UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

BEATRIZ MARTINES DE SOUZA

Effect of an experimental paste with hydroxyapatite nanoparticles and fluoride on dental demineralization and remineralization *in situ*

Avaliação do efeito de uma pasta experimental com nanopartículas de hidroxiapatita e fluoreto sobre a desmineralização e remineralização dentária *in situ*

BAURU
2015

BEATRIZ MARTINES DE SOUZA

Effect of an experimental paste with hydroxyapatite nanoparticles and fluoride on dental demineralization and remineralization *in situ*

Avaliação do efeito de uma pasta experimental com nanopartículas de hidroxiapatita e fluoreto sobre a desmineralização e remineralização dentária *in situ*

Dissertation presented to the Bauru School of Dentistry of the University of São Paulo to obtain the degree of Master in Science in the Applied Dental Science Program, Stomatology and Oral Biology concentration area.

Supervisor: Prof. Dr. Ana Carolina Magalhães

Dissertação apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Mestre em Ciências no Programa de Ciências Odontológicas Aplicadas, área de concentração Estomatologia e Biologia Oral.

Orientadora: Profa. Dra. Ana Carolina Magalhães

Versão corrigida

BAURU

2015

Souza, Beatriz Martines de

So89e

Effect of an experimental paste with hydroxyapatite nanoparticles and fluoride on dental demineralization and remineralization *in situ*/ Beatriz Martines de Souza. – Bauru, 2015.

73p. : il. ; 31cm.

Dissertação (Mestrado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientadora: Prof. Drª Ana Carolina Magalhães

Nota: A versão original desta dissertação encontra-se disponível no Serviço de Biblioteca e Documentação da Faculdade de Odontologia de Bauru – FOB/USP.

Comitê de Ética da FOB-USP

Protocolo nº: 15272613.6.0000.5417

Data: 29/05/2013

DEDICATÓRIA

Dedico esta dissertação à minha família, pelo apoio e amor incondicional.

Aos meus pais **Milton** e **Ana Marisa**, pelo exemplo de amor, perseverança e dignidade.

A minha irmã **Juliana**, pela amizade e companheirismo em todos os momentos desde o meu nascimento.

Meu amor por vocês é eterno!

"O amor desculpa tudo, crê tudo, espera tudo, suporta tudo."

Coríntios, 13.7

AGRADECIMENTOS

À **Deus**,

Por me propiciar nascer no amor de uma família maravilhosa, com bons ensinamentos, e fé. Obrigada pelas oportunidades colocadas na minha vida, e por me dar o discernimento de enxergá-las e aproveitá-las. Obrigada por não permitir desistir diante das dificuldades surgidas. Pela força, garra e determinação para enfrentar os problemas.

Obrigada, Senhor, por estar sempre presente em minha vida!

Ao meu pai **Milton,**

Pelo grande exemplo de homem que é, por tudo que enfrentou sem nunca perder a dignidade, honestidade e o caráter. Por me oferecer sempre o abraço acolhedor em todos os momentos sem me julgar. Por todo o amor que sempre tive desde que nasci. Pelo porto seguro que encontro nos seus braços.

À minha mãe **Ana Marisa**,

Por me apoiar em todos os momentos e por acreditar em mim até mesmo quando duvidei. Por ser minha mãe, amiga e cúmplice. Por segurar em minha mão desde o meu nascimento até hoje, sem nunca soltá-la, apesar da distância imposta algumas vezes. Por toda paciência, pela palavra que acalma a alma. Meu exemplo de força sem perder a doçura. Obrigada por não me deixar desistir.

À minha irmã **Juliana,**

Por estar ao meu lado sempre, desde que nasci. Pela amizade e cumplicidade que só irmãos podem propiciar. Obrigada pelo carinho, amor e cuidado que sempre teve comigo. Alguém em quem posso confiar tudo. Você sempre me inspirou, meu exemplo desde criança até hoje!

Obrigada por demonstrar que sempre posso contar com você!

Aos meus **familiares**,

Avós, tios, tias, primos e cunhado, pelo apoio e incentivo que me deram e dão até hoje. O amor e carinho de vocês é o que me motiva a ser melhor, e não desistir dos meus objetivos.

À minha amiga e companheira de trabalho **Lívia**,

Por estar presente em todos os momentos importantes, desde a minha iniciação científica, passando segurança em cada passo que dava durante a pesquisa. Grande parte do que sei fazer no laboratório foi você quem me ensinou. Foi você meu exemplo e inspiração pra entrar no mestrado. Obrigada pela amizade conquistada, pela cumplicidade, pela companhia nas madrugadas do laboratório, pelas risadas, pelos conselhos e pelos desabafos. Sem você, seu apoio e amizade, não teria graça.

Aos meus amigos: **Layla, Leticia Prestes, Rafael, Samuel, Marina Giacomini, Tiago, Luciana e Isabela Sabino,**

Obrigada por fazerem parte da minha vida, por terem feito parte desse processo, seja colando uns bloquinhos, usando aparelho, ou simplesmente escutando meus desabafos e me acalmando nos momentos de crise. Cada um de vocês estará para sempre guardada num lugar especial do meu coração.

Aos colegas do laboratório de Bioquímica: **Priscila, Heloisa, Senda, Cíntia, Cristiane, Polliana, Flávia Levy, Flávia Iano, Aline Dionísio, Mileni, Vinícius, Luiza, Talita, Amanda, Juliana,**

Pela companhia e amizade durante todo esse tempo. Pelo convívio diário, pelas conversas e risadas. A presença de vocês torna o ambiente mais agradável.

Aos alunos de Iniciação Científica: **Mariele, Constantino** e **Victor,** Pela ajuda nas pesquisas, pela troca de conhecimento, pelos momentos engraçados vividos. Vocês sempre vão ser lembrados com muito carinho, meus primeiros alunos, onde comecei a aprender a ensinar e pôr em prática isso. Obrigada pela oportunidade de ter convivido com vocês.

Aos **voluntários** que participaram desta pesquisa,

Agradeço imensamente aos voluntários que aceitaram a participar desta pesquisa por livre e espontânea vontade. Obrigada por se dedicarem ao experimento, por atender as instruções e por sempre estarem prontos a ajudar. Obrigada por terem aguentado firme por longas quatro fases, por não terem desistido. Sem vocês minha pesquisa de mestrado não poderia acontecer. Vocês foram e são partes importantes disso tudo.

Muito obrigada!

Aos professores da discilpina de Bioquimica, **Profª Drª Marília Afonso Rabelo Buzalaf** e **Prof. Dr. Rodrigo Cardoso de Oliveira**

Obrigada pelo convívio de trabalho no departamento de Bioquímica, por sempre serem tão atenciosos e educados, e estarem sempre dispostos a ensinar e ajudar no que fosse possível.

Aos funcionários do departamento de Ciências Biológicas: **Thelma**, **Larissa, Aline, Vera e Dalva,**

Vocês sempre foram muito prestativos, principalmente quando precisei da ajuda de vocês. Obrigada pela atenção e pelo esforço em ajudar.

Às secretárias da pós-graduação: **Fátima**, **Meg** e **Letícia** e à **Maristela**,

Por ajudarem sempre no que fosse preciso em relação a documentos, prazos, relatórios. Muito obrigada pela atenção e dedicação.

À **Faculdade de Odontologia de Bauru-USP**, na pessoa da senhora diretora **Profa. Dra. Maria Aparecida Andrade Moreira Machado**, e do senhor presidente da **Comissão de Pós-Graduação, Prof. Dr. Guilherme dos Reis Pereira Janson,**

Pela oportunidade em realizar pós-graduação em nível de Mestrado nesta instituição, ao qual tenho orgulho e profundo respeito, desde março de 2009, data em que iniciei meu curso de graduação em Odontologia.

Muito obrigada, tenho muito orgulho de ser FOB-USP.

À **Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP)**,

Pelo apoio financeiro concedido durante a realização deste estudo de Mestrado (Processo nº 2013/03942-7), e também ao estudo de Iniciação Científica, realizado anteriormente (Processo nº 2010/07001-4).

