Uma das formas para aumentar sua efetividade seria a adição de agentes alternativos que proporcionem uma ação anticariogênica adicional

(Roberts, 1995; Zero, 2006). Entretanto, a combinação de F e outros suplementos deve ser realizada de maneira ponderada para evitar que o F torne-se indisponível.

A suplementação com sais de cálcio (Ca) e fosfato tem sido uma das alternativas para aumentar a efetividade do F. Embora o processo da remineralização seja intensificado pela presença do F, depende primariamente da disponibilidade desses íons na saliva (Schemehorn et al., 1999a,b). A presença desses íons no ambiente bucal durante o desafio cariogênico proporciona uma diminuição no processo de desmineralização e um aumento no processo de remineralização do esmalte dentário (ten Cate et al., 2000). O processo de remineralização pode ocorrer de duas maneiras: através da precipitação de fosfatos de cálcio ou pelo crescimento dos cristais de esmalte remanescentes através do Ca e P presentes na saliva (Ingram e Edgar, 1994). Entretanto, a concentração desses íons é limitada. A utilização de dentifrícios suplementados poderia aumentar a disponibilidade desses íons na saliva e ao mesmo tempo proporcionar reserva residual no biofilme, fluido da placa e no esmalte dentário, a partir da qual o F pode ser incorporado aos cristais minerais do esmalte ou se depositar como CaF_2. A concentração desses íons, F, Ca e fosfato no biofilme bacteriano tem apresentado uma relação inversa na incidência de cárie (Shaw et al., 1983). Esses íons são liberados no fluido da placa causando uma redução na desmineralização e aumento da remineralização pela supersaturação em relação ao esmalte dentário (Tanaka & Margolis, 1999). A suplementação tem a função de suprir F como agente ativo e/ou cálcio e fosfato.

A associação de fosfatos à redução da cárie dentária teve início na década de 60 (Nizel e Harris, 1964; Harris et al., 1967; Henry and Navia, 1969; Gonzalez, 1971; Larson et al., 1972; Gonzalez et al., 1973; Städtler et al., 1996), quando foram adicionados em dietas (Harris et al., 1967; Henry & Navia, 1969; Larson et al., 1972), gomas de mascar (Finn et al., 1978, Vogel et al., 2000) e mais tarde em dentifrícios (Ständtler et al., 1996; O´Mullane et al., 1997; Takeshita et al. 2009). Dentre os vários sais de fosfato, o trimetafosfato de sódio (TMP) apresentou maior ação anticariogênica (Harris et al., 1967). Entretanto, seu mecanismo de ação ainda não está bem esclarecido (Roberts, 1995). Estudos sugerem que a ação do TMP pode estar relacionada com uma ação local (Harris et al., 1967, Henry and Navia, 1969), sendo adsorvido à superfície do esmalte (Gonzalez, 1971; Larson et al., 1972;

Gonzalez et al., 1973), causando uma redução na solubilidade da hidroxiapatita (Mcgaughey and Stowell, 1977) e reduzindo as trocas minerais entre o meio e o esmalte (Gonzalez, 1971; Gonzalez et al., 1973). Além disso, a ação local do TMP poderia influenciar a colonização dos microorganismos no biofilme (Henry and Navia, 1969) e a adesão de proteínas salivares (Briscoe and Pruitt, 1975). Um estudo clínico demonstrou uma alteração no biofilme dentário exposto ao TMP, ocorrendo uma diminuição dos *Streptococci* e aumento de *Veillonella* (Dennis et al., 1976). Por outro lado, outro estudo clínico observou que o TMP não altera a quantidade de biofilme formado nem sua composição microbiológica (Finn et al., 1978). Portanto, ainda há discussões controversas sobre o mecanismo de ação do TMP tanto sobre o esmalte como sobre o biofilme dentário.

A suplementação com TMP em dentifrícios teve início na década de 90. Trabalhos clínicos utilizando dentifrícios com 3% TMP não associado ao F mostraram uma ligeira redução nos incrementos de cárie quando comparados a um dentifrício placebo (Ständtler, 1996). Outro, porém, mostrou que a adição de 3% TMP a um dentifrício de 1500 µg F/g não mostrou diferenças significantes quando comparado ao mesmo dentifrício sem a suplementação (O´Mullane et al., 1997), indicando que na presença de altas concentrações de F a suplementação de TMP não traz um efeito adicional na ação anticariogênica. Entretanto, Takeshita et al. (2009) demonstraram, *in vitro*, que a suplementação com concentrações acima de 0,25% TMP em um dentifrício de reduzida concentração de F (500 µg/g) mostrou uma ação semelhante a um dentifrício padrão (1100 µg/g). A partir disso, levantou-se a hipótese pela qual é possível aumentar a ação anticariogênica de dentifrícios de baixa concentração de F através da suplementação com TMP.

A combinação de TMP e F no mesmo dentifrício tem demonstrado ser estável, entretanto, para atestar sua eficácia bem como validar novas formulações é necessário verificar sua capacidade em inibir a desmineralização e ativar a remineralização do esmalte (ADA, 1985). Um dos modelos *in vitro* utilizados na avaliação desses produtos é a ciclagem de pH que permite simular o desenvolvimento de cárie sob condições controladas (White, 1995). Em estudos *in situ* o modelo mais utilizado é o “teste intra-oral de cariogenicidade” (ITC), capaz de avaliar o efeito anticariogênico de produtos fluoretados com a presença de fatores interferentes como saliva e placa bacteriana (Zero, 1995). A análise de alguns

parâmetros como: incorporação de F pelo esmalte, progressão da lesão, desmineralização e remineralização do esmalte pode indicar a eficácia desses produtos (Faller, 1995). Ao contrário de estudos clínicos que necessitam de grande número amostral, uma das maiores vantagens dos modelos de estudo *in situ* é a utilização de pequeno número de voluntários (Featherstone e Zero, 1992).

A partir da utilização de diferentes modelos de estudo, tanto *in vitro* como *in situ*, o objetivo do presente trabalho foi avaliar a ação do TMP sobre o processo des-remineralização do esmalte, e sobre o biofilme formado *in vitro* e *in situ*, em associação ou não ao F, na tentativa de se obter mais informações sobre o mecanismo de ação em que estão envolvidos. Os trabalhos foram divididos em 4 capítulos, nos quais os capítulos 1 e 2 estão relacionados com os trabalhos *in vitro*, e os capítulos 3 e 4, com os trabalhos *in situ.*

Capítulo 1

2 Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization.*

2.1 ABSTRACT

Objective: The aim of this study was to evaluate the effects of TMP added to different concentrations of fluoride (F) on the de- and remineralization of enamel lesions.

Methods: Bovine enamel specimens were submitted to 15 days of pH-cycling with a daily schedule of 6 cycles, each 0.5 h demineralization and 2.5 h remineralization. The treatment was performed for 5 min in individual 5 mL aliquots of 30wt% dilutions of 0, 500, 1500 or 3000 µg F/g with or without the addition of 3% sodium trimetaphosphate (TMP). After the pH-cycling period the enamel specimens were analyzed by microradiography (TMR).

Results: The TMR data showed the formation of a second lesion (at depths beyond the original lesion) in the low F groups. This phenomenon, which might lead to lesion progression, was not found in the TMP groups, which also showed lower remineralization of the original lesions.

Conclusion: We conclude that TMP affects both de- and remineralization, resulting in different remineralization patterns inside the lesion.

Key words: pH cycling, fluoride, sodium trimetaphosphate, demineralization, remineralization.

*Capítulo escrito de acordo com as instruções do periódico Archives of Oral Biology (ANEXO B)

2.2 INTRODUCTION

The cariostatic effects of phosphate salts have been studied since the 1960s. Questions regarding the mode of action and efficacy of phosphates has led to numerous laboratory, animal and clinical studies comparing different compounds, such as ortho-, meta-, pyro- and poly-phosphates.[1,2,3,4,5,6] Among all these phosphate salts, sodium trimetaphosphate (TMP) came out as the most effective anticaries agent, with an effectivity in rodents similar to fluoride.[2] While many studies were positive, most reports still question the precise mechanism of action of TMP.

Phosphate salts generally act as buffer at values around neutral pH. However, the effectiveness of TMP does not appear to be related to this characteristic: TMP is a ring structure which does not provide hydrogen ions for buffering. One of the proposed hypotheses regards its capacity to improve the stability of hydroxyapatite.[7] Caries results from the selective mineral dissolution by low pK acids at the enamel surface and diffusion of acids through enamel porosities. TMP probably replaces phosphate groups and these stronger adsorbing groups protect the surface against dissolution during the caries attack,[3] as it does at the crystallite level.

Most studies suggest a mode of action for TMP different from fluoride (F), and propose TMP as an alternative or additional agent which might even be synergistic with fluoride. Gonzalez et al.[5] reported that when both agents were present during acid challenge a less soluble fluorapatite mineral was formed.

In spite of many *in vitro* studies demonstrating favorable F plus TMP results, the most recent clinical study involving TMP failed to show an overall additional effect when TMP was added to (1000 or 1500 µg F/g) fluoride toothpastes.[8] Although it was not specifically discussed, this study did show reduced caries increments when TMP was added to a 1000 µg F/g. Similarly, Takeshita et al.[9] reported an additional effect of TMP when it was added to a 500 µg F/g dentifrice, and studied in a pH-cycling study of initially sound enamel. They hypothesized that fluoride used at higher concentrations lead to a maximum in ‘caries’ inhibition, where favorable TMP effects cannot be separated.

Considering the above, this study aimed to further explore the effects of TMP added to a wide range of fluoride concentrations on enamel lesion de- and remineralization, and specifically focusing on the de/remineralization patterns inside the lesion.

2.3 MATERIALS AND METHODS

Experimental Design

Bovine enamel discs (n = 45) with artificial caries lesions were allocated in 8 groups (n=5) after a 3 day pH-cycling regime to determine baseline characteristics of the lesions. The calcium uptake and loss data were used to form groups with similar average de-/remineralization. The enamel specimens were then submitted to 15 days pH-cycling with daily treatments. Calcium was measured in the daily refreshed de- and remineralization solutions. After the pH-cycling period the enamel specimens were analyzed by microradiography and compared to the remaining 5 enamel lesions that were not used during the experimental period (baseline group).

Enamel Specimens and Lesion Formation

Enamel discs (22 mm^2 each) were cut from bovine incisors with a hollow drill and embedded in resin. The enamel specimens were ground flat to remove approximately 200µm surface enamel on 600 grit silicon carbide abrasive paper under running tap water. Lesions were formed in a methylcellulose gel and 0.1 mol/L lactate buffer system at pH 4.6[10,11] for 10 days.

pH-cycling

The experimental period involved 3 days of pre-cycling and 15 days pH-cycling with treatments. It was performed with a daily schedule of 6 cycles, each 0.5h demineralization (1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, and 50 mmol/L acetate, pH 4.6, 3 mL aliquots/specimens) and 2.5h remineralization (1.5 mmol/L $CaCl_2$, 0.9 mmol/L KH_2PO_4, 130 mmol/L KCl, and 20 mmol/L HEPES, pH 7.0, 3 mL aliquots/specimens), followed by a ‘night’ period of 6h remineralization. During the weekends, the specimens were kept in the remineralization solution at room temperature. The solutions were analyzed and refreshed daily.

Treatment Groups

Daily treatments were given after the six-hour remineralization ‘night’ period: Specimens were immersed for 5 min in individual 5 mL aliquots of 30wt% dilutions of 0, 500, 1500 or 3000 µg F/g sodium fluoride (NaF, Merck, Germany) with or without 3% sodium trimetaphosphate (TMP, Sigma-Aldrich Co., USA) solutions in distilled

water. Fluoride contents were confirmed by gas chromatography (data not shown). Thirty percent dilutions were used for treatments, as is done in toothpaste studies. After treatment, the specimens were rinsed (in tap and distilled water) in a standardized way to remove excess treatment solution.

Transverse Microradiography

After the pH-cycling process, two 400µm sections were cut with a water-cooled diamond-coated wire saw (Well type 3242, Ebner, Mannheim, Germany). Sections were ground to 100µm thickness by means of 3µm Al_2O_3 particles (Logitech PM4, Glasgow, Scotland). Sections were microradiographed on high-resolution plates (K1A Photoplates, Microchrome Technology Products, San Jose, CA, USA) with soft (20 kV) X-rays, together with a reference stepwedge (13 steps, 0–300µm aluminum foil). Microradiograms were scanned with dedicated software (TMR software 1.25, Inspektor Research Systems, Amsterdam, The Netherlands), to produce the standard TMR output parameters: Mineral content versus depth profiles and Integrated Mineral Loss (IML).[12]

Statistics

For statistical analysis, GMC software[13] was used and the significance limit was set at 5%. First, the normality and homogeneity of the samples was tested using Cochran test. Calcium data presented normal and homogeneous distribution and were submitted to two-way analysis of variance followed by Tukey's test. IML values were heterogeneous and were subjected to Kruskal-Wallis test, followed by Miller's test.