AGRADECIMENTOS ESPECIAIS

Agradeço em especial minha **Profª Orientadora Ana Carolina Magalhães,**

Por acreditar na minha competência e confiar em meu trabalho. Tudo começou com uma aula de Bioquímica, onde uma professora entusiasmada se esforçava em ensinar uma disciplina que no auge do meu primeiro ano era extremamente complexa, com toda sua didática, bom humor e jeito acessível e amigável tornava tudo mais fácil, e assim, com o fim da disciplina veio o convite para o estágio no Departamento de Bioquímica, passando minhas primeiras férias (de muitas) em meio ao laboratório, dentes, soluções e artigos, e para alguns isso poderia desestimular, mas com seu jeito de único de lidar com o aluno, só me motivou a querer fazer Iniciação Cientifica na Bioquímica, e isso não foi fácil. Muitas pessoas do primeiro ano se interessaram pelas vagas de Iniciação Científica na Bioquímica, e entre os selecionados não estava meu nome. Isso poderia ter me feito desistir, e talvez não estivesse hoje aqui, mas com coragem e um pouco de medo, lembro-me de marcar uma reunião com você para saber a razão de não ter conseguido e no que eu poderia melhorar, e você me mostrou minha redação, mostrou os pontos que poderiam ser melhores, mostrou a diferença das outras redações, e falou que se eu mantivesse o interesse, no começo do segundo ano teria outra vaga, que eu deveria procurá-la. E não é que em fevereiro eu já estava na FOB e num encontro no corredor você me ofereceu minha primeira Iniciação Científica. Não foi fácil, sei que dei muita dor de cabeça pra você, principalmente quando seu telefone tocava e era eu, chorando falando que tinha quebrado um disco, ou dois, mas mesmo assim não desistimos. Você estava lá pra esclarecer cada dúvida, pra corrigir e incentivar, assim, quando minha pesquisa acabou eu já sabia que iria tentar fazer minha segunda iniciação, com você, é claro. A partir desse momento, minha paixão pela pesquisa, por querer ver os resultados de um trabalho só crescia, junto com minhas dúvidas sobre o futuro, fazer mestrado, especialização, prestar um concurso. Conversar com você, ver sua paixão pela docência, pela pesquisa, por ensinar, só me ajudou a tomar essa decisão tão importante em minha vida, fazer mestrado e optar por seguir essa carreira. Obrigada por todas as explicações prontamente respondidas, pela paciência em me ajudar e corrigir os meus erros, por me ensinar a amar a vida de pesquisadora e a de docência. Você é meu grande exemplo de dedicação e sua paixão pelo que faz é um grande estímulo e me faz seguir à diante. Obrigada por ser minha orientadora, conselheira e amiga.

“Não sei se a vida é curta ou longa para nós, mas sei que nada do que vivemos tem sentido, se não tocarmos o coração das pessoas.”

Cora Coralina

RESUMO

Avaliação do efeito de uma pasta experimental com nanopartículas de hidroxiapatita e fluoreto sobre a desmineralização e remineralização dentária *in situ*

Este estudo avaliou o potencial de uma pasta experimental contendo nano-HAP/fluoreto sobre a redução da desmineralização e o aumento da remineralização dentária *in situ*. Treze indivíduos participaram de 4 fases cruzadas e duplo cegas (14 dias cada). Quatro amostras hígidas (H) e quatro pré-desmineralizadas (MB) foram utilizadas intraoralmente por fase correspondente aos seguintes tratamentos: Nanop Plus (10% de hidroxiapatita + 900 ppm F), MI Paste Plus (CPP-ACP + 900 ppm F) , F (900 ppm F) e placebo (sem ingrediente ativos). Para isso, 480 amostras (240 de esmalte e 240 de dentina) foram selecionadas com base nos valores de microdureza de superfície, sendo a metade submetida à desmineralização (MB, solução desmineralizante com pH 5, durante 6 dias e 7 dias, respectivamente) e a outra metade permaneceu hígida (H). As amostras H foram protegidas por tela plástica para o acúmulo de biofilme; enquanto sobre as amostras MB, biofilme não foi acumulado para possibilitar a remineralização. As amostras H foram posteriormente expostas a um desafio cariogênico (20% de sacarose, 8x5min/dia para esmalte e 4x5min/dia para dentina). Os tratamentos foram feitos 2x4 min/dia, extra-oralmente. A des-remineralização foi quantificada por microradiografia transversal. Os dados foram analisados estatisticamente por ANOVA de medida repetidas seguida pelo teste de Tukey ($p < 0,05$). Em relação à desmineralização de dentina, a Nanop Plus apresentou o melhor efeito sobre a redução da ΔZ (% minxμm), enquanto que todos os tratamentos foram capazes de reduzir similarmente a profundidade da lesão (μm) em comparação ao placebo: Nanop Plus (780,5 ± 212,0; 98,8 ± 26,2); MI Paste Plus (876,0 ± 268,4; 95,7 ± 30,5); F (900,5 ± 236,3; 96,0 ± 26,1); Placebo (1188,2 ± 502,5; 142,7 ± 28,0), respectivamente ($p<0,05$). Para a desmineralização do esmalte, nenhum tratamento foi capaz de reduzir o ΔZ e a profundidade da lesão em comparação ao placebo: Nanop Plus (1.000,9 ± 249,5; 45,0 ± 15,3); MI Paste Plus (883,6 ± 431,7; 60,7 ± 26,4); F (985,5 ± 313,4; 53,4 ± 21,1); Placebo (1369,6 ± 988,3; 57,2 ± 30,6), respectivamente. No que diz respeito à remineralização da dentina, todos os tratamentos foram igualmente capazes de aumentar o ganho

mineral (ΔΔZ) em comparação ao placebo: Nanop Plus (910,1 ± 328,8); MI Paste Plus (964,2 ± 446,4); F (902,1 ± 606,8); Placebo (337,9 ± 408,2) ($p<0,05$). No esmalte, entretanto, apenas os tratamentos com Nanop Plus e F foram eficazes no aumento do ganho mineral (ΔΔZ) em comparação ao placebo: Nanop Plus (549,9 ± 405,4); MI Paste Plus (370,8 ± 230,6); F (555,5 ± 264,1); Placebo (200,4 ± 186,8) ($p<0,05$). A Nanop Plus é mais eficiente que a MI Paste Plus na redução da desmineralização na dentina e no aumento da remineralização do esmalte. Nenhum tratamento foi capaz de reduzir a desmineralização no esmalte, enquanto todos os tratamentos aumentaram a remineralização da dentina.

Palavras-chave: Cárie Dentária. Dentina. Desmineralização do dente. Esmalte dentário. Nanotecnologia. Remineralização Dentária

ABSTRACT

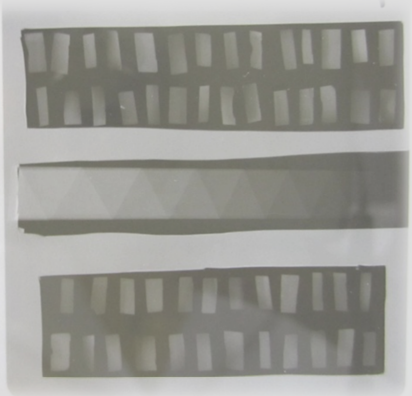
Effect of an experimental paste with hydroxyapatite nanoparticles and fluoride on dental demineralization and remineralization *in situ*