2.4 RESULTS

The microradiographs can be analyzed by the mineral depth profiles (Figure 1) and when the profiles are cumulated by the Integrated Mineral Loss (IML) values (Table 1). Water treatments resulted in an increase of the lesion depth and a lower mineral content in the lesion body (at a depth around 40µm). Together this resulted in an increase of IML of 676 vol%.µm. The mineral profiles show distinct differences when groups with the same fluoride levels with and without TMP are compared. In the original lesion (0-75µm) mineral content values are higher in the outer part of the

lesion (0-40µm) in the F-only groups, but at greater depth (40-75µm) the mineral content values are higher in the F+TMP groups. A general observation was also that in the F+TMP groups no second lesion was found in the originally sound enamel (beyond 75µm), this again was in contrast to the F only groups. In the low F group the second lesion was more evident than in high F concentrations. The cumulated IML data (Table 1) show that TMP addition to 500 and 1500 µg F/g resulted in significantly higher remineralization ($p<0.05$), while no significant differences were observed for the 3000 µg F/g groups with and without TMP.

2.5 DISCUSSION

The data show that TMP affects the processes of enamel de- and remineralization and also that the mode of action of TMP and fluoride in lesion de-/remineralization are different. As a consequence the remineralization patterns, in the depth of the remineralizing lesion, are different in the presence and absence of TMP.

The overall mineral loss (IML) showed that in presence of TMP the values were lower than in F only groups, mainly in 500 µg F/g group. The microradiographs revealed interesting findings. The mineral profiles are very different between the groups. The condition of pH-cycling with fluoride treatments gave a significant remineralization of the original lesion, but also the formation of a second lesion beyond the original lesion front, as has been observed previously.[14,15] In contrast, in the TMP+F groups no second lesion was found and also the mineral profiles in the former lesion body were different. The TMP+F profiles showed significantly more remineralization close to the original lesion front. This suggests that this depth zone is favorably affected by a treatment involving TMP, as also evidenced by the lack of second lesion. But considering the outer part of the lesion (0-40*µm*) the remineralization was lower in TMP+F groups.

Explaining this, the data support that fluoride and TMP have different mode of action in the dynamics of enamel de- and remineralization. TMP is particularly effective in inhibiting demineralization. However, by binding to the crystallite surface, replacing orthophosphate ions, TMP hampers crystal growth and hence remineralization, similar to pyrophosphate. In this respect the current study with enamel lesions confirms earlier findings on surface enamel by Gonzalez[3]. The different remineralization patterns throughout the lesion can be explained with the

same phenomenon. As TMP inhibits remineralization in the outer parts of the lesion, and considering that ion diffusion is rate limiting in remineralization,[16] there will be a greater calcium flux inside the lesions with enhanced remineralization in the deeper layers of the lesion. This precipitation will more effectively block lesion pores at the lesion front and thereby hinder acid diffusion to the underlying sound enamel (hence second lesion formation).

The pH-cycling approach provides an interesting method to simulate caries development under controlled conditions[17] and mimic de- and remineralization process *in vitro.* In that sense it was originally developed to assess the effects of agents on dental hard tissues[18] and has been used and modified extensively. The pH-cycling method, as used in this study, consists of a microradiographic assessment of the lesions after pH-cycling. In the current study, microradiography was instrumental to disclose how treatments modified the mineralization patterns throughout the lesion and how these were influenced by fluoride and particularly TMP treatments. We conclude that the findings reveal aspect of the working mechanism of TMP that were questioned by numerous authors since the 1960s.

Until today the formation of a second lesion occurring under the same conditions (and simultaneously) when preformed lesions remineralize has been a challenging observation. Although this was first observed in laboratory experiments, it parallels the findings in vivo of lesion progression underneath seemingly sound surfaces in the case of fluoride administration.[19]

Extrapolating our current findings to the clinical situation we conclude that the use of TMP could, under optimal conditions, prevent such undesirable lesion progression.

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Table

Table 1 – Integrated Mineral Loss (IML) values calculated from the mineral content depth profiles.

	IML (vol%.µm)
Baseline	3610 ± 257 [a]
Water- negative control	4286 ± 342 [a,b]
3% TMP	3397 ± 152 [b,c]
500ppm F	3035 ± 247 [c]
500ppm F 3% TMP	2677 ± 182 [d]
1500ppm F	2584 ± 232 [d]
1500ppm F 3% TMP	2290 ± 172 [e]
3000ppm F	2230 ± 256 [e]
3000ppm F 3% TMP	2213 ± 388 [e]

Means followed by distinct letters are significantly different. Kruskal-Wallis ($p<0.001$).

Figure

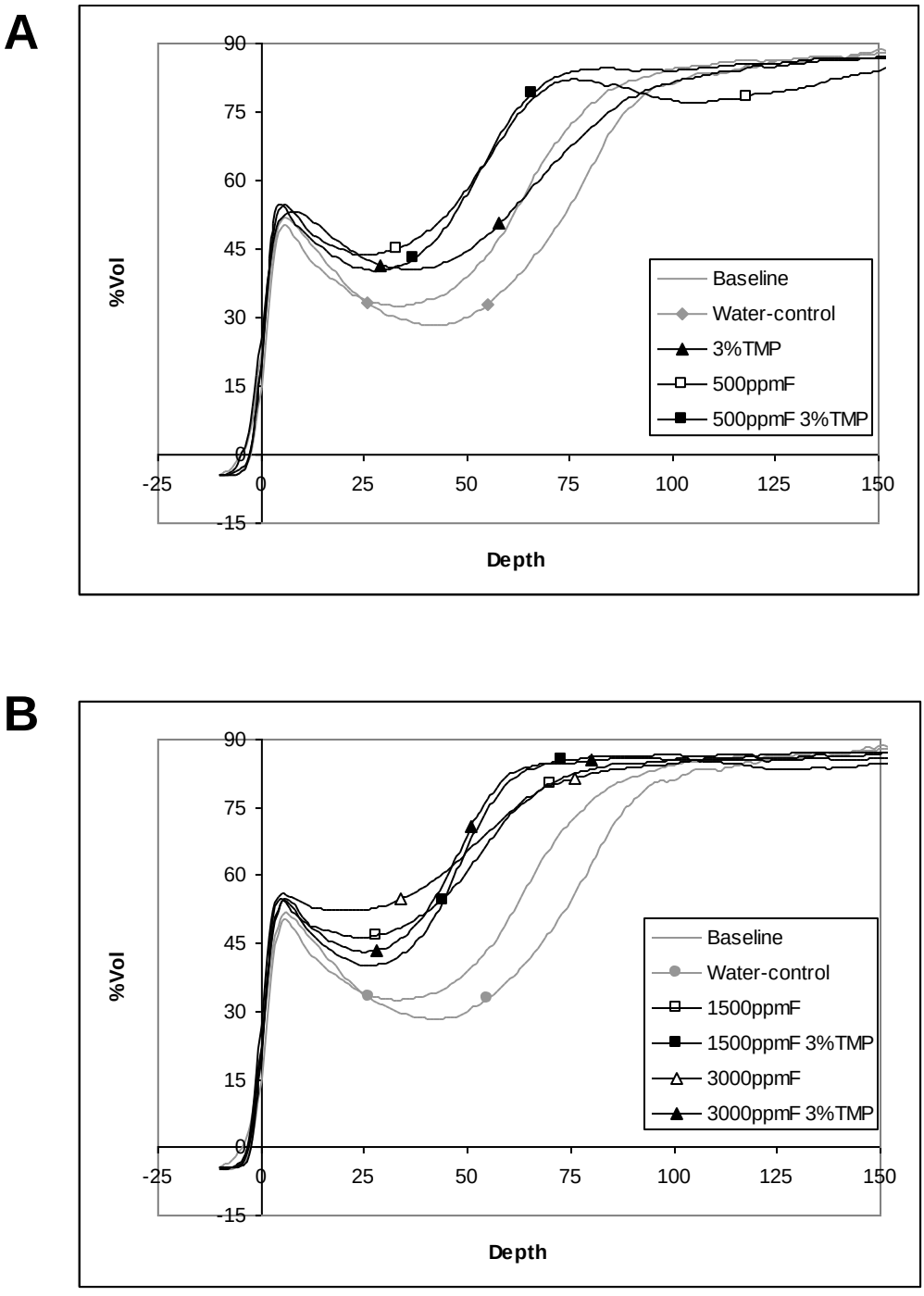

Figure 1 – Average mineral content profiles (vol% mineral versus depth) recorded from microradiographs taken from specimens of lower (Panel A) and higher fluoride groups (Panel B), both with and without TMP.

Capítulo 2

3 Evaluation of trimetaphosphate effect on microcosm and single species biofilm in vitro.*

3.1 ABSTRACT

Objective: Sodium trimetaphosphate (TMP) is considered the most effective anticaries agent among phosphates. The mode of action on hard tissues and biofilm is still unclear. New *in vitro* models have been developed to help explain how it could act. The aim of this study was to evaluate the effect of TMP on microcosm and single species biofilm formed in an *in vitro* biofilm model and its efficacy when combined to different antimicrobials.

Methods: This study was carried out in three experiments. In experiment 1 (microcosm biofilm), glass and enamel were used as substrata. The treatment groups were control, sterile solution with 3% TMP pre-treatment and treatment after 8 hours *in vitro* biofilm formation. In experiment 2 (single species biofilm), only glass samples were used as substratum. The treatment groups were control, sterile solution with 1% TMP and 0.11% NaF solution with or without 1% TMP. And in experiment 3 (single species biofilm), glass samples were used as substratum. The treatment was done with combination of TMP and antimicrobials solutions. For all experiments, effect on biofilm was analyzed by Colony Forming Unit (CFU) counts and lactic acid production.

Results: The results showed that in both microcosm and single species biofilm TMP did not alter lactic acid production and CFU counts. And the combination of TMP to others antimicrobials did bring an additional effect on reducing acid production and CFU counts.

Conclusion: We can conclude that in the present study TMP had no action on biofilm.

Key words: antimicrobials, sodium trimetaphosphate, biofilm, microcosm biofilm, *S. mutans*.

*Capítulo escrito de acordo com as instruções do periódico Archives of Oral Biology (ANEXO B)

3.2 INTRODUCTION

It is well established that dental caries is a multifactorial disease in which the fermentation of food sugars by bacteria from the biofilm can lead to tooth surface demineralization. Antimicrobial agents have been studied to modify the biofilm to reduce the cariogenic challenge. Nowadays, biofilm models have been developed to simulate plaque activities *in vitro* and to test antimicrobials effect on biofilm.[1,2,3,4,5]

After 60 decades, fluoride (F) has become the most important agent to reduce dental caries. However, the widespread use has been related to dental fluorosis in some countries. For that reason, developing effective alternative agents would make caries prevention less dependent on fluoride.[6]

Phosphate salts have been considered alternative anticaries agent.[7,8] Harris et al.[9] demonstrated that sodium trimetaphosphate (TMP) is the most effective phosphate salt against dental caries. Studies carried out in the past demonstrated the affinity of TMP on enamel surface and/or hydroxyapatite resulting in a reduction of enamel solubility.[10,11,12] A recent study[13] showed an additional effect of TMP in prevent enamel demineralization when added to dentifrice with low F concentration.

However, there is a lack of information about phosphates action against oral bacteria. Generally, they are used as food additives to inhibit bacterial growth.[14] It is suggested that antibacterial action of some condensed phosphate is related to their chelating capacity.[15] However, regarding TMP literature is controversial. TMP is a cyclic phosphate and in terms of mechanism of action is different from others phosphate compounds. Since TMP has affinity to enamel surface, a new biofilm model developed by Exterkate et al.[5] was suitable to attest TMP action on biofilm formed on different substrates. This way, the aim of this study was to verify the effect of TMP on biofilm and its efficacy when combined to different antimicrobials.

3.3 MATERIAL AND METHODS

Experimental design

The present study was carried out in three experiments:

Experiment 1: enamel and glass samples were used as substratum to grow *in vitro* biofilm. The treatment groups were control, sterile solution with 3% TMP pre-treatment and treatment after 8 hours *in vitro* biofilm formation.

Experiment 2: glass samples were used as substratum. The treatment groups were control, sterile solution with 1% TMP and 0.11% NaF solution with or without 1% TMP.

Experiment 3: glass samples were used as substratum. The treatment groups were control, sterile solution with 1% TMP and chlorhexidine 0.05 and 0.1% solution with or without 1% TMP.

Experiment 4: glass samples were used as substratum. The treatment groups were control, sterile solution with 1% TMP and cetylpyridinium chloride 0.05 and 0.1% solution with or without 1% TMP.

For all experiments, effect on biofilm was analyzed by Colony Forming Unit (CFU) counts and lactic acid production.

Microcosm Biofilm growth conditions and harvesting

A growth medium was used as described by McBain et al.[16], which contained mucin (type II, porcine, gastric), 2.5 g/L; bacteriological peptone, 2.0 g/L; tryptone, 2.0 g/L; yeast extract, 1.0 g/L; NaCl, 0.35 g/L, KCl, 0.2 g/L; CaCl2, 0.2 g/L; cysteine hydrochloride, 0.1 g/L; haemin, 0.001 g/L; vitamin K1, 0.0002 g/L, pH 7. The biofilm model used is described by Exterkate et al.[5]. Sterilized glass or enamel discs were used as substrata that were positioned vertically in the wells of polystyrene, 24-well flat-bottomed microtiter plates. Each well was filled with 1.5 mL of growth medium with 0.2% (v/v) sucrose supplement. As inoculum a sample (400 µL) saliva-glycerol stock was added and the plate was incubated anaerobically at 37 °C (80% N_2, 10% CO_2 and 10% H_2). The medium was refreshed after 8, 16 and 24 h.

After growth the enamel and glass discs were removed from the wells, put into tubes with 2 mL of cysteine peptone water (CPW) (8.5 g NaCl, 5 g Bacto yeast extract, 1 g Bacto peptone, 0.5 g cysteine HCl per liter, pH 7.3) and dispersed by sonication on ice for 2 min at an amplitude of 40W (Vibra cell™, Sonics & Materials INC, USA). Serially diluted samples were plated onto TSA blood agar and incubated anaerobically at 37 °C for 72 h. The viability of cells grown in biofilms was determined by CFU counts.

Single species biofilm growth conditions and harvesting

S. mutans C180-2 kept at –80 ° C in 60% w/v glycerol was incubated overnight on brain heart infusion (BHI) and grown at 37 ° C anaerobically (80% N_2 , 10% CO_2 and 10% H_2). 10 fold diluted overnight culture in half-strength BHI supplemented with 50 mM PIPES (medium) was inoculated anaerobically at 37 °C in 24-well flat-bottomed microtiter plates (bio-one; Greiner, Frickenhausen, Germany). The biofilm model used is described by Exterkate et al.[5]. After 8 h the medium was refreshed and the biofilm incubated anaerobically for an additional 16 h.

After growth the glass discs were removed from the wells, put into tubes with 2 mL of CPW and dispersed by sonication on ice for 2 min at an amplitude of 40W (Vibra cell™, Sonics & Materials INC, USA). Serially diluted samples were plated onto BHI agar and incubated anaerobically at 37 °C for 72 h. The viability of cells grown in biofilms was determined by CFU counts

Treatments

Experiment – 1: A sterile solution of TMP 3% (Sigma, St Louis, MO, USA) was freshly prepared on the day of use. The treatment was carried out during 1 hour according to the experimental design.

Experiment – 2: Sterile solutions with TMP 1% and 0.11% sodium fluoride (NaF, Merck, Germany) and one solution containing both were freshly prepared on the day of use. The samples were stored overnight in treatment solutions, before starting incubation. Following, the treatments were carried out between fresh medium changes during 5 minutes.

Experiment – 3: Sterile solution of chlorhexidine, 0.05% and 0.1%, with or without 1% TMP was freshly prepared on the day of use. The treatment was carried out at the end of 24h biofilm growth.

Experiment – 4: Sterile solution of cetylpyridinium chloride, 0.05% and 0.1%, with or without 1% TMP was freshly prepared on the day of use. The treatment was carried out at the end of 24h biofilm growth.

Lactic acid production

After 24h and 48h, the samples were immediately placed on a 24-well plate containing PBW with 0.2% sucrose. The plate was incubated anaerobically for 3 hrs at 37º C. After that, the PBW was transferred into microtubes and placed in a water bath at 80ºC for 5 min, to stop all acid production. The samples were stored at −20 °C for lactic acid estimations. The concentration of lactic acid was measured with an enzymatic–spectrophotometric method. The principle of the method is based on the enzymatic conversion of L-lactate to pyruvate with concomitant conversion of NAD^+ to NADH, the increase in absorbance at 340 nm being proportional to NADH formation.

Statistics

For statistical analysis, GMC software[17] was used and the significance limit was set at 5%. First, the normal distribution of the data and the homogeneity of the variances were tested using, respectively, the Kolmogorov-Smirnov and Cochran tests. The lactic acid data from experiment 1, 2 and 3 and CFU data from experiment 1 were heterogeneous and were subjected to Kruskal-Wallis test, followed by Miller's test. The lactic acid data from experiment 4 were subjected to ANOVA, followed by Tukey test. The CFU data of the experiment 2, 3 and 4 were log transformed and were subjected to ANOVA, followed by Tukey test.

3.4 RESULTS

Lactic acid production

The lactic acid production data are shown in figure 1 and table 1 to 3. In experiment 1, different substrata and treatments were compared. Both glass and enamel substrata showed similar behavior to TMP treatments. There were no differences between the two treatment groups and the control ($p<0.05$). In experiment 2, there were no differences among the groups ($p>0.05$). In experiment 3 and 4, CHX and CPC were effective in reduce acid production, but the combination with TMP did not bring an additional effect in both concentration.

Microorganism Growth on biofilm

The CFU counts are shown in table 1 to 4. In experiment 1, the treatment with TMP 3% did not induce decrease in CFU counts in microcosm biofilm. Even when TMP

treatments were carried out during overnight period before starting the experiment and at every change of medium no differences were found when compared to the control group (data not shown). Since experiment 1 showed no differences between the substrata, only glass discs were used in experiment 2 to 4. In experiment 2, NaF decreased *S. mutans* growth when compared to control group ($p<0.05$), but the addition of TMP did not interfere on it ($p>0.05$). In experiment 3, the results from chlorhexidine and cetylpyridinium chloride showed effective on reducing *S. mutans*. In both experiment TMP did not reduce total CFUs after biofilm established on substratum surface, since it was done after 24h. In both antimicrobials, TMP caused an increase of microorganism growth.

3.5 DISCUSSION

The data presented in this study demonstrated that the biofilm model used was suitable to grow biofilm on different substratum both in microcosm and single species biofilm. The model allows for the evaluation of antimicrobials agent, and TMP, on biofilm survival and metabolism.

Nowadays, the search for alternative anticaries agent has been increased to make caries prevention less dependent on fluoride. TMP is one of them but the mode of action remains unclear. Studies suggested in the past that TMP had local action that could influence the colonization of microorganisms responsible for dental caries[18] and the binding of TMP to the enamel surface could inhibit proteins such as phosphatase, or perhaps cariogenic microorganisms and then they would be no longer available for enamel destruction.[19]

In a clinical study TMP had significant alterations in human plaque components. After TMP exposure numbers of total *streptococci* decreased and *Veillonella* increased. However, *S. mutans* did not alter.[20] Other clinical study observed that TMP did not alter either the amount of plaque formed or its microbiological composition.[21]

Since *in vitro* and *in vivo* studies remained controversial, in the present study the use of a new *in vitro* biofilm model played an important role to show that TMP in two tested concentrations, 1 and 3%, did not interfere on biofilm composition. When TMP was added to antimicrobials no positive effect was observed, TMP caused an increase of microorganism growth. Even literature suggested that TMP adsorbed to

enamel inhibits the binding of proteins to enamel surface, in the present study we observed that treatment of TMP on enamel surface did not avoid biofilm growth. In single species biofilm experiment *S. mutans* did not change comparing TMP and control group. And regarding microcosm biofilm the results are in agreement to that of Finn et al.[21].

Therefore, the development of new biofilm models has made possible new studies evaluating the action of antibacterial agents on biofilm formed *in vitro*. The results from the present study can contribute to the discussion about TMP outcome from *in vitro* and *in vivo* studies by some authors.[7,8] Although *in vivo* results are more remarkable, *in vitro* studies play an important role as well. The biofilm model used in this study based on Exterkate et al.[5] was suitable to attest TMP influence on biofilm in different substrata. Therefore, we can conclude that in the present study TMP alone or combined to antimicrobials had no direct action on biofilm.

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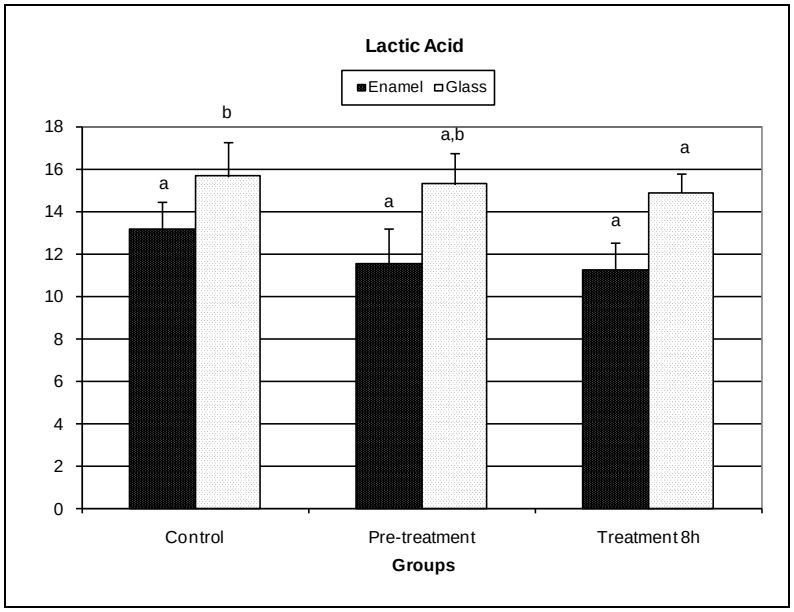
Figure



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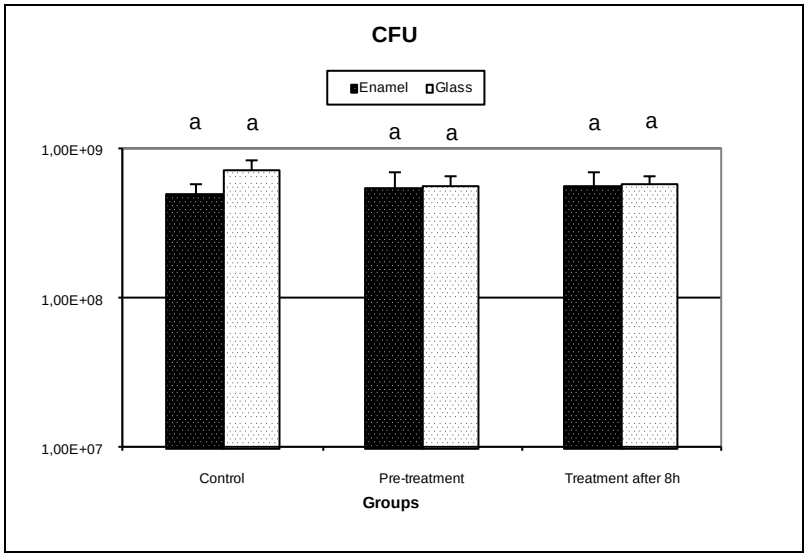

B

Figure 1 – Lactic acid data (mmol/L) (A) (mean ± SD) and Colony Forming Unit (CFU counts) (B) (mean ± SD) from experiment 1. Distinct letters indicate statistical significance ($p<0.05$).

Tables

Table 1 - Lactic acid production (mean ±SD) and Colony Forming Unit (CFU) counts (mean ±SD) from experiment 2.

	Treatments			
	Control	TMP 1%	NaF 0.11%	NaF 0.11% TMP 1%
Lactic Acid	26.4±2.6[a]	29.1 ±1.6[a]	24 ±3.5[a]	23.5 ±3.6[a]
CFU (10^8)	49.5 ±3.4[a]	44.4 ±1.6[a]	36.8 ±3.4[b]	36.4 ±2.3[b]

Within rows, distinct superscript letters indicate statistical significance ($p<0.05$).

Table 2 - Lactic acid production (mean ±SD) and Colony Forming Unit (CFU) counts (mean ±SD) from experiment 3.

	Treatments				
	Control	CHX 0.05%	CHX 0.05% TMP 1%	CHX 0.1%	CHX 0.1% TMP 1%
Lactic Acid	27.2 ±0.5[a]	11.7 ±0.8[b]	18.5 ±0.4[c]	7.9 ±1.2[d]	11 ±0.8[b]
CFU (10^8)	9.1 ±1.8[a]	2.6 ±1.0[b,c]	4.7 ±3.3[c]	0.8 ±0.0[b]	1.5 ±0.8[c]

Within rows, distinct superscript letters indicate statistical significance ($p<0.05$).

Table 3 - Lactic acid production (mean ±SD) and Colony Forming Unit (CFU) counts (mean ±SD) from experiment 4.

	Treatments				
	Control	CPC 0.05%	CPC 0.05% TMP 1%	CPC 0.1%	CPC 0.1% TMP 1%
Lactic Acid	27 ±1.8[a]	11.5 ±2.8[a,b,d]	12.5 ±0.2[b]	8.7 ±1.5[c,d]	9.1 ±1.5[d]
CFU (10^8)	3.9 ±1.5[a]	0.9 ±0.2[b]	0.8 ±0.2[b]	1.2 ±0.5[b]	1.0 ±0.1[b]

Within rows, distinct superscript letters indicate statistical significance ($p<0.05$) indicate statistical significance (ANOVA, Bonferroni; $p < 0.001$).

Capítulo 3

4. Effectiveness of a dentifrice with low fluoride content supplemented with trimetaphosphate on enamel demineralization *in situ*.*

4.1 ABSTRACT

Objective: This study evaluate whether the supplementation with sodium trimetaphosphate (TMP) of a dentifrice with low fluoride (F) content (500 µg/g) would provide similar effect to that of a standard dentifrice (1100 µg F/g).

Methods: In this crossover double-blind study, 10 volunteers wore acrylic palatal appliance containing four enamel bovine blocks selected through surface hardness. The enamel blocks (n=160) were allocated to four treatment groups: placebo, dentifrice with 500 µg F/g, dentifrice with 500 µg F/g TMP 1% and dentifrice with 1100 µg F/g. Dentifrices treatment was performed 2x/day, and 20% sucrose solution was applied 6x/day. After each experimental period, the biofilm was collected for F, calcium (Ca), phosphorus (P) and insoluble extracellular polysaccharides (EPS) analysis. In each enamel block, Ca, F and P content was determined and surface hardness (SH_2) was again assessed to calculate the percentage change of surface hardness (%SH) and cross section hardness test was performed to calculate the integrated loss of subsurface area (ΔKHN). The data of dental biofilm analysis were homogeneous and were submitted to ANOVA, followed by Bonferroni test. Enamel data were heterogeneous and were subjected to Kruskal-Wallis test, followed by Miller's test.