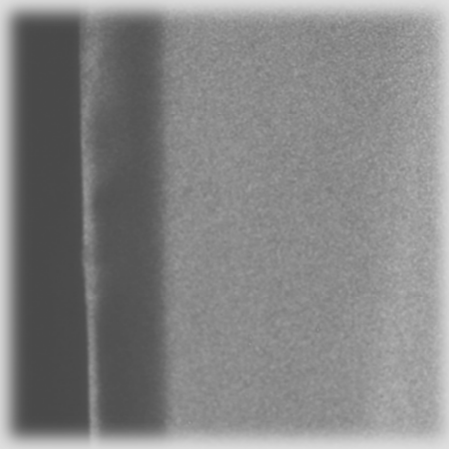
This study evaluated the potential of an experimental paste containing nano-HA/fluoride on the reduction of dental demineralization and on the increase of dental remineralization *in situ*. Thirteen subjects took part in this crossover and double-blind study performed in 4 phases (14 days each). Four sound (S) and 4 pre-demineralized (WS) specimens were worn intraorally at each phase corresponded to the following treatments: Nanop plus (10% hydroxyapatite + 900 ppm F), MI Paste Plus (CPP-ACP + 900 ppm F), F (900 ppm F) and placebo (without active ingredients). For that, 480 specimens (240 enamel and 240 dentin) were selected by using surface microhardness; half of the samples were subjected to demineralization (WS, demineralizing solution at pH 5, for 6 and 7 days, respectively) and the other half remained sound (S). S specimens were protected from disturbance by using plastic mesh to allow biofilm accumulation; while on WS no biofilm accumulation was allowed to facilitate remineralization. S specimens were further exposed to severe cariogenic challenge (20% sucrose, 8x5min/day for enamel and 4x5min/day for dentin). The treatments were done 2x4 min/day, extraorally. The de-remineralization was quantified by transversal microradiography. The data were statistically analyzed using Repeated-Measures ANOVA followed by Tukey's test ($p<0.05$). In respect to dentin demineralization, Nanop Plus had the best effect on the reduction of ΔZ (%minxμm), while all treatments were similarly able to reduce to the lesion depth (μm) compared with placebo: Nanop Plus (780.5 ± 212.0, 98.8 ± 26.2); MI Paste Plus (876.0 ± 268.4; 95.7 ± 30.5); F (900.5 ± 236.3; 96.0 ± 26.1); Placebo (1188.2 ± 502.5; 142.7 ± 28.0), respectively ($p<0.05$). For enamel demineralization, no treatment was able to reduce ΔZ and lesion depth compared to placebo: Nanop Plus (1000.9 ± 249.5, 45.0 ± 15.3); MI Paste Plus (883.6 ± 431.7; 60.7 ± 26.4); F (985.5 ± 313.4, 53.4 ± 21.1); Placebo (1369.6 ± 988.3, 57.2 ± 30.6), respectively. In respect to dentin remineralization, all treatments were similarly able to improve mineral uptake (ΔΔZ) compared to placebo: Nanop Plus (910.1 ± 328.8); MI Paste Plus (964.2 ± 446.4); F (902.1 ± 606.8); Placebo (337.9 ± 408.2) ($p<0.05$). For enamel, only the Nanop Plus and F were effective in increasing mineral uptake (ΔΔZ) compared to

placebo: Nanop Plus (549.9 ± 405.4); MI Paste Plus (370.8 ± 230.6); F (555.5 ± 264.1); Placebo (200.4 ± 186.8) ($p<0.05$). Nanop Plus is more effective than MI Paste Plus on the reduction of dentin demineralization and the increase of enamel remineralization. No treatments were able to reduce enamel demineralization, while for dentin remineralization all treatments were effective.

Key-words: Demineralization. Dental Caries. Dental Enamel. Dentin. Nanotechnology. Tooth Remineralization

SUMÁRIO





1 INTRODUÇÃO

1 INTRODUCTION

Dental caries is a disease affecting teeth due to demineralization provoked by exposure to acids from microorganism metabolism, which is established in patients with cariogenic biofilm and high sugar intake, especially of sucrose (TEN CATE et al., 2003; TAKAHASHI; NYVAD, 2011). The main acid produced by the bacteria is lactic acid, which when released reduces the biofilm pH below 5.5 (between 4.5 to 5.5), which associated with the concentration of fluoride, calcium and phosphate in biofilm, can generate a surface (in initial stages) and a subsurface demineralization (more advanced stages) that is clinically seen as white spot lesion (not cavitated lesion). During this stage, it is still possible reverse the process, and consequently the need of restorative treatment (TEN CATE et al., 2003, AMMARI et al., 2014; ASSUNÇÃO; DA COSTA; BORGES, 2014).

In the last decades, increase in oral health levels in most industrialized and developing countries, like Brazil, has been observed with a significant reduction in the prevalence and severity of dental caries (NARVAI et al., 2006; BÖNECKER et al., 2010; BOING et al., 2014). Face to this change in public oral health, factors associated with the development of the disease as social and behavioral factors, have been widely discussed (NARVAI et al., 2006; BOING et al., 2014).

The decline in dental caries prevalence worldwide was accompanied by a phenomenon known as polarization of the disease, in which the disease and the need of treatment is concentrated in a small portion of the population (HOFFMANN et al., 2004; MARTINS et al., 2006; SALES-PERES et al., 2008; BOING et al., 2014). In the places with little access to fluoride and/or preventive programs, the worst case of the disease is seen, characterizing significantly health inequities (CARDOSO et al., 2003; NARVAI et al., 2006; LEMOS et al., 2014), which requires the attention of authorities and appropriate interventions in health (HUGO et al., 2007; SALES-PERES et al., 2008; BOING et al., 2014). As a result of this phenomenon, treatment must be focused on this specific population (high-risk groups).

For the high-risk population, the regular fluoride exposure (from water and toothpaste) fails to reduce the development of new lesions and the progression of pre-existing lesions, especially when oral health instruction programs are not present

(LEMOS et al., 2014). More specific strategies associated with educational activities and adequate oral hygiene practices can significantly contribute to changes in the epidemiology of dental caries (GUSHI et al., 2008).

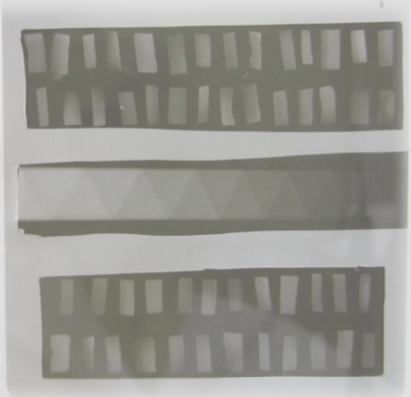
Currently new technologies and biofunctional materials have been developed to positively interfere in the process of tooth de-remineralization, bringing new perspectives in the treatment of initial caries lesions. Whereas hydroxyapatite (HA) is the main constituent of the inorganic phase of the tooth, products with similar chemical and structure characteristics able to provide calcium and phosphate ions in adequate concentrations and velocity could reflect on promising results in remineralization by replacement of calcium and phosphate ions into demineralized tissue and the formation of a protective layer of HA on the tooth surface with cohesive and acid resistance similar to the natural tooth (COCHRANE et al., 2010; HUANG et al., 2011; NONGONIERMA; FITZGERALD, 2012).

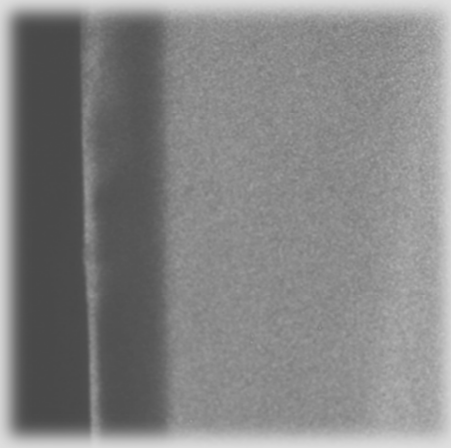
Accordingly, pastes containing different concentrations of nano-hydroxyapatite (Nano-HA) have shown able to remineralize enamel carious lesion *in vitro*, in particular those containing 10% Nano-HA (HUANG et al., 2011; TSCHOPPE et al., 2011; NAJBFARD et al., 2011), but we still have limited information on their potential on dentin and its effect in preventing tooth demineralization (TSCHOPPE et al., 2011) The advantage of using nano-sized particles is that they present similar morphology, crystal structure, solubility and biocompatibility compared with dental apatite, playing an essential role in the formation and remineralization of hard tissue (BALASUNDARAM; SATO; WEBSTER, 2006).

Commercial products containing other calcium phosphate salts, with similar mechanism of action as Nano-HA, have also been tested. Pastes containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) have shown potential to prevent tooth demineralization and hypersensitivity and to increase remineralization *in vitro* (KUMAR; ITTHAGARUN; KING, 2008, PULIDO et al., 2008; CAO et al., 2013) as well to repair initial enamel caries lesion *in vivo* (YENGOPAL; MICKENAUTSCH, 2009; BAILEY et al., 2009; LI et al., 2014).

Nano-HA (10% or 20%) has been compared to CPP-ACP, and both were not effective in reducing tooth demineralization *in vitro* (COMAR et al., 2013). However, we still have doubts if in the clinical situation the results would be the same. Saliva

and Biofilm seem to have an important interaction with Nano-HA paste in contact with the tooth (ZHANG et al., 2015). An *in situ* model would help us to understand the effect of Nano-HA paste in a situation closer to the *in vivo* condition compared to *in vitro* study, where Nano-HAP could interact with saliva and biofilm. Therefore, the aim of this study was to evaluate the potential of an experimental paste containing hydroxyapatite nanoparticles on the reduction of dental demineralization and the increase of dental remineralization compared to CPP-ACP paste (commercial control) *in situ*. The null hypothesis is that experimental Nano-HA paste has the same effect compared with CPP-ACP paste on enamel and dentin demineralization and remineralization.





2 ARTIGO

2 ARTICLE

Article formatted according to Caries Research Guidelines.

Effect of an experimental paste with hydroxyapatite nanoparticles and fluoride on dental demineralisation and remineralisation *in situ*

Souza BM[1], Comar LP[1], Vertuan M[1], Fernandes Neto C[1], Buzalaf MAR[1], Magalhães AC[1*]

[1]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil.

Short title: Effect of Nano-HA/Fluoride on dental caries *in situ*

Keywords: Demineralization, Dental Caries, Dental Enamel, Dentin, Hydroxyapatite, Nanotechnology, Tooth remineralization

***Corresponding author:** Present address: Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo. Al Octávio Pinheiro Brisolla 9-75. 17012-901, Bauru-SP, Brazil. Phone/Fax: +55 14 32358497. E-mail: acm@fob.usp.br

Declaration of Interest: the authors declare no conflict of interests.

Abstract

This study evaluated the effect of an experimental paste containing nano-HA/fluoride on dental de-remineralisation *in situ*. Thirteen subjects took part in this crossover/randomised/double-blind study performed in 4 phases (14 days each). Four sound (S) and 4 pre-demineralised (PD) specimens were worn intraorally at each phase corresponded to the following treatments: Nanop Plus (10% hydroxyapatite, 0.2% NaF, nano-HA/Fluoride), MI Paste Plus (CPP-ACP, 0.2% NaF), F (0.2% NaF) and placebo. Two-hundred and forty enamel and 240 dentine specimens were selected by using surface microhardness; half of them were subjected to pre-demineralisation (PD) and the other half remained sound (S). S specimens were further exposed to severe cariogenic challenge (20% sucrose in biofilm) *in situ*, while PD was not. All specimens were exposed to fluoride dentifrice's slurry 2x1 min/day. Thereafter, the treatments were done for 4 min. The de-remineralisation was quantified by transversal microradiograph. The data were statistically analysed by Repeated-Measures ANOVA/Tukey's tests ($p<0.05$). In respect to dentine demineralisation, Nanop Plus had the best effect on the reduction of integrated mineral loss (ΔZ), while all treatments were similarly able to significantly reduce the lesion depth (LD) compared to placebo. For enamel, no treatment was able to reduce ΔZ and LD compared to the placebo. For dentine remineralisation, all treatments were similarly able to improve integrated mineral uptake ($\Delta\Delta Z$) compared to the placebo; while for enamel, only Nanop Plus and F were effective in increasing $\Delta\Delta Z$ compared to the placebo. Nanop Plus seems to be a promising agent to positively influence dental de-remineralisation.

Introduction

New biofunctional materials have been developed to positively interfere in dental caries development and progression, bringing new perspectives for high-risk patients. Accordingly, materials containing hydroxyapatite (HA), the main constituent of the inorganic phase of the tooth, may be able to provide calcium and phosphate ions for reducing tooth demineralisation and/or improving tooth remineralisation. HA in nanoparticles (nano-HA) may penetrate tooth porosities and produce a protective layer on the tooth surface similar to natural tooth [Cochrane et al., 2010; Huang et al., 2011; Nongonierma and Fitzgerald, 2012]. The advantage of using nano-HA compared to micro-HA is that the former has similar morphology, size, crystal structure, solubility and biocompatibility compared to dental apatite [Balasundaram et al., 2006].

Accordingly, materials containing different concentrations of nano-HA, in particular those containing 10% nano-HA, have shown the ability to remineralise bovine and human enamel and dentine carious lesion *in vitro* and *in situ* [Huang et al., 2011; Tschoppe et al., 2011; Najbfard et al., 2011]. However, we still have limited information on their effect on dentine and their potential to prevent tooth demineralisation.

Commercial materials containing other calcium phosphate salts, with a similar mode of application and indication as nano-HA, have also been tested. Pastes containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) have shown potential to prevent tooth demineralisation and hypersensitivity and to increase tooth remineralisation *in vitro* [Kumar et al., 2008; Cao et al., 2013; Zhou et al., 2014]. Furthermore, CPP-ACP paste is able to increase remineralisation of initial enamel caries lesion based on *in situ* studies and randomised clinical trials, although it does not appear to be significantly different from that of fluorides [Yengopal and Mickenautsch, 2009; Bailey et al., 2009; Li et al., 2014; Meyer-Lueckel et al., 2015].

We have compared experimental nano-HA pastes (10% or 20%, with or without F) and CPP-ACP pastes (with or without F), and both were ineffective in reducing tooth demineralisation *in vitro* [Comar et al., 2013]. However, we still have doubts about whether the results would be the same in the clinical situation. Saliva and biofilm seem to interfere in the interaction between nano-HA paste and the tooth

[Zhang et al., 2015]. Therefore, an *in situ* model would help us to improve understanding about the effect of nano-HA paste compared to our *in vitro* study, since the model allows for the presence of human saliva and the formation of dental biofilm in the oral cavity, better simulating the clinical condition.

Therefore, the aim of this study was to evaluate the potential of an experimental paste containing nano-HA/fluoride on the reduction of dental demineralisation and the increase of dental remineralisation compared to CPP-ACP paste (commercial control) *in situ*. The null hypothesis is that our experimental nano-HA paste has the same effect compared with CPP-ACP paste on enamel and dentine demineralisation and remineralisation.

Material and Methods

Ethical aspects and volunteers selection

The study followed a double-blind, randomised and crossover protocol, comprising 4 phases of 14 days each, with an interval of 5 days among them. Thirteen healthy adults (11 women and two men, 19-28 yr old) were included according to the inclusion and exclusion criteria. The inclusion criteria included: good oral health (no active caries or acute gingivitis or periodontics); stimulate physiological salivary flow rate of > 1 ml/min; and non-stimulated physiological salivary flow rate of > 0.25 ml/min. The exclusion criteria included: systemic illness; pregnancy or breastfeeding; use of orthodontics appliances; professional fluoride application in the last 2 months; and smokers.

A number of 12 volunteers was previously calculated based on the results (percentage of remineralisation, 29.4% MI Paste Plus and 3.7% placebo) of a previous *in situ* study [Shen et al., 2011], considering a power of 80% (α=0.05).

This study was conducted according to the guidelines of good clinical practice and conformed to the Declaration of Helsinki. Ethical approval for this study involving human subjects was granted by the local Ethics Committee (no. 15272613.6.0000.5417; Ethics Committee of the Bauru School of Dentistry, University of São Paulo, SP, Brazil).

The subjects received written instructions, including schedules, and were trained for all procedures required during the study. Informed consent was obtained from all subjects before starting the study.