Results: The results showed that the dentifrice with 500 µg F/g TMP 1% showed the lowest percentage change of surface hardness ($p<0.05$). Regarding F and Ca in enamel and biofilm there were no differences between dentifrice with 500F TMP 1% and positive control ($p>0.05$), but they were different when compared to dentifrice with 500 F ($p<0.05$).

Conclusion: It is concluded that the dentifrice with 500 µg F/g supplemented with TMP 1% had similar effect when compared to a standard dentifrice in an *in situ* model.

Keywords: dentifrice, fluoride, trimetaphosphate, dental enamel, *in situ*, demineralization, biofilm.

*Capítulo escrito de acordo com as instruções do periódico Archives of Oral Biology (ANEXO B)

4.1 INTRODUCTION

Recently, the use of fluoridated products has led to a decline in the prevalence dental caries. However, the widespread use of fluoride has caused an increase of dental fluorosis.[1,2] The reduction of F concentration in dentifrices would be one of the possibilities to reduce the F ingestion by children under 6 years old.[3] However, dentifrices with low F concentration (e.g. 500 µg F/g) have shown to be less effective than dentifrices with 1100 µg F/g dentifrice.[4,5]

One method that can be used to increase the effectiveness of F dentifrices is by supplementing them with calcium and/or phosphate. In 60 decades, studies have demonstrated that some phosphate (P) salts have an anticariogenic activity.[6,7] Sodium trimetaphosphate (TMP) seems to be the most effective against dental caries (6); however, the mechanism of action remains unclear.[8] Studies suggest that TMP is adsorbed to the enamel surface, thereby reducing enamel demineralization,[7, 9, 10] hydroxyapatite solubility,[11] mineral exchange, and changing the affinity between the enamel surface and salivary proteins.[12]

In clinical studies, a decrease of caries was observed after the addition of 1.5% and 3 % TMP in gum and dentifrice, respectively.[13,14] However, it seems that TMP does not bring an additional effect in reducing caries when added to high fluoride dentifrices (1000 and 1500 µg F/g).[15]

On the other hand, Takeshita et al.[16] showed that the addition of TMP in low F dentifrices resulted in a similar effectiveness to that of a standard dentifrice (1100 µg F/g) in an *in vitro* study. Those authors showed that, at concentrations above 1%, TMP increased surface hardness, Ca and F content in enamel treated with low F dentifrices.

Since the previous study[16] was carried out using a pH cycling model that has some limitations in predicting clinical effectiveness,[8] those results should not be considered definitive. In situ models have been used to evaluate fluoride containing dental products[17] and may be considered as an intermediate stage between in vivo and in vitro studies, allowing the control of clinical conditions related to the development of caries.[18,19] Considering the lack of information about the effects on enamel and biofilm of dentifrices with low F concentration supplemented with TMP, the aim was to evaluate whether the supplementation with sodium trimetaphosphate

(TMP) of a dentifrice with low-F content (500 µg/g) would provide similar effect to that of a standard dentifrice (1100 µg F/g).

4.2 MATERIAL AND METHODS

Experimental Design

This study was previously approved by the Human Research Ethical Committee (protocol#2007/01367) and all participants read and signed informed consent statements prior to study initiation. The experimental design is presented in figure 1. This crossover double blind study was performed in four phases of 14 days each. Ten volunteers wore acrylic palatal appliances with enamel bovine teeth that were previously stored in 2% formaldehyde solution (pH 7) for 30 days at room temperature.[20] Sound enamel blocks (4x4 mm, n=160) were sequentially polished and selected using a surface hardness test (SH) (330.0 up to 370.0 KHN). Then, the specimens were allocated to four treatments: placebo (negative control), dentifrice with 500 µg F/g, dentifrice with 500 µg F/g TMP 1% and a dentifrice with 1100 µg F/g (positive control). The cariogenic challenge was produced by dripping a 20% sucrose solution 6x/day. Dentifrices treatments were carried out 2x/day. After each experimental period, the biofilm was collected for F, calcium (Ca), phosphorus (Pi) and insoluble extracellular polysaccharides (EPS) analysis. The enamel surface hardness (SH_2) was again assessed to calculate the percentage change of surface hardness (%SH). The blocks were sectioned longitudinally and one of the halves underwent a cross-section hardness test to calculate the integrated loss of subsurface area (ΔKHN) while the other one had the F, Ca and Pi content in enamel determined.

Dentifrice formulation and Fluoride and Phosphorus Assessment

The experimental dentifrices were prepared in a laboratory and had the following ingredients: carboxymethylcellulose, sodium methyl-p-hydroxybenzoate, sodium saccharin, peppermint oil, glycerol, hydrated silica, sodium lauryl sulfate and water. Fluoride (NaF, Merck, Germany) concentration in experimental dentifrice was 500 µg F/g and it had 1% sodium trimetaphosphate (TMP, Sigma-Aldrich Co., St. Louis, MO, USA) added. A dentifrice without F and P (placebo, negative control) and one with 500 µg F/g and other with 1100 µg F/g (Crest™, Proctor & Gamble, USA, positive control) were also used. To ensure the F and Pi concentrations in the experimental

dentifrices, F in dentifrices was determined using a specific electrode for F ion (9609 BN – Orion Research Inc, Beverly, MA, USA) attached to an ion analyzer (Orion 720 A+- Orion Research Inc), which was calibrated with standards containing 0.125 to 2.000 µg F/g. Pi concentration, and measured the levels of F according to the colorimetric method as described by Fiske and Subbarow,[21] after being submitted to a previous acid hydrolysis.

Palatal appliance preparation and treatments

The oral appliances were prepared in acrylic resin (Jet - Articles Classic Odontológico, São Paulo, Brazil) in accordance with Hara et al.[22]. Four enamel blocks were fixed in the intraoral device in each phase. In order to allow accumulation of biofilm on the enamel blocks, a piece of gauze was fixed to the acrylic appliance leaving a space (1 mm) from the block surface.[23] To provide the cariogenic challenge, the volunteers were instructed to remove the device and drip 20% sucrose solution on each enamel block 6x/day. Five min later, the device was re-inserted into the mouth. The treatments with dentifrices were performed 2x/day, during the volunteers' habitual oral hygiene routine. The device was removed and dentifrice slurries (1:3 w/w) were dripped onto the blocks. The device was then washed in deionized water and re-inserted in the mouth. During the 7-day pre-experimental period and washout periods (7-days between each phase), the volunteers brushed their teeth with non-fluoridated dentifrices. The volunteers received all the instructions prior to the experimental procedures.

Microhardness Analysis

The enamel surface hardness was determined before (SH_1) and after each phase (SH_2) in each specimen using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25g load for 10s. 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. The percentage change of surface hardness (%SH) was calculated (%SH = 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 and gradually polished. Three sequences of eight indentations at different distances (10, 30, 50, 70, 90, 110, 220 and 330 µm) were made from the surface of the enamel in the central region spaced 100µm from each other, under a 25g load for 10s. The means were calculated for each distance and the integrated area (KHN x µm) of sound enamel and after each treatment was calculated up to a depth of 90 µm to calculate ΔKHN, through trapezoid rule: ΔKHN = ΔX*(Y1+Y2)/2 (GraphPad Prism, version 3.02). Lesion depth was measured from the enamel surface up to the distance where hardness represented 95% of the value for sound enamel.

Fluoride, Calcium, and Phosphorus in Enamel

Enamel biopsy was performed according to the study by Weatherell et al.[24] Blocks measuring 2X2mm were obtained from one of the halves of the longitudinally sectioned blocks. The blocks were fixed to a mandrel and fixed to the top of a modified microscope. Self-adhesive polishing discs (13 mm of diameter) of 400 grit silicon carbide (BUEHLER) were fixed to the bottom of polystyrene crystal tubes (J-10, Injeplast, Sao Paulo, SP, Brazil). A layer of 50 µm deep of each enamel block was removed and 8 mL HCl 0.5 mol/L was added to the enamel powder. The tubes were kept under agitation for 15 minutes and 0.8 mL NaOH 0.5 mol/L was then added. For F analysis, the specific electrode for the Orion 9690 connected to an ion analyzer (Orion 720^{+}) and TISAB II (total ionic strength adjustment buffer) at 1:1 ratio (TISAB:sample) were used. For Ca analysis, the cresolftalein colorimetric method (Katal, Belo Horizonte, MG, Brazil) was used. The Pi was measured according to Fiske and Subbarow.[21]

Analysis of Dental Biofilm Composition

After each experimental period, the biofilm formed on the enamel surface from each slab was collected and stored in microcentrifuge tubes. The biofilm samples were dried under vacuum with phosphorus pentoxide for 12 h at room temperature. Hydrochloric acid (0.5 mol/L) was added to the tubes in the proportion of 250 µL/mg plaque wet weight. After extraction for 3 h at room temperature under constant agitation, the same volume NaOH 0.5 mol/L was added. The samples were then centrifuged (11,000*g*) for 1 min and the supernatant retained for F, Ca and Pi

determination. EPS were extracted by adding NaOH 1.0 mol/L (10 µL/mg dry weight) to the biofilm. The samples were vortex for 1 min and after 3 h under agitation at room temperature they were centrifuged (1 min, 11.000*g* at room temperature).[25] EPS were precipitated from supernatants with 75% ethanol overnight, centrifuged and re-suspended in 1.0 mol/L NaOH.[26] EPS were determined by the phenol-sulfuric acid method.[27] The results were expressed as µg/mg dry weight.

Statistical Analysis

For statistical analysis, GMC software[28] was used and the significance limit was set at 5%. First, the normal distribution of the data and the homogeneity of the variances were tested using, respectively, the Kolmogorov-Smirnov and Cochran tests. The data of the dental biofilm analysis (F, Ca, Pi and IP) were normal and homogeneous and were submitted to ANOVA, followed by Bonferroni test. The variable Ca and IP were homogeneous after log transformation. Enamel data (%SH, ΔKHN, F, Ca and Pi) were heterogeneous and were subjected to Kruskal-Wallis test, followed by Miller's test.

4.3 RESULTS

The analysis of F in dentifrice is presented in Table 1. The Pi in dentifrice with 1% TMP and positive control were, respectively, 3374.34 ± 55.3 (mean±SD) and 1735.11 µg P/g.

Table 2 shows the mean of (%SH) after each treatment. The dentifrice supplemented with F and TMP 1% showed the lowest %SH ($p<0.05$). The order of effect in reducing %SH was dentifrice with 500 µg F/g TMP 1% > positive control > 500 µg F/g > placebo. The same results were observed for ΔKHN, however, the order of effect in reducing was 500 µg F/g TMP 1% ~ positive control > 500 µg F/g > placebo.

Regarding F and Ca in enamel after each treatment in a layer of 50 µm removed from the enamel surface, there were no differences between dentifrice with 500 µg F/g TMP 1% and positive control ($p>0.05$), but they were different when compared to the dentifrice with 500 µg F/g and placebo ($p<0.05$).

The analysis of dental biofilm composition, presented in Table 3, showed that 500 µg F/g TMP 1% and the positive control dentifrice presented equal F and Ca

concentrations ($p>0.05$). The placebo dentifrice showed the lowest concentrations of F and Ca in the biofilm ($p<0.05$) and the dentifrice with 500 µg F/g differed from the others ($p<0.05$). The Pi concentration was similar in all groups, either in biofilm or enamel.

Similar results of EPS in dental biofilm were observed between 500 µg F/g TMP 1% and the positive control (Table 3, $p>0.05$). This EPS formation was lower when compared to other dentifrices ($p<0.05$).

4.5 DISCUSSION

The present study evaluated the possibility of reducing F concentration in a dentifrice in order to make it less toxic (dental fluorosis), while at the same time, maintaining a similar efficacy to a standard dentifrice (1,100 µg F/g) using an *in situ* model.

The overall data (ΔKHN, Ca and F) showed that the positive control and the 500 µg F/g TMP 1% dentifrice demonstrated similar effects on enamel demineralization and biofilm composition. Only the %SH data showed the lowest mineral loss with the 500 µg F/g TMP 1% dentifrice. The findings of the present study revealed how TMP promotes an additional effect on F action when 500 µg F/g and 500 µg F/g TMP 1% groups are compared. F and Ca content in enamel in 500 µg F/g TMP1% were ~33% and ~24% (respectively) higher than with the 500 µg F/g dentifrice. This suggests that calcium fluoride deposition might be enhanced by the presence of TMP and during the cariogenic challenge more F and Ca were released to biofilm. Moreover, TMP causes retention of the surface zone, resulting in a more pronounced surface layer that is further enhanced by the presence of fluoride.[16,29] Although these results do not show a TMP influence on F and/or Ca mobility into enamel, based on biochemical analysis performed in the present study; it can be suggested that TMP adsorbed on enamel alters the selective permeability and ion diffusion into the lesion.[30,31,32] These effects could contribute to a lower mineral loss in enamel treated with TMP.