Preparation of the specimens

Two-hundred and forty enamel and dentine specimens (4mm x 4mm x 3mm) were prepared from the labial surface of the bovine crown and labial/lingual surfaces of the cervical portion of bovine roots, respectively. The teeth were stored in 0.1% buffered thymol solution (pH 7.0) at 4ºC. The specimens were cut using an ISOMET Low Speed Saw cutting machine (Buehler Ltd., Lake Bluff, USA) separated by a 4-mm-thick spacer. The specimens' surfaces were ground flat with water-cooled silicon-carbide discs (320, 600 and 1200 grades of Al_2O_3 papers; Buehler, Lake Bluff,

USA) and polished with felt paper wetted with diamond spray (1 µm; Buehler Ltd., Lake Bluff, USA), resulting in removal of approximately 200 µm depth of the enamel and dentine. This was controlled with a micrometer. After polishing, the specimens were cleaned in an ultrasonic device with deionized water for 10 minutes. The initial surface hardness was measured at the beginning of the study (SH baseline) by the mean of five indentations (Knoop diamond, 25 g /10 s for enamel and 10 g /15 s for dentine) using a microhardness tester (HMV-2; Shimadzu Corporation, Tokyo, Japan). Specimens with microhardness values 10% lower or 10% higher than the mean of all specimens were excluded from the study.

Half of enamel and dentine specimens (n=120) were then demineralised using a solution containing 3 mM $CaCl_2.2H_2O$ (Labsynth, São Paulo, Brazil), 3 mM KH_2PO_4 (Sigma-Aldrich, St Louis, USA), 50 mM lactic acid (Sigma-Aldrich), 6 µM Tetraethyl methylhydroxidiphosphonate (Sigma-Aldrich), and traces of thymol (Sigma-Aldrich), pH 5.0 (10 M KOH to adjust pH) (30 mL per specimen), for 7 days (dentine) and 6 days (enamel) at 37 ºC [Buskes et al., 1985; Magalhães et al., 2009; Moron et al., 2013]. Before demineralisation, one-third of the surface was covered with nail varnish to create a sound-control surface. After demineralisation, a second third of the surface was covered with nail varnish (demineralised control surface), leaving free a central band of demineralized surface (1 mm x 4 mm) that was further treated *in situ*. The surface microhardness of the pre-demineralised enamel/dentine (SH lesion, %SHC, percentage of surface microhardness change) was measured immediately after demineralisation.

The other half of the specimens (n=120) remained sound. Two-thirds of the surface was also covered with nail varnish in order to create control areas (two sound surfaces) at either side of the central band of surface (1 mm x 4 mm) that was further demineralised and treated *in situ*. SH baseline (for S) and % SHC (for PD) means were used for the computerised random allocation sequence of the specimens in the experimental phases, volunteers and position in the appliance.

In Situ Protocol

Acrylic palatal appliances were prepared individually for each subject and for each phase (author L.P.C.). They were made with eight cavities (5 mm x 5 mm x 4

147 mm) and distributed on the left and right sides. Before the specimens' insertion in the
148 palatal appliances, they were disinfected by dipping in 70% alcohol solution for 30
149 min, in addition to the previous immersion in thymol solution, and then re-hydrated in
150 distilled water per 30 min [Schlueter et al., 2009; Comar et al., 2012]. Enamel and
151 dentine specimens were randomly accommodated into the cavities and fixed in place
152 with wax, totalizing two sound (S) and two pre-demineralised (PD) enamel and
153 dentine specimens per appliance (in each phase). For S specimens, a 4 mm-deep
154 space was created in the acrylic appliance, leaving a 1.0 mm space for biofilm
155 accumulation. In order to enhance the growth of dental biofilm, the samples were
156 protected from mechanical disturbance by plastic mesh (pores area of 1mm^2,
157 Sanremo, São Paulo, Brazil) fixed with wax and acrylic resin on the acrylic surface
158 [Magalhães et al., 2007]. The PD specimens were placed on the same level as the
159 acrylic, with no space for biofilm accumulation, allowing remineralisation. The S and
160 PD specimens were replaced after each phase.

161 The study comprised 4 crossover phases of 14 days each, with an interval
162 period of 5 days. In each phase, three or four volunteers were randomly assigned to
163 one of the four treatments, as following: Nanop Plus (10% HA + 0.2% NaF, nano-
164 HA/Fluoride), MI Paste Plus (Recaldent technology, CPP-ACP + 0.2% NaF; GC
165 America, St. Alsip, USA), F (0.2% NaF) and placebo (paste without active
166 ingredients). The experimental pastes (Nanop Plus, F and placebo) were prepared
167 by FGM-Denstcare (Joinvile, Brazil) with the following composition: nanometer
168 calcium phosphate (hydroxyapatite, 100 nm size, for Nanop Plus), sodium fluoride
169 (Nanop Plus and F), potassium nitrate, distilled water, thickener, surfactant, moist,
170 flavour, sweetener and preservative.

171 During the *in situ* phase, the S specimens were exposed to severe cariogenic
172 challenge (20% sucrose, 8x5min/day for enamel and 4x5min/day for dentine,
173 extraorally, 1 drop/specimen) [Ccahuana-Vasquez et al., 2007]. The sucrose solution
174 was renewed every 3 days of the experiment [Aires et al., 2006; Comar et al., 2012].
175 No sucrose solution was applied to PD specimens.

176 The subjects applied 1 drop of fluoride dentifrice slurry (1 g dentifrice: 3 ml
177 water), 2 times x 1 minute per day, extraorally, and then washed the specimens.
178 Thereafter, 1 drop of the treatment slurries (1 g paste: 3 ml water) was applied per

specimen, extraorally, 2 times x 4 minutes per day, once in the morning (before the first cariogenic challenge) and once in the evening (after the last cariogenic challenge). After 4 minutes of treatment, the volunteers repositioned the appliance in the mouth and were instructed not to rinse or take it off for the next 30 minutes. All treatment slurries were placed in similar tubes to allow blindness (authors C.F.N. and M.V.).

During the *in situ* phases, the appliance was only removed for the main meals (four times a day, maximum 1-hour duration each, 2-3 h interval between meals) and for the extraoral treatment times (sucrose, dentifrice and paste applications). The subjects were instructed to keep their usual eating habits and to perform oral hygiene using a toothbrush (Colgate, São Bernardo, Brazil), dental floss and fluoridated dentifrice (1500 ppm F, NaF, Crest, Procter & Gamble, Cincinnati, USA) provided by the researchers during all stages of the study. Only the palatal surface of the appliance could be brushed. The subjects were also instructed not to use any other type of fluoride or antibacterial product during the study. At the end of each phase, the appliances of all subjects were collected and washed with deionised water, and the specimens were prepared for transverse microradiography (TMR) analysis (author L.P.C.).

TMR analysis

The specimens were perpendicularly sectioned to the orientation of the protective nail varnish, allowing all areas to be included in the TMR specimen (sound, demineralised and de-remineralised surfaces). The fragments were then handily polished to obtain a specimen of an approximate thickness of 100 µm using water-cooled silicon-carbide discs (600-grade papers ANSI grit; Buehler, Lake Bluff, USA). The final thickness of each specimen was checked with a micrometer (author L.P.C.). The dentine specimens were immersed in ethylene glycol (Sigma-Aldrich, Steinheim, Germany) for 24 h before the analysis to avoid shrinkage during X-ray exposure due to dehydration [Buchalla et al., 2003].

Microradiographs were made of each tooth fragment in combination with “stepwedge” (14 slices, ± 30 µm thick, 99.9% Al) for calibration, using glass plates that were exposed to X-ray Cu Ka (20 KV and 20 mA) for 13 min. Each plate

contained 32 enamel and 60 dentine specimens per exposure. The X-ray was directed perpendicularly to the lesion at a distance of 30 cm. After each exposure, the glass plate was developed (7 min, 20 ° C), fixed (7 min, 20 ° C) and washed with running water (10 min).