The biofilm formation on the enamel surface did not impair the effect of dentifrice treatment on enamel. However, it should be considered that treatment with dentifrice started in the first day of the experiment, when biofilm was not formed. It is assumed that dentifrice treatment was performed directly on enamel during the initial

days of each experimental phase. For this reason, this in situ study demonstrated the overall results considering all steps during a cariogenic challenge – starting with sound enamel until lesion and biofilm formation. F and Ca concentrations in biofilm showed that TMP increased the amount of these ions and decreased the amount of EPS, which was similar to the positive control. The highest calcium values in 500 µg F/g TMP 1% group and positive control can also be related to the lowest amount of EPS in biofilm. Ca concentration varying between $1x10^{-3}$ and $1x10^{-4}$ $mol.L^{-1}$ (0.04 and 0.004 µg/mg) reduces EPS concentration.[33] However, even EPS is related to bacterial adhesion and biofilm virulence.[34] In a microbiological study (data not published), TMP did not interfere on bacterial growth and lactic acid production on microcosm biofilm formed *in vitro*. Thus, the TMP action on biofilm could be related only to biochemical changes, with these ions interfering on biofilm, since the highest F and Ca concentrations in biofilm alter the bacterial metabolism and act in the bacterial biological activity, i.e., EPS formation.[35,36]

Phosphorus content in biofilm seems to be not affected by sucrose frequency.[37,38] Even though the biofilm was treated with a phosphate salt, the concentration of Pi in enamel and biofilm did not increase. Although TMP is soluble in water; it is a cyclic phosphate and seems to not be hydrolyzed in saliva or spontaneously. The amount of Pi theoretically expected in dentifrices was detected only after acid hydrolysis and it was not observed in enamel and biofilm treated with TMP. Consequently, TMP does not increase the phosphate ions availability in the oral environment. These results are similar to those demonstrated by Takeshita et al.,[16] who found that a range of concentrations for TMP from 1% to 3% did not change the Pi content in enamel.

The *in situ* model used in the present study allowed evaluation of the interaction between biofilm and enamel. Even the volunteers were selected based on their enamel demineralization and biofilm formation capacity, the variables of the present study demonstrated large variation. Unlike clinical studies that require a large sample, one of the biggest advantages of in situ models is the use of small numbers of volunteers, 10-20, able to provide answers to questions about the caries preventive agents.[39] Therefore, using this in situ model, it can be assumed that the action of TMP can be similar between biofilm and enamel mineral changes. Therefore, the results obtained in this *in situ* study suggest that it is possible to obtain

a greater reduction in mineral loss through the supplementation of 1% TMP in a 500 μg F/ g dentifrice, maintaining a similar effect of a standard dentifrice (1100 μg F/g).

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Figure

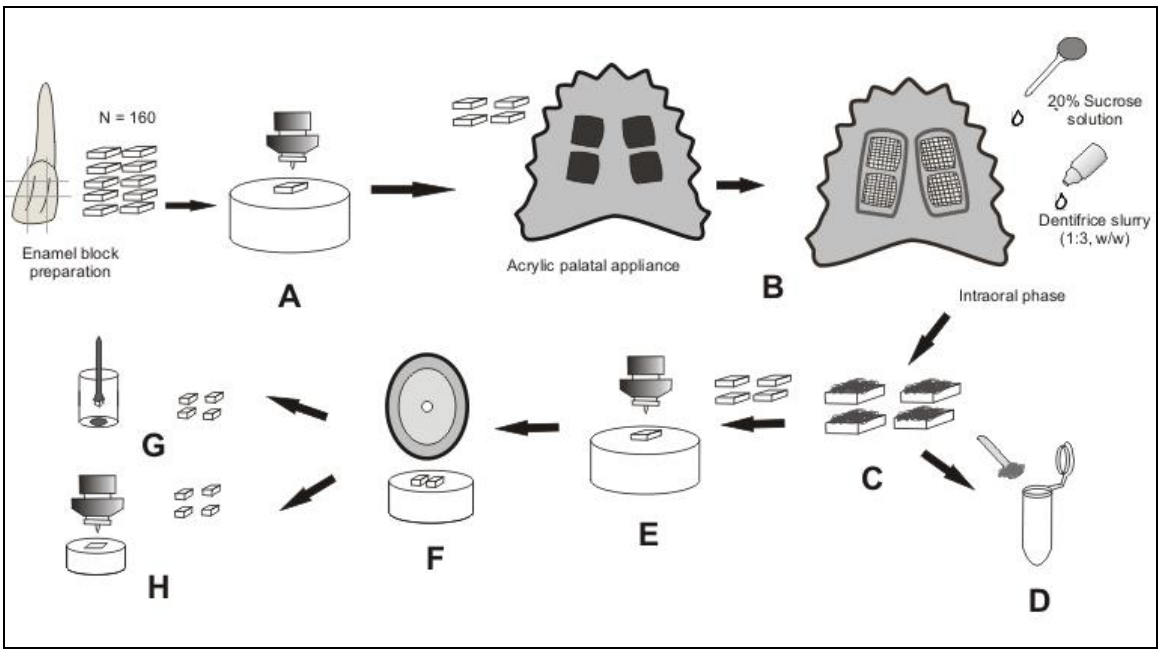

Figure 1 – **A** Enamel blocks selection through surface hardness test. **B** Acrylic palatal appliance preparation and intraoral phase. **C** After 14 days, enamel blocks and biofilm were colected from each slot of palatal appliance. **D** F, Ca, P and EPS content in biofilm analysis. **E** Surface hardness test after each phase. **F** Enamel blocks were sectioned longitudinally. **G** F, Ca and P content in enamel analysis in one of the halves of enamel block. **H** Cross-sectional hardness test in the other half of enamel block.

Tables

Table 1 - Total fluoride (FT) and ionic fluoride (FI) concentrations (mean ± SD, n=3) in dentifrices.

Groups	FT, µg/g	FI, µg/g
Placebo	12.2 ± 1.1	12.3 ± 1.7
500 µg F/g	556.7 ± 7.5	526.0 ± 19.6
500 µg F/g TMP 1%	555.4 ± 4.9	552.1 ± 3.8
Positive control	1086.2 ± 34.2	1106.3 ± 49.3

Table 2 - Fluoride (F), calcium (Ca) and phosphorus (P) concentration in enamel and percentage of change in surface hardness (%SH) and integrated loss of subsurface hardness (ΔKHN) (mean ± SD) of the enamel blocks according to the treatments.

Variable	Treatments (Dentifrices)			
	Placebo	500 µg F/g	500 µg F/g TMP 1%	Positive control
F, µg/mm^3	0.58 ±0.15^a n=8	0.84 ±0.30^b n=8	1.26 ±0.47^c n=8	1.31 ±0.63^c n=8
Ca, µg/mm^3	787.2 ±118.8^a n=8	793.6 ±272.0^a n=8	1,038.9 ±96.2^b n=8	1,160.2 ±164.8^b n=8
P, µg/mm^3	250.2 ±117.0^a n=8	251.7 ±87.6^a n=8	322.4 ±51.0^a n=8	344.9 ±61.9^a n=8
%SH	-67.1 ±6.1^a n=10	-30.8 ±13.8^b n=10	-9.9 ±5.9^c n=18	-18.0 ±7.6^d n=18
ΔKHN	7,451.3 ±1685.3^a n=10	4,717.8 ±596.5^b n=10	3,027.0 ±790.7^c n=10	3,207.2 ±720.8^c n=10

Within rows, distinct superscript letters indicate statistical significance (Kruskal-Wallis, $p < 0.05$).

Table 3 - Fluoride (F), calcium (Ca), phosphorus (P) and extracellular polysaccharides (EPS) concentration (means ± SD) in dental biofilm according to the treatments.

Variable	Treatments (Dentifrices)			
	Placebo	500 µg F/g	500 µg F/g TMP 1%	Positive control
F, µg/mg	19.7 ± 5.0[a] n=10	82.0 ± 70.2[b] n=10	117.8 ± 79.7[c] n=10	151.2 ± 91.0[c] n=10
Ca, µg/mg	2.2 ± 0.5[a] n=10	5.1 ±1.6[b] n=10	7.3 ± 2.2[c] n=10	9.4 ± 4.2[c] n=10
P, µg/mg	5.2 ± 1.9[a] n=10	5.8 ± 2.1[a] n=10	6.8 ± 3.4[a] n=10	8.5 ± 4.1[a] n=10
EPS, µg/mg	298.2 ± 102.6[a] n=10	231.1 ± 77.2[b] n=10	134.7 ± 39.7[c] n=10	170.8 ± 52.4[c] n=10

Within rows, distinct superscript letters indicate statistical significance (ANOVA, Bonferroni, $p < 0.001$).

Capítulo 4

5 Remineralizing potential of a dentifrice with low fluoride content supplemented with non-fluoride alternative agent - *in situ* study.*

5.1 ABSTRACT

Objective: The use of dentifrices with low fluoride (F) content has been a way to reduce F ingestion as well as dental fluorosis. The aim of this *in situ* study was to evaluate whether a dentifrice with low F content supplemented with sodium trimetaphosphate would provide a similar remineralization potential as a standard dentifrice (1100µgF/g).

Methods: In this crossover double-blind study , eleven volunteers were selected and wore palatal appliance containing four bovine enamel blocks with artificial caries lesion. This study was performed in 4 phases during 3 days each. The treatment was carried out, *in vivo*, 3x/day during 1 min with dentifrices: placebo (negative control), 500 µgF/g, 500 µgF/g and TMP 1%, and 1100 µgF/g (positive control). After each phase, the percentage of surface hardness recovery (%SH_R) and the integrated loss of subsurface area (ΔKHN) were calculated. F, calcium (Ca) and phosphorus (P) content in enamel was determined.

Results: The results showed that the positive control and dentifrice with 500 µgF/g and TMP 1% showed similar %SH_R (ANOVA; $p>0.05$), but they were different when compared to placebo and dentifrice with low F ($p<0.05$). Regarding F and P in enamel there were no differences between dentifrice with 500 µgF/g and TMP 1% and positive control ($p>0.05$). Ca content in enamel was higher in positive control ($p<0.05$), followed by dentifrice 500 µgF/g and TMP 1%.

Conclusion: It is concluded that the dentifrice with low F supplemented with TMP 1% had similar effect as compared with a standard dentifrice in an *in situ* model.

Key-words: dentifrice, fluoride, trimetaphosphate, dental enamel, *in situ*, remineralization

*Capítulo escrito de acordo com as instruções do periódico Archives of Oral Biology (ANEXO B)

5.2 INTRODUCTION

Mineral ions in oral environment play an important role to promote enamel remineralization. They enter the oral cavity with duct saliva, foodstuffs, and crevicular fluid.[1] However, the concentration of these ions in saliva is limited and depends on individual human physiological characteristics and health conditions. Fluoride (F), calcium (Ca) and phosphate are the main minerals related to remineralization process.

Supplementation of manufactured products such as dentifrices, mouth rinses, infant formula and diet came out as a way to increase the bioavailability of these ions in oral environment. Phosphorus containing agents represent the greatest number of potential non-fluoride anticaries agents and the majority of them are phosphates.[2] Studies in 60´s decades have shown the efficacy of phosphate salts against dental caries.[3,4] Sodium trimetaphosphate (TMP) came out as the greatest cariostatic effect among all the phosphates tested. The order of effectiveness was: trimetaphosphate > hexametaphosphate > tripolyphosphate ~ pyrophosphate ~ orthophosphate.[3]

However, the mode of action of TMP on enamel remineralization remains unclear.[5] Studies suggested that this salt is adsorbed to enamel surface reducing enamel demineralization,[4,6,7] reducing hydroxyapatite solubility[8] and mineral exchange,[6,7] and changing the affinity between enamel surface and salivary proteins.[9]

Since the enhanced efficacy of F dentifrice is related to dental caries reduction as well as dental fluorosis in some countries[10] the combination of low F content in dentifrice with alternative agents (i.e. phosphates salts) could be a way to maintain dentifrice efficacy similar to that of high F content (1100 µg F/g) and reduce the risk of dental fuorosis. Takeshita et al.[11] showed positive results testing this combination in an *in vitro* study.

Once a new formulation dentifrice has been developed, the dentifrice needs to be evaluated to ensure that the components remain active.[12] *In situ* models have been developed to test caries-preventive agents and try to replace clinical trials.[1] Since it does not have in literature *in situ* studies evaluating the effect of dentifrices with low fluoride concentration supplemented with TMP, the present study evaluated if the remineralization potential of this dentifrice would be similar to a standard dentifrice using an *in situ* model.

5.3 MATERIAL AND METHODS

Experimental Design

This crossover double blind study was previously approved by the Human Ethical Committee (protocol#2009/0807). The experimental design is displayed in figure 1. Eleven volunteers wearing acrylic palatal appliance with four enamel bovine blocks were subjected to four phases of 3 days each with 7 days washout period between each phase. Enamel blocks (4x4 mm, n=176) of bovine incisive teeth were stored in 2% formaldehyde solution (pH 7) for 30 days at room temperature.[13] They were sequentially polished and selected through surface hardness test (330.0 up to 370.0 KHN). The blocks were demineralized and submitted to post demineralization surface hardness (SH_1). Based on the percentage of surface hardness loss the enamel blocks were allocated to four treatments: placebo (negative control), dentifrice with 500 µg F/g, dentifrice with 500 µg F/g and TMP 1% and a dentifrice with 1100 µg F/g (positive control). After each experimental period, the surface hardness (SH_2) was again assessed to calculate the percentage of surface hardness recovery ($\%SH_R$). The blocks were sectioned to perform cross-sectional hardness test to calculate the integrated loss of subsurface area (ΔKHN). Fluoride, calcium (Ca) and phosphorus (Pi) content in enamel were also determined.

Dentifrice formulation and Fluoride and Phosphorus Assessment

The experimental dentifrices were prepared in a laboratory and had the following ingredients: carboxymethylcellulose, sodium methyl-p-hydroxybenzoate, sodium saccharin, peppermint oil, glycerol, hydrated silica, sodium lauryl sulfate and water. Fluoride (NaF, Merck, Germany) concentration in experimental dentifrice was 500 µg F/g and it was added 1% of sodium trimetaphosphate (TMP, Sigma-Aldrich Co., USA). Dentifrice without F and P (placebo, negative control) and one with 500 µg F/g and other with 1100 µg F/g (Crest™, Procter & Gamble, USA, positive control) were also used. Fluoride in dentifrices was determined using a specific electrode for F ion (9609 BN – Orion Research Inc, Beverly, MA, USA) attached to an ion analyzer (Orion 720 A+- Orion Research Inc) and calibrated with standards containing 0.125 to 2.000 µg F/g. Pi in dentifrices was measured according to the colorimetric determination as described by Fiske and Subbarow[14], submitted to a previous acid hydrolysis.[11]

Subsurface enamel demineralization

The cut surface of each enamel block was covered with a protective coating (nail varnish), except enamel. The subsurface enamel demineralization[15] was produced by immersing each enamel block in 32 ml of a solution with 1.3 mmol/L Ca, 0.78 mmol/L P in 0.05 mol/.L acetate buffer, pH 5.0; 0.03 ppm F; for 16h at 37°C.[16]

Palatal appliance preparation and treatments

The oral appliance was prepared in acrylic resin (Jet - Articles Classic Odontológico, São Paulo). Four enamel blocks with artificial caries lesion were fixed in the intraoral device in each phase. The treatments with the dentifrices were performed 3x/day, during the volunteers' habitual oral hygiene routine. The volunteers were instructed to wear their appliances 24 hours a day during each treatment period and brush their teeth with the appliance in place, using the toothbrush provided to them. Volunteers brushed for one minute during each brush and actively “swished” the dentifrice slurry around their entire mouth before expectorated. During 7-day pre-experimental period and washout periods, the volunteers brushed their teeth with nonfluoridated dentifrices. The volunteers received all the instructions previously.

Hardness Analysis

The enamel surface hardness was determined before (SH), post subsurface enamel demineralization (SH_1) and after each experimental phase (SH_2) using a Shimadzu HMV-2000 microhardness tester (Shimadzu Corp., Kyoto, Japan) under a 25g load for 10s. Five indentations spaced 100 µm from each other were made at the center of the enamel surface (SH). Indentations for post demineralization surface hardness (SH_1) and for post experiment surface hardness (SH_2) spaced 100 µm from each other and from the baseline. The percentage of surface hardness recovery ($\%SH_R = ((SH_2 – SH_1) / (SH – SH_1)) \times 100$) was calculated.

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 and gradually polished. Three sequences of fourteen indentations at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 and 330 µm) from the outer enamel 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 5 g load for 10 s (X1000). The mean values at all three measuring points at each distance from the surface were then averaged.[17]

The integrated area above the curve (cross-sectional profiles of hardness into the enamel), using the hardness values (KHN), was calculated by trapezoidal rule (GraphPad Prism, version 3.02) in each depth (µm) from the lesion up to sound enamel. This value was subtracted from integrated area of sound enamel, to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN).[11]

Fluoride, Calcium, and Phosphorus Content in Enamel

Enamel biopsy was performed according to the study by Weatherell et al.[18] One of the halves of the longitudinally sectioned blocks was used obtaining blocks measuring 2X2mm. The blocks was fixed to a mandrel and fixed to the top of a modified microscope with a micrometer (Pantec, Sao Paulo, SP, Brazil) to measure the depth. Self-adhesive polishing discs (13 mm of diameter), 400 grit silicon carbide (Buehler,Lake Buff, Il, USA) was fixed to the bottom of polystyrene crystal tubes (J-10, Injeplast, Sao Paulo, SP, Brazil). A layer of 50 µm deep of each enamel block was removed. The enamel powder was stored in tubes and 0.8 mL HCl 0.5 mol/L was added. The tubes were kept under agitation during 30 minutes and 0.8 mL NaOH 0.5 mol/L was then added. For F analysis, specific electrode Orion 9690 connected to an ion analyser (Orion 720^{+}) and TISAB III (total ionic strength adjustment buffer) at 1:10 ratio (TISAB:sample) were used. For Ca analysis, the cresoftalein colorimetric method (Katal, Belo Horizonte, MG, Brazil) was used. The P was measured according to Fiske and Subbarow.[14]

Statistical analysis

The analysis was performed by GMC software[20], with a significance level of 5%. Experimental groups (placebo, 500 F, 500 F TMP 1%, positive control) were considering as variation factors and the variables were SH, SH_1, SH_2, $\%SH_R$, ΔKHN, F, Ca and Pi. First, the normality and homogeneity of the samples were tested using, respectively, the Kolmogorov-Smirnov and Cochran tests. The data were homogeneous and were submitted to ANOVA, followed by Bonferroni test.

5.4 RESULTS

The analysis of F in dentifrice was in accordance to the concentration added. Total fluoride and ionic fluoride (µg/g) in placebo, 500 µg F/g, 500 µg F/g TMP 1% and positive control, were respectively, 12.2 ± 1.1; 556.7 ± 7.5; 555.4 ± 4.9; 1086.2 ± 34.2 (total fluoride) and 12.3 ± 1.7, 526.0 ± 19.6, 552.1 ± 3.8, 1106.3 ± 49.3 (ionic fluoride). The phosphorus in dentifrice with TMP 1% and positive control are respectively, 3374.34 ± 55.3 (mean ± SD) and 1735.11 µg P/g.

Table 1 shows the baseline surface hardness (SH), post demineralization surface hardness (SH_1), *in situ* remineralization surface hardness (SH_2), percentage of surface hardness recovery ($\%SH_R$) according to experimental groups. No statistical differences were observed among the groups regarding SH and SH_1. Table 2 shows the average of ΔKHN after each treatment and from the blocks that were only submitted to post demineralization surface hardness (artificial caries). The results showed that the positive control and dentifrice supplemented with F and TMP 1% showed similar $\%SH_R$ and ΔKHN ($p>0.05$), but they were different when compared to placebo and 500 µg F/g group.

Figure 2 shows the profiles of hardness in the groups according to the depth. The blocks submitted only to post demineralization surface hardness (artificial caries) showed a demineralization area up to 120 um. For the others groups the subsurface demineralization was more evident up to a depth of 50 um. All groups showed enamel remineralization wherein 500 µg F/g TMP 1% and positive control groups showed the highest hardness values.

Regarding F and P in enamel after each treatment there were no differences between dentifrice with 500 µg F/g TMP 1% and positive control ($p>0.05$), but they were different when compared to dentifrice 500 µg F/g and placebo ($p<0.05$). Ca content in enamel was higher in positive control ($p<0,05$), followed by dentifrice 500 µg F/g TMP 1% (Table 2).

5.5 DISCUSSION

The present study evaluated the remineralization potential of a dentifrice with low F content and supplemented with TMP 1% comparing it to a standard dentifrice (1100 µg F/g) using an *in situ* model. Since our previous *in vitro*[11] and an *in situ* (data not shown) studies demonstrated TMP effect avoiding enamel demineralization, the

evaluation of remineralization promoted by TMP simultaneously with F is interesting in view the fact that the mechanism of action is still unclear.[5,21]

The subsurface demineralization produced in enamel blocks was standardized and based on the percentage of mineral loss the specimens were selected and allocated in each treatment group. By this way, all the groups started with the same mean of the percentage of mineral loss. The $\%SH_R$ and ΔKHN showed that dentifrice with 500 µg F/g and 1% TMP was similar to that of positive control ($p>0.05$). The same results were observed in F and P content in enamel. Differently, Ca content in enamel in positive control was slightly higher (only ~11%) than in 500 µg F/g TMP 1% group, but statistically significant ($p<0.05$). Comparing 500 µg F/g F and the respective supplemented group, TMP increased F, Ca and Pi in enamel, resulting in more remineralization ($p<0.05$). However, we have to consider that these data is related to mineral content in 50 µm layer removed from enamel surface. Above this depth, it can be observed a large remineralization area up to 120 µm when all groups are compared to the baseline.

In the solution used to produce enamel subsurface demineralization, low concentration of F (0.03 µg F/g) is added in order to form a thin surface layer allowing hardness analysis after demineralization as observed in depth profiles in the blocks submitted to subsurface demineralization only. TMP could be adsorbed to this layer reducing Ca diffusion inside the lesion[6] and facilitating F and Pi deposition in enamel; this way, improving the quality of minerals formed[6] and reducing subsurface lesions. TMP can cause retention of the surface zone resulting in a more pronounced surface layer that is further enhanced by the presence of F.[11,21] By the addition of TMP 1% to 500F a more mineralized surface layer up to 10 µm depth was promoted, as shown in surface hardness and cross-sectional hardness analysis.

Since the *in situ* model designed in the present study was performed during 3 days and no additional cariogenic challenge was promoted, (for example, sucrose solution application), only remineralization was evaluated. Therefore, the calcium-phosphate solution (pH 5) used to promote subsurface demineralization, 3 days experiments were enough to evaluate differences among the groups. A recent study demonstrated that this *in situ* model was able to show verify dose-response relationship and suitable for testing low F concentration dentifrices.[23] However, considering the fact that TMP can cause slow mineral exchanges to the environment,

[6,7] long period of experiments should be tested, and perhaps show different outcomes in Ca uptake.

Therefore, the use of low F dentifrices combined to alternative agents capable of enhance F action is an interesting way to reduce F exposure to 3 - 6 years old children, thereby reducing the risk of dental fluorosis. Further accurate studies should be carried out to evidence how TMP improves low F dentifrices efficacy. In the present *in situ* study we concluded that the supplementation with TMP in a low F dentifrice showed a similar efficacy to that of a standard dentifrice (1100 µg F/g).

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Figures

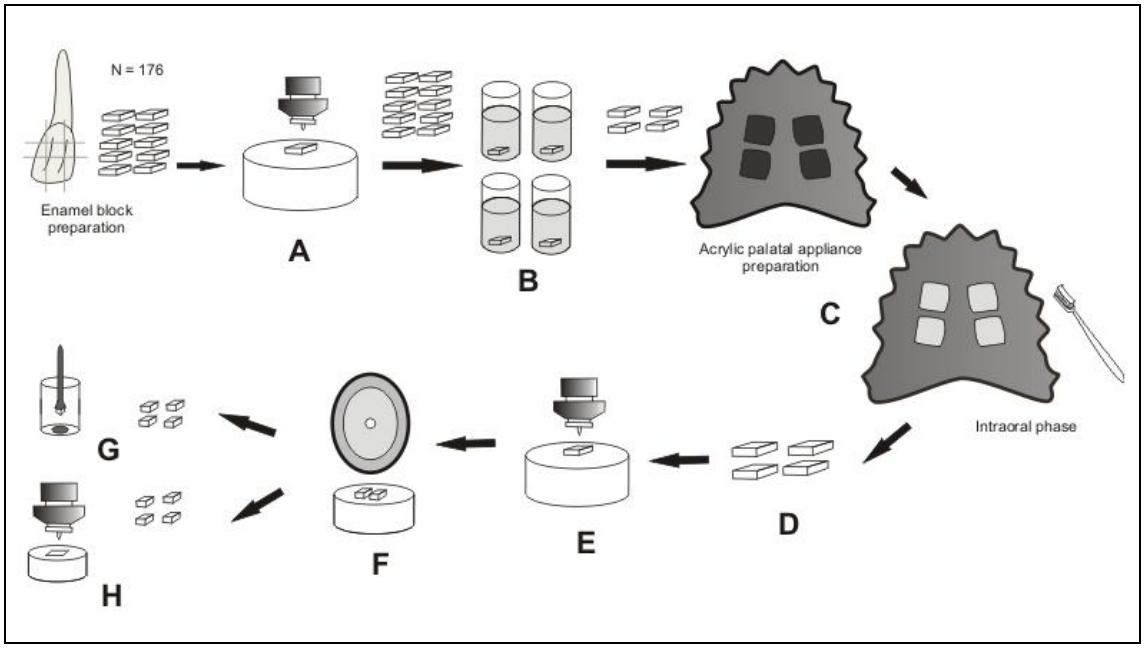

Figure 1 – **A** Enamel blocks selection through surface hardness test. **B** Subsurface enamel demineralization – Enamel block immersion in acid Ca-P solution during 16h at 37^{o}C. **C** Acrylic palatal appliance preparation and intraoral phase – in vivo dentifrice treatment. **D** After 3-days experiment, enamel blocks are removed from palatal appliance. **E** Surface hardness test after each phase. **F** Enamel blocks were sectioned longitudinally. **G** F, Ca and P content in enamel analysis. **H** Cross-sectional hardness test.