The developed plate was analysed using a transmitted light microscope fitted with a 20x objective (Axioplan, Zeiss, Oberkochen, Germany) and a CCD camera (XC-77 CE, Sony, Tokyo, Japan) coupled to a computer with software for calculations (TMR 2012 and TMR 2006 software, Inspector Research BV, Amsterdam, Netherlands) (author B.M.S.). The microradiography were saved as images with a 640x480 pixels resolution and 256 grey scales [Buchalla et al., 2008; Magalhães et al., 2009].

We calculated the integrated mineral loss (ΔZ) and lesion depth (μm) using the formula by Angmar et al. [1963]. The ΔZ (%min.volxμm) is the product of the difference between the percentage of mineral volume of sound tooth (87% and 50% for enamel and dentine, respectively) and the percentage of mineral volume of demineralised tooth (%min.vol) in relation to the lesion depth (μm). The lesion depth (LD) is defined by the distance from the surface (0 vol% min) to the depth at which the enamel/dentine again has a mineral content equal to or greater than 95% of the mineral content of sound tooth (> 82.5% and > 47.5% mineral content, for enamel and dentine, respectively). For PD specimens, we further calculated the difference between demineralised area and de-remineralised area: ΔΔZ/ΔL was calculated by the difference between ΔZ/LD lesion and ΔZ/LD effect after the treatments *in situ*. The maximum mineral content of the surface layer (Z max, %min.vol), the thickness of the pseudo-intact surface layer (SL, μm) and R-value (ΔZ/depth, %min.vol, representing the average mineral loss) were also calculated [Schirrmeister et al., 2007].

Statistical analysis

Data were statistically analysed using the software Graph Pad Instat for Windows (GraphPad Software, San Diego, USA). Repeated measures ANOVA followed by Tukey's test were applied to compare the different treatments, considering separately each substrate (S and PD, enamel and dentine) (author

A.C.M.). The sample size was 13 (considering the number of subjects as sample), and the significance level for all tests was set at 5%.

Results

No volunteers dropped out or were excluded from the study. However, we lost some specimens during TMR preparation (around 28% enamel and 10% dentine specimens).

Generally, the treatments were more effective on dentine than on enamel. In respect to dentine demineralisation, Nanop Plus was the unique treatment with significant effect on the reduction of ΔZ and R when compared with placebo, while all treatments were similarly able to reduce the LD compared with the placebo (Table 1, $p<0.05$). Fluoride alone had a superficial effect by increasing SL and Z max parameters of dentine. For enamel demineralisation, no treatment was able to reduce ΔZ and LD compared to the placebo (Table 2). Figures 1 and 2 show a representative microradiography picture and the mineral profile for dentine and enamel S (after demineralisation), respectively.

In respect to remineralisation, the pre-demineralised specimens did not have significant differences among them with respect to the baseline integrated mineral loss (Enamel: [Nanop Plus ΔZ= 2724.08±501.21; MI Paste Plus ΔZ= 2937.08±545.35; F ΔZ= 2688.77±633.15; Placebo ΔZ= 2453.77±757.15 %min.volxμm] dentine: [Nanop Plus ΔZ= 2554.17±750.36; MI Paste Plus ΔZ= 2254.55±624.50; F ΔZ= 2325.00±623.30; Placebo ΔZ= 2160.46±393.99 %min.volxμm]).

For dentine, all treatments were similarly able to improve the integrated mineral uptake (ΔΔZ) compared to the placebo ($p<0.05$); F alone also improved mineral uptake mean (ΔR); no effect was found among the treatments for ΔLD (Table 3). For enamel, only Nanop Plus and F were effective in significantly increasing ΔΔZ compared to the placebo; however, only Nanop Plus significantly reduced ΔLD ($p<0.05$). Fluoride alone significantly increased the mineral content at the enamel surface layer (Zmax). For ΔR and ΔSL parameters, there were no differences among the treatments. Figures 3 and 4 show a microradiograph picture and mineral profile of dentine and enamel PD (before and after remineralisation), respectively.

Discussion

Previous studies have shown that pastes and toothpastes containing nano-HA have some potential to increase enamel and dentine remineralisation *in vitro* and *in situ* [Huang et al., 2011; Tschoppe et al., 2011; Najibfard et al., 2011; Pepla et al., 2014]. However, there are few studies about its effect on dentine de-remineralisation, and most of them analysed the desensitising effect or morphological changes on dentine [Wang et al., 2012; Cao et al., 2013; Orsini et al., 2013; Wang et al., 2014].

Products containing CPP-ACP have also shown some potential to prevent dental demineralisation and hypersensitivity as well as to increase remineralisation *in vitro* [Kumar et al., 2008; Cao et al., 2013; Zhou et al., 2014]. Furthermore, CPP-ACP paste is able to increase remineralisation of initial enamel caries lesion based on *in situ* studies and randomised clinical trials, although it does not appear to be significantly different from fluorides [Yengopal and Mickenautsch, 2009; Bailey et al., 2009; Li et al., 2014; Meyer-Lueckel et al., 2015]. There is no scientific evidence for the benefit of the application of CPP-ACP, according to Systematic Reviews [Chen et al., 2013; Li et al., 2014].

Despite this clinical limitation, CPP-ACP is a commercial product whose modes of application and indication are similar to nano-HA. Therefore, we have chosen this product as a commercial control. The placebo paste was produced without active ingredients but with the same basic components as the experimental pastes containing nano-HA/Fluoride and F. Except the placebo, all pastes have the same fluoride concentration (0.2% NaF, 900 ppm F).

In the present study, the experimental pastes were applied twice a day per 4 minutes, following the company's guidelines, and as slurry, to simulate its dilution by saliva. We decided to include the application of dentifrice slurry before the treatment to simulate the residual effect of fluoride after toothbrushing as done by Meyer-Lueckel et al. [2015]. It is also important to highlight that the experimental pastes are not dentifrices; therefore, they must be applied after oral hygiene by the patient 2 times a day (in early morning and late evening before sleeping) [Behnan et al., 2010; Comar et al., 2013]. After finger application, the patient is advised to wait 4 minutes to remove the excess of paste.

While enamel is a highly mineralised tissue containing more than 95% of minerals (such as hydroxyapatite) organised in nanostructure crystals arranged in parallel arrays-enamel rods [Baldassarri et al., 2008], dentine is a calcified collagen matrix, in which hydroxyapatite is classified as intrafibrillar crystallites found within the hole zones and pore spaces of collagen fibrils [Cao et al., 2013].

Due to the morphological characteristic, the diffusion process in dentine is more facilitated than it is with enamel; larger and more-porous lesions can be more easily remineralised than smaller and less-porous lesions [Strang et al., 1987]. This might be the reason for a better effect of the nano-HA/Fluoride on dentine (both sound and pre-demineralised) than on enamel, in which the paste was only effective for remineralisation of PD specimens due to the presence of large pores compared to S specimens.

Initial caries lesions can be re-hardened by the deposition of hydroxyapatite that first happens near the surface layer but then is gradually transferred inward and finally precipitated in the dark zone during the long-term remineralisation [Huang et al., 2010]. We expected that nano-HA acts in depth, because of its size, infiltrating in the pores of the lesion [Huang et al., 2011]. In our study, nano-HA/Fluoride improved in 3-folders and 2-folders dentine and enamel remineralisation compared to the placebo, respectively. Figure 3 shows that the mineral volume of dentine specimens remineralised by nano-HA/Fluoride was increased up to 110 μm; while for the placebo, it was restricted to 10-μm depths. For enamel, the remineralisation was very superficial, as shown by Figure 4.

Fluoride, on the other hand, acts mainly in the surface layer, which is incorporated into the crystal structure, forming fluorapatite more resistant to acid dissolution [Buzalaf et al., 2011]. We have confirmed this statement since F paste had a significant effect on the tooth surface (SL and Zmax), especially on the sound dentine subjected to demineralisation (Table 1). Figure 1 also shows a microradiography picture, where the thickness of the surface layer is greater for the specimen treated with F compared to the other groups.

Our *in situ* results were more promising than those found for the *in vitro* study [Comar et al., 2013], as saliva and biofilm may have modulated the effect of paste on

334 the tooth. Salivary proteins may stabilise the particles and precipitates on tooth
335 surface *in situ*, while these particles may be lost to de-remineralising solutions *in vitro*
336 [Wang et al., 2012; Comar et al., 2013].