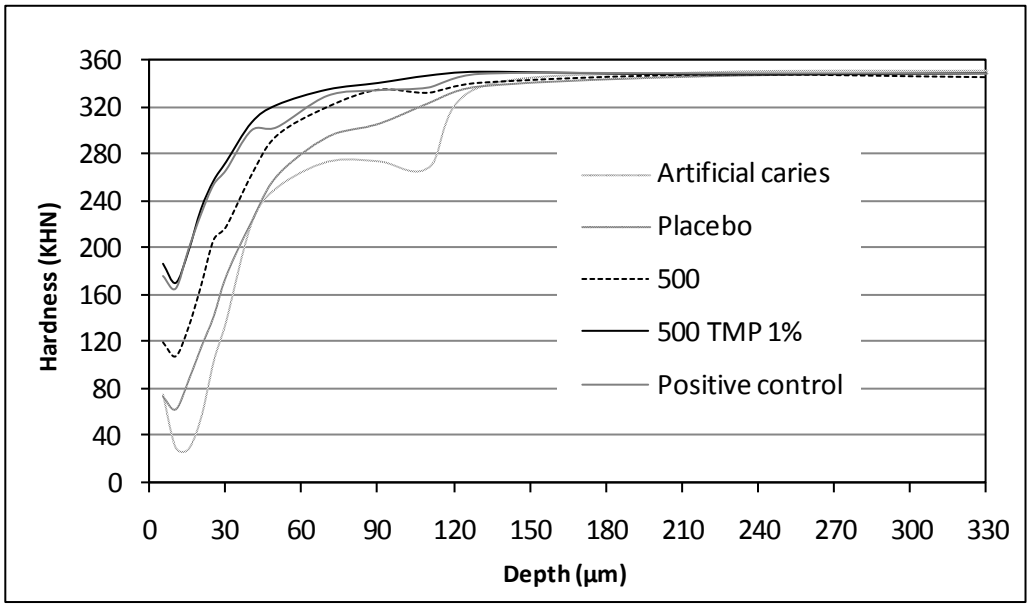

Figure 2 - Cross-sectional hardness profiles (mean KHN, n = 11) at different depths in enamel blocks according to the treatments.

Tables

Table 1 – Means (± SD) of SH, SH_1, SH_2 and percentage of surface hardness recovery ($\%SH_R$) and integrated loss of subsurface area (ΔKHN) in enamel blocks according to the treatments.

Variable	Treatments (Dentifrices)			
	Placebo	500 µg F/g	500 µg F/g TMP 1%	Positive control
SH	358.89 ±4.44[a]	359.8 ± 6.85[a]	359.26 ±5.09[a]	360.25 ± 4.81[a]
SH_1	59.39 ± 2.85[a]	66.93 ± 3.2[a]	68.3 ±2.72[a]	67.5 ± 3.54[a]
SH_2	118.17 ± 20.14[a]	142.93 ± 22.85[a]	176.41 ± 23.77[b]	175.5 ± 24.43[b]
$\%SH_R$	17.97 ± 6.54[a]	25.32 ± 7.67[a]	37.42 ± 7.41[b]	37.39 ± 8.03[b]

Within rows, distinct superscript letters indicate statistical significance (ANOVA, Bonferroni; SH ($p=0.937$), SH_1 ($p=0.193$), SH_2 ($p<0.05$), $\%SH_R$ ($p<0.05$).

Table 2 – Means (± SD) of integrated loss of subsurface area (ΔKHN) in enamel blocks according to the treatments.

Variable	Treatments (Dentifrices)				
	Artificial caries	Placebo	500 µg F/g	500 µg F/g TMP 1%	Positive control
ΔKHN	9647.6 ± 521.5[a]	7828.1 ± 1032.2[b]	5818.5 ± 1009.6[c]	3427.6 ± 503.5[d]	4123.6 ± 760.1[d]

Within rows, distinct superscript letters indicate statistical significance (ANOVA, Bonferroni; $p < 0.001$).

Table 3 – Means (± SD) of fluoride (F), calcium (Ca) and phosphorus (P) in enamel blocks according to the treatments.

Variable	Treatments (Dentifrices)			
	Placebo	500 µg F/g	500 µg F/g TMP 1%	Positive control
F, $\mu g/mm^3$	0.61 ±0.14[a] n=11	0.95 ±0.18[b] n=11	1.21 ±0.32[c] n=11	1.44 ±0.43[c] n=11
Ca, $\mu g/mm^3$	749.9 ±92.6[a] n=11	813.9 ±119.2[a] n=11	995.6 ±132.5[b] n=11	1126.0 ±151.3[c] n=11
P, $\mu g/mm^3$	190.7 ±16.3[a] n=11	197.5 ±34.6[a] n=11	227.8 ±23.3[b] n=11	244.1 ±31.3[b] n=11

Within rows, distinct superscript letters indicate statistical significance (ANOVA, Bonferroni; $p < 0.001$).

Anexos

ANEXO A

REFERÊNCIAS BIBLIOGRÁFICAS – INTRODUÇÃO GERAL

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ANEXO B

INSTRUÇÕES AOS AUTORES

Guide for Authors

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ANEXO C

COMITÊ DE ÉTICA – CAPÍTULO 1

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"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMITÊ DE ÉTICA EM PESQUISA –CEP-

OF. 105/2007
CEP
SFCD/bri

Araçatuba, 08 de agosto de 2007.

Referência Processo FOA 2007-01367

O Coordenador do Comitê de Ética em Pesquisa desta Unidade, tendo em vista o parecer favorável da relatora que analisou o projeto **"AVALIAÇÃO "IN SITU" DA AÇÃO ANTICARIOGÊNICA DE DENTIFRÍCIOS DE BAIXA CONCENTRAÇÃO FLUORETO E SUPLEMENTADO COM FOSFATO"** expede o seguinte parecer:

Aprovado:

Informamos a Vossa Senhoria que de acordo com as normas contidas na resolução CNS 215, **deverá ser enviado relatórios parciais em 02/08/2008; 02/08/2009; 02/08/2010 e o relatório final em 02/08/2011.**

Prof. Dr. Stefan Fiúza de Carvalho Dekon
Coordenador do CEP

Ciente/De acordo.
17/08/07
Dr. Alberto Carlos Botazzo Delbem

Ilmo. Senhor
Dr. ALBERTO CARLOS BOTAZZO DELBEM
Araçatuba-SP-

Faculdade de Odontologia e Curso de Medicina Veterinária –
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba – SP
Tel (18) 620-3203 E-mail: diretor@foa.unesp.br

ANEXO D

COMITÊ DE ÉTICA – CAPÍTULO 2

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"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

COMITÊ DE ÉTICA EM PESQUISA – CEP

C E R T I F I C A D O

Certificamos que o Projeto **"Avaliação in situ da ação anticariogênica de dentifrícios de baixa concentração de fluoreto e suplementados com fosfato sobre remineralização do esmalte"**, sob a responsabilidade de Alberto Carlos Botazzo Delbem está de acordo com os Princípios Éticos em Pesquisa e foi aprovado em 16/4/09, de acordo com o Processo FOA-0807/09.

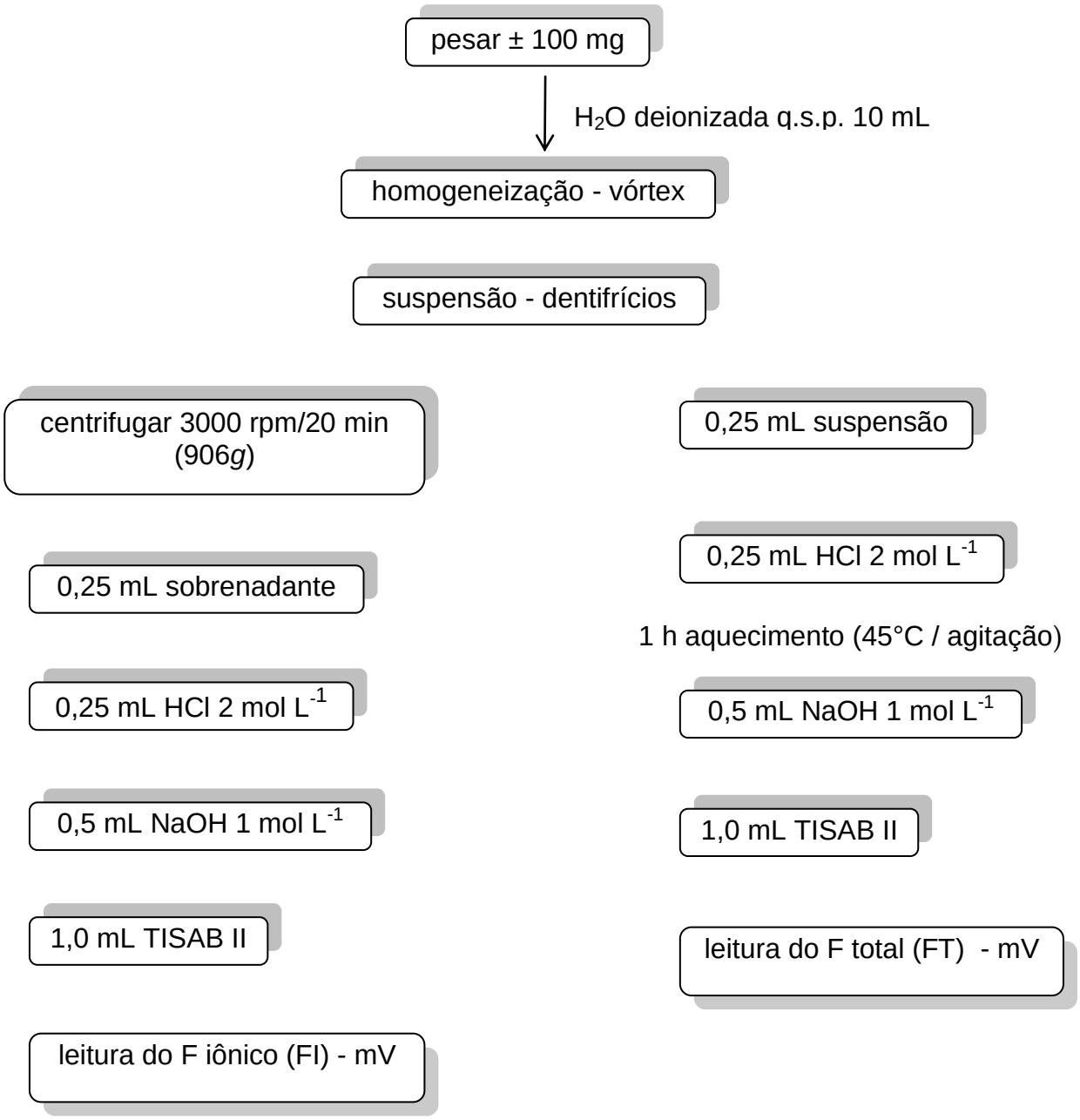
Araçatuba, 24 de abril de 2009.

ALESSANDRA MARCONDES ARANEGA
Vice-Coordenadora do CEP

Faculdade de Odontologia e Curso de Medicina Veterinária - Seção Técnica Acadêmica
Rua José Bonifácio, 1193 CEP 16015-050 Araçatuba – SP
Tel (18) 3636-3225 E-mail: cep@foa.unesp.br