337 Dental biofilm is a diffusion barrier for calcium and phosphate from saliva, but
338 at the same time it is a good mineral reservoir. A recent study has shown that in the
339 presence of a biofilm the efficacy of nano-HA was improved, while the opposite
340 happens for F [Zhang et al. 2015]. In the case of dentine demineralisation in our
341 study, the percentage of preventive effect was about 33% for nano-HA/Fluoride
342 compared to the placebo. Figure 1 also confirmed the effect in depth of nano-
343 HA/Fluoride compared to F paste, considering the mineral volume from 50 to 110 μm
344 depth. Despite that no treatment was effective against enamel demineralisation,
345 Table 2 and Figure 2 show that the lesions treated with nano-HA/Fluoride are more
346 superficial than those from MI Paste Plus and placebo.

347 In respect to CPP-ACP, Vanichvatana and Auychai [2013] in their *in situ* study
348 demonstrated that CPP-ACP with F has a remineralising effect on the enamel lesions
349 as well as F, but no additional benefit of CPP-ACP with F associated with fluoride
350 dentifrice was seen compared to fluoride dentifrice only. Another *in situ* study using
351 CPP-ACP without fluoride showed that this product was less effective than fluoride
352 dentifrice (long-term use) in remineralising caries lesions [Meyer-Lueckel et al.,
353 2015]. These studies are in agreement with our results, showing limited benefit of the
354 application of CPP-ACP with F (MI Paste Plus).

355 In conclusion, Nanop Plus (nano-HA/Fluoride) is more effective than MI Paste
356 Plus on the reduction of dentine demineralisation and the improvement of enamel
357 remineralisation. No treatments were able to reduce enamel demineralisation, while
358 for dentine remineralisation all treatments were effective when compared to the
359 placebo. Additional randomised clinical trials are needed to confirm our findings. If it
360 is proven that Nanop Plus has a good clinical effect, it can be further prescribed for
361 patients with a high risk for caries.

362 **Acknowledgments:** We thank FAPESP for the concession of a scholarship to the
363 first author (Proc. 2013/03942-7). We are also grateful to the subjects who
364 participated in the *in situ* phases. This publication is a thesis submitted by the first

365 author to Bauru School of Dentistry, University of São Paulo, in fulfillment of the
366 requirements for a MS degree in Oral Biology.

367

368 **References**

369 Aires CP, Tabchoury CP, Del Bel Cury AA, Koo H, Cury JA: Effect of sucrose
370 concentration on dental biofilm formed in situ and on enamel demineralization. Caries
371 Res 2006;40(1):28-32.

372 Angmar B, Carlstromd D, Glas JE: Studies on the ultrastructure of dental enamel. IV.
373 The mineralization of normal human enamel. J Ultrastruct Res 1963;8:12-23.

374 Bailey DL, Adams GG, Tsao CE, Hyslop A, Escobar K, Manton DJ, Reynolds EC,
375 Morgan MV: Regression of post-orthodontic lesions by a remineralizing cream. J
376 Dent Res 2009;88:1148-1153.

377 Balasundaram G, Sato M, Webster TJ: Using hydroxyapatite nanoparticles and
378 decreased crystallinity to promote osteoblast adhesion similar to functionalizing with
379 RGD. Biomaterials 2006;27(14):2798-2805.

380 Baldassarri M, Margolis HC, Beniash E: Compositional determinants of mechanical
381 properties of enamel. J Dent Res 2008;87(7):645-649.

382 Behnan SM, Arruda AO, González-Cabezas C, Sohn W, Peters MC: In-vitro
383 evaluation of various treatments to prevent demineralization next to orthodontic
384 brackets. Am J Orthod Dentofacial Orthop 2010;138(6):712.e1-7.

385 Buchalla W, Attin T, Roth P, Hellwig E: Influence of olive oil emulsions on dentin
386 demineralization in vitro. Caries Res 2003;37:100-107.

387 Buchalla W, Imfeld T, Attin T, Swain MV, Schmidlin PR: Relationship between
388 nanohardness and mineral content of artificial carious enamel lesions. Caries
389 Res 2008;42(3):157-163.

390 Buskes JA, Christoffersen J, Arends J: Lesion formation and lesion remineralization
391 in enamel under constant composition conditions. A new technique with applications.
392 Caries Res 1985;19(6):490-496.

393 Buzalaf MA, Pessan JP, Honório HM, ten Cate JM: Mechanisms of action of fluoride
394 for caries control. Monogr Oral Sci 2011;22:97-114.

395 Cao Y, Mei ML, Xu J, Lo EC, Li Q, Chu CH: Biomimetic mineralisation of
396 phosphorylated dentine by CPP-ACP. J Dent 2013;41(9):818-825.

397 Ccahuana-Vasquez RA, Tabchoury CP, Tenuta LM, Del Bel Cury AA, Vale GC, Cury
398 JA: Effect of frequency of sucrose exposure on dental biofilm composition and
399 enamel demineralization in the presence of fluoride. Caries Res 2007;41(1):9-15.

400 Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC: New approaches to
401 enhanced remineralization of tooth enamel. J Dent Res 2010;89:1187-1197.
402
403 Chen H, Liu X, Dai J, Jiang Z, Guo T, Ding Y: Effect of remineralizing agents on white
404 spot lesions after orthodontic treatment: a systematic review. Am J Orthod
405 Dentofacial Orthop 2013;143(3):376-382.

406 Comar LP, Souza BM, Gracindo LF, Buzalaf MAR, Magalhães AC: The impact of
407 experimental pastes containing hydroxyapatite nanoparticles, with or without fluoride,
408 on the dental demineralization in vitro. Braz Dent J 2013;24(3):273-278.

409 Comar LP, Wiegand A, Moron BM, Rios D, Buzalaf MA, Buchalla W, Magalhães AC:
410 In situ effect of sodium fluoride or titanium tetrafluoride varnish and solution on
411 carious demineralization of enamel. Eur J Oral Sci 2012;120(4):342-348.

412 Huang S, Gao S, Cheng L, Yu H: Combined effects of nano hydroxyapatite and Galla
413 chinensis on remineralisation of initial enamel lesion in vitro. J Dent 2010;38:811-
414 819.

415 Huang S, Gao S, Cheng L, Yu H: Remineralization potential of nano- hydroxyapatite
416 on initial enamel lesions: an in vitro study. Caries Res 2011;45:460-468.

417 Kumar VLN, Itthagarun A, King NM: The effect of casein phosphopeptide-amorphous
418 calcium phosphate on remineralization of artificial caries-like lesions: an in vitro
419 study. Aust Dent J 2008;53:34-40.

420 Li J, Xie X, Wang Y, Yin W, Antoun JS, Farella M, Mei L: Long-term remineralizing
421 effect of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on early
422 caries lesions in vivo: a systematic review. J Dent 2014;42(7):769-777.

423 Magalhães AC, Moron BM, Comar LP, Wiegand A, Buchalla W, Buzalaf MA:
424 Comparison of cross-sectional hardness and transverse microradiography of artificial
425 carious enamel lesions induced by different demineralising solutions and gels. Caries
426 Res 2009;43(6):474-483.

427 Magalhães AC, Furlani T de A, Italiani F de M, Iano FG, Delbem AC, Buzalaf MA:
428 Effect of calcium pre-rinse and fluoride dentifrice on remineralisation of artificially
429 demineralised enamel and on the composition of the dental biofilm formed in situ.
430 Arch Oral Biol 2007;52(12):1155-1160.

431 Meyer-Lueckel H, Wierichs RJ, Schellwien T, Paris S: Remineralizing efficacy of a
432 CPP-ACP cream on enamel caries lesions in situ. Caries Res 2015;49(1):56-62.

433 Moron BM, Comar LP, Wiegand A, Buchalla W, Yu H, Buzalaf MA, Magalhães AC:
434 Different protocols to produce artificial dentine carious lesions in vitro and in situ:
435 hardness and mineral content correlation. Caries Res 2013;47(2):162-170.