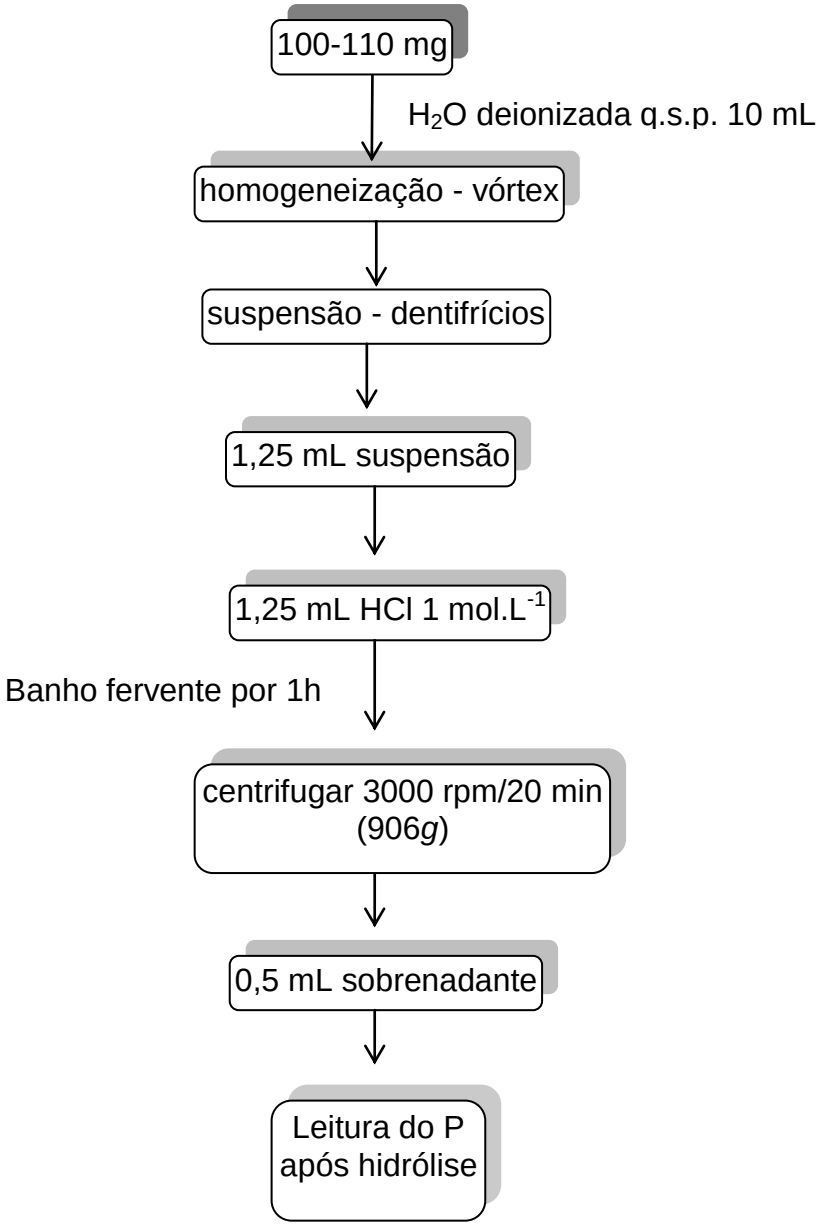
ANEXO E

DOSAGEM DE F NOS DENTIFRÍCIOS – CAPÍTULO 1 E 2



ANEXO F

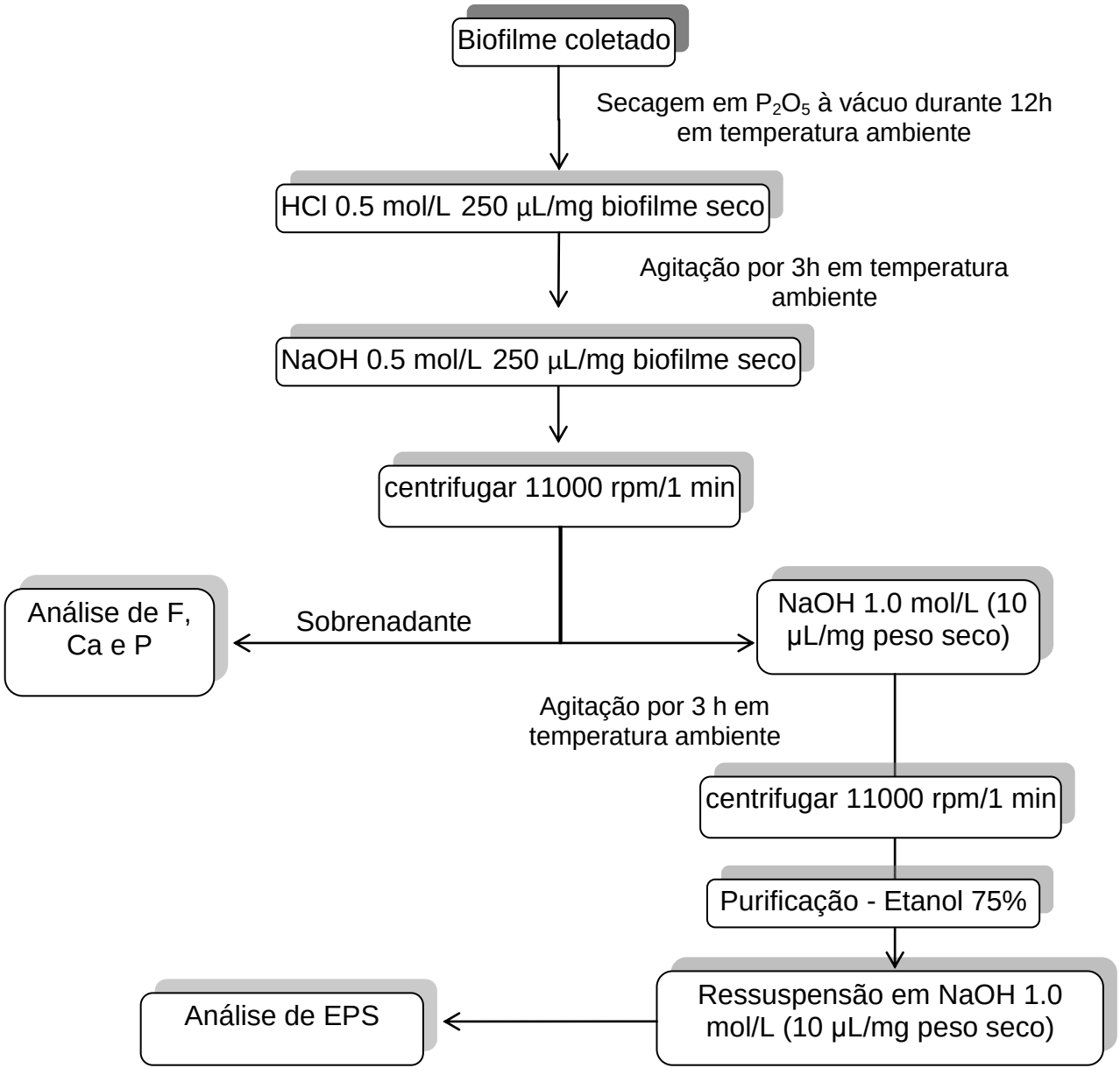
DOSAGEM DE P NOS DENTIFRÍCIOS – CAPÍTULO 1 E 2



ANEXO G

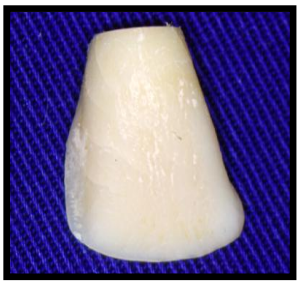
DETERMINAÇÃO DE F, Ca, P e EPS NO BIOFILME – CAPÍTULO 1

1. Após 14 dias, foi realizada a coleta do biofilme formado. O biofilme de cada slots foi analisado separadamente e depois calculado a média para cada fase de cada voluntário.

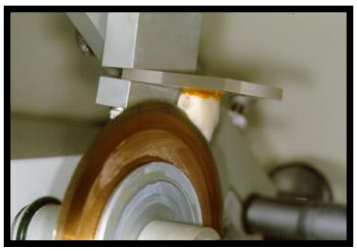


ANEXO H

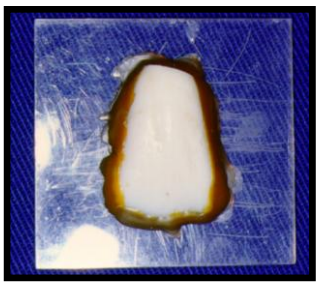
PREPARO E SELEÇÃO DOS BLOCOS DE ESMALTE (4 x 4 mm)



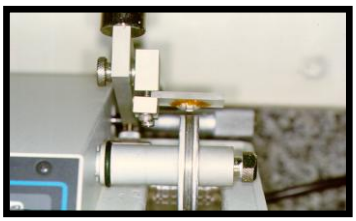
2. Coroa do dente bovino incisivo central inferior, separada da raiz através de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob refrigeração (água destilada/deionizada).



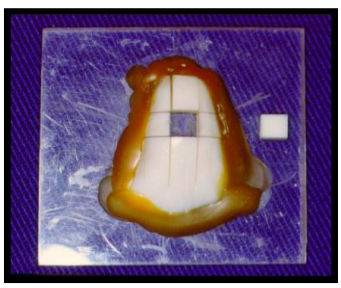
3. Secção da coroa utilizando disco diamantado (série 15 HC Diamond - n. 11-4244 Buehler) separando a superfície vestibular da lingual.



4. Face vestibular fixada na placa de acrílico.



5. 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.

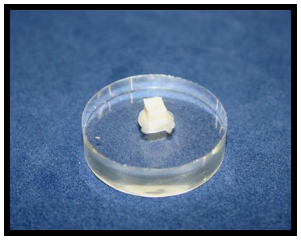


6. Fragmento vestibular do dente bovino, fixado sobre placa de resina. Ao lado, bloco de esmalte dentário.

ANEXO I

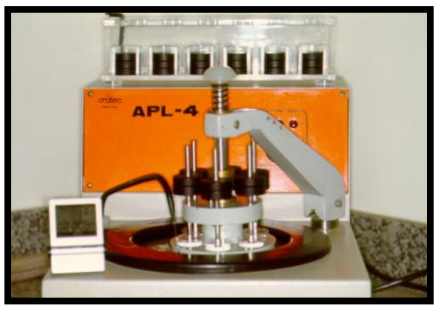
PREPARO E SELEÇÃO DOS BLOCOS DE ESMALTE

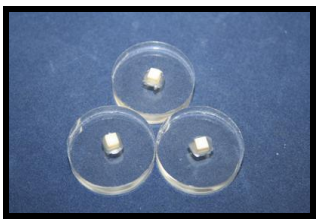
Planificação da dentina e polimento do esmalte



1. 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.

2. 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.





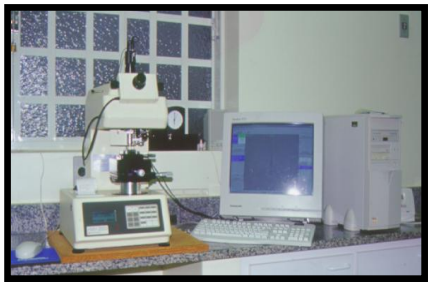
3. Blocos fixados com a superfície do esmalte voltada para cima, a qual será polida.

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;
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.

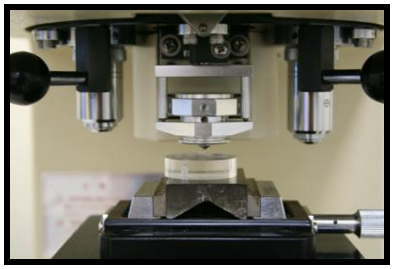
ANEXO J

ANÁLISE DE MICRODUREZA



1. Microdurômetro Shimadzu Micro Hardness Tester HMV-2.000 (Shimadzu Corporation - Kyoto-Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA).

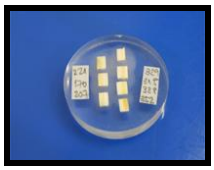
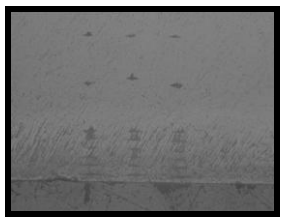
2. Bloco de esmalte sendo submetido à leitura no microdurômetro, carga estática de 25 gramas e tempo de 10 segundos, para análise da microdureza de superfície.



3. Fotomicrografia das impressões para análise de microdureza de superfície inicial (SMH-inicial), e final (SMH-final) (Aumento: 100x).

ANEXO K

MICRODUREZA EM SEÇCÃO LONGITUDINAL

	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/cm2, 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. Corpo de prova – plano longitudinal voltado para a superfície da resina acrílica.
	3. Microdurômetro Shimadzu Micro Hardness Tester HMV-2.000 (Shimadzu Corporation - Kyoto-Japan), com penetrador tipo Knoop, acoplado ao Software para análise de imagem CAMS-WIN (NewAge Industries, USA).
	4. Fotomicrografia das impressões para análise de microdureza de secção longitudinal nas diferentes distâncias. (Aumento: 100x).

Seqüência do polimento de esmalte:

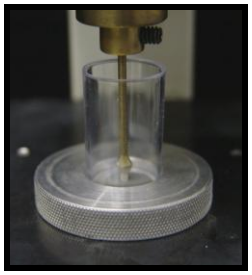
1. Na Politriz APL-4 AROTEC - lixa de granulação 320 (1 minuto – 2 pesos), 600, 800 e 1200 (2minutos – 2 pesos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/deionizada por 2 minutos, entre cada lixa;
2. Na Politriz APL-4 AROTEC - acabamento final com disco de papel feltro MICROCLOTH SUPREME PSA (Buehler Polishing Cloth) (2 minuto – 2 pesos) e suspensão de diamante 1/4 micron base-água (Buehler);
3. Limpeza em lavadora ultrassônica utilizando solução detergente (Ultramet Sonic Cleaning Solution - Buehler) diluída 20:1 em água destilada/deionizada (3minutos);
4. Lavagem durante 30 segundos com jato de água destilada/deionizada.

ANEXO L

MICROABRASÃO

1. Micrômetro acoplado ao aparelho de microscópio modificado.

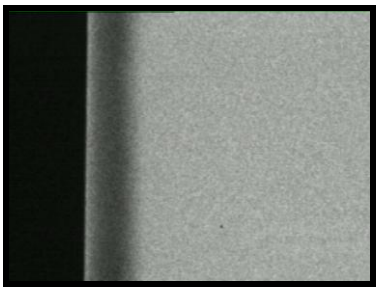
2. Bloco de esmalte adaptado ao mandril, sendo submetido à microabrasão, com desgaste de 50 µm, para análise da incorporação de F, Ca e P no esmalte.



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

ANEXO M

CICLAGEM DE pH e MICRORRADIOGRAFIA TRANSVERSAL – CAPÍTULO 3

	1. Sistema robótico para ciclagem de pH.
	2. Secção dos espécimes em espessura aproximada de 400µm em cortadeira - Well type 3242, Ebner, Mannheim, Germany.
	3. Desgaste bilateral das secções até espessura de aproximadamente 100µm. Abrasivo - partículas de 3µm de Al_2O_3 (Logitech PM4)
	4. Análise: TMR software 1.25, Inspektor Research Systems

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