436 Najibfard K, Ramalingam K, Chedjieu I, Amaechi BT: Remineralization of early caries
437 by a nano-hydroxyapatite dentifrice. J Clin Dent 2011;22(5):139-143.

438 Nongonierma AB, Fitzgerald RJ: Biofunctional properties of caseinophosphopeptides
439 in the oral cavity. Caries Res 2012;46(3):234-267.

440 Orsini G, Procaccini M, Manzoli L, Sparabombe S, Tiriduzzi P, Bambini F, Putignano
441 A: A 3-day randomized clinical trial to investigate the desensitizing properties of three
442 dentifrices. J Periodontol 2013;84(11):e65-73.

443 Pepla E, Besharat LK, Palaia G, Tenore G, Migliau G: Nano-hydroxyapatite and its
444 applications in preventive, restorative and regenerative dentistry: a review of
445 literature. Ann Stomatol (Roma) 2014;5(3):108-114.

446 Schirrmeister JF, Seger RK, Altenburger MJ, Lussi A, Hellwig E: Effects of various
447 forms of calcium added to chewing gum on initial enamel carious lesions *in situ*.
448 Caries Res 2007;41(2):108-14. Erratum in: Caries Res. 2007;41(3):243.

449 Schlueter N, Klimek J, Ganss C: Efficacy of an experimental tin-F-containing solution
450 in erosive tissue loss in enamel and dentine in situ. Caries Res 2009;43(6):415-421.

451 Shen P, Manton DJ, Cochrane NJ, Walker GD, Yuan Y, Reynolds C, Reynolds EC:
452 Effect of added calcium phosphate on enamel remineralization by fluoride in a
453 randomized controlled in situ trial. J Dent 2011;39(7):518-525.

454 Strang R, Damato FA, Creanor SL, Stephen KW: The effect of baseline lesion
455 mineral loss on in situ remineralization. J Dent Res 1987;66(11):1644-1646.

456 Tschoppe P, Zandim DL, Martus P, Kielbassa AM: Enamel and dentin
457 remineralization by nano-hydroxyapatite toothpastes. J Dent 2011;39(6):430-437.

458 Vanichvatana S, Auychai P: Efficacy of two calcium phosphate pastes on the
459 remineralization of artificial caries: a randomized controlled double-blind *in situ* study.
460 Int J Oral Sci 2013;5(4):224-228.

461 Wang L, Magalhães AC, Araujo D, Francisconi LF, Calabria MP, Buzalaf MA, Lauris,
462 JR, Pereira JC: Clinical Performance of new agents on reduction of dentinal
463 hypersensitity. J Dent Res 2012;91B:1384.

464 Wang R, Wang Q, Wang X, Tian L, Liu H, Zhao M, Peng C, Cai Q, Shi Y:
465 Enhancement of nano-hydroxyapatite bonding to dentin through a collagen/calcium
466 dual-affinitive peptide for dentinal tubule occlusion. J Biomater Appl 2014;29(2):268-
467 277.

468 Yengopal V, Mickenautsch S: Caries preventive effect of casein phosphopeptide-
469 amorphous calcium phosphate (CPP-ACP): a meta-analysis. Acta Odontol Scand
470 2009;67:321-332.

471 Zhang M, He LB, Exterkate RA, Cheng L, Li JY, Ten Cate JM, Crielaard W, Deng
472 DM: Biofilm Layers Affect the Treatment Outcomes of NaF and Nano-hydroxyapatite.
473 J Dent Res 2015;94:602-607.

474 Zhou C, Zhang D, Bai Y, Li S: Casein phosphopeptide-amorphous calcium
475 phosphate remineralization of primary teeth early enamel lesions. J Dent
476 2014;42(1):21-29.

Table 1. Mean and standard deviation of the values obtained in TMR for sound dentin subjected to demineralization and the treatments *in situ*

Treatments	ΔZ (%vol.µm)	LD (µm)	R (%vol)	SL (µm)	Z max (%vol)
Nanop Plus	780.46±212.01[a]	98.76±26.19[a]	8.58±3.48[a]	14.89±4.51[ab]	49.67±10.09[ab]
MI Paste Plus	875.91±268.44[ab]	95.65±30.53[a]	11.68±3.52[ab]	12.89±4.20[ab]	58.28±5.61[a]
F	900.46±236.30[ab]	96.02±26.06[a]	10.54±3.76[ab]	24.56± 5.48[a]	54.06±8.69[a]
Placebo	1188.18±502.52[b]	142.67±27.93[b]	12.60±2.69[b]	15.94±3.92[b]	43.22±4.15[b]

Different superscript letters indicate significant differences among the treatments for each column (ANOVA, $p<0.022$). n = 13 for all parameters except SL and Z max (n = 9). Positive values indicate demineralisation.

Table 2. Mean and standard deviation of the values obtained in TMR for S enamel subjected to demineralisation and the treatments *in situ*

Treatments	ΔZ (%min.volx µm)	LD (µm)	R (%min.vol)	Presence of Surface Layer (%)
Nanop Plus	1000.91±249.51[a]	45.01±15.31[a]	24.87±6.49[a]	0%
MI Paste Plus	883.63±431.67[a]	60.72±26.43[a]	24.23±6.20[a]	23.1%
F	985.45±313.37[a]	52.36±21.06[a]	25.15±3.71[a]	0%
Placebo	1369.55±988.34[a]	57.23±30.55[a]	25.80±7.29[a]	15.4%

Similar superscript letters indicate no significant differences among the treatments for each column (repeated measures ANOVA, $p>0.1890$). n = 13 for all parameters. Positive values indicate demineralisation.
The parameters of the surface layer were not evaluated (SL and Z max), since we could analyse only few samples in this regard.

Table 3. Mean and standard deviation of the values obtained in TMR for the PD dentine subjected to remineralisation and the treatments *in situ*

Treatments	ΔΔZ (%min.volxμm)	ΔLD (μm)	ΔR (%min.vol)	Presence of surface Layer (%)
Nanop Plus	910.09±328.75[a]	22.28±17.56[a]	5.82±3.11[ab]	69.2%
MI Paste Plus	964.15±446.36[a]	19.64±18.77[a]	6.10±3.54[ab]	53.8%
F	902.08±606.83[a]	18.54±34.05[a]	8.85±5.19[a]	61.5%
Placebo	337.92±408.24[b]	4.85±13.41[a]	3.99±4.07[b]	53.8%

Different superscript letters indicate significant differences among the treatments for each column (ANOVA, $p<0.029$). n=13 *ΔΔZ= ΔZ baseline – ΔZ final (the same for the other parameters). Positive values indicate remineralisation and negative values mean demineralization.

The parameters of the surface layer were not evaluated (ΔSL and ΔZ max), since we could analyse only few samples in this regard.

Table 4. Mean and standard deviation of the values obtained in TMR for the PD enamel subjected to remineralisation and the treatments *in situ*

Treatments	ΔΔZ (%min.volxμm)	ΔLD (μm)	ΔR (%min.vol)	ΔSL (μm)	ΔZ max (%min.vol)
Nanop Plus	549.92±405.40[a]	24.30±14.02[a]	1.13±5.66[a]	0.05±2.23[a]	4.86±5.31[ab]
MI Paste Plus	370.83±230.58[ab]	8.63±12.50[b]	1.30±6.10[a]	-0.06±3.07[a]	-0.78±4.50[b]
F	555.54±264.12[a]	13.11±8.28[ab]	2.74±4.85[a]	-0.52±1.53[a]	8.91±5.93[a]
Placebo	200.35±186.78[b]	7.99±6.45[b]	-1.34±5.97[a]	0.07±1.66[a]	2.19±10.36[b]

Different superscript letters indicate significant differences between the groups for each column (repeated measures ANOVA, $p<0.0019$). n = 13 *ΔΔZ= ΔZ baseline – ΔZ final (the same for the other parameters). Positive values indicate remineralisation and negative values mean demineralisation.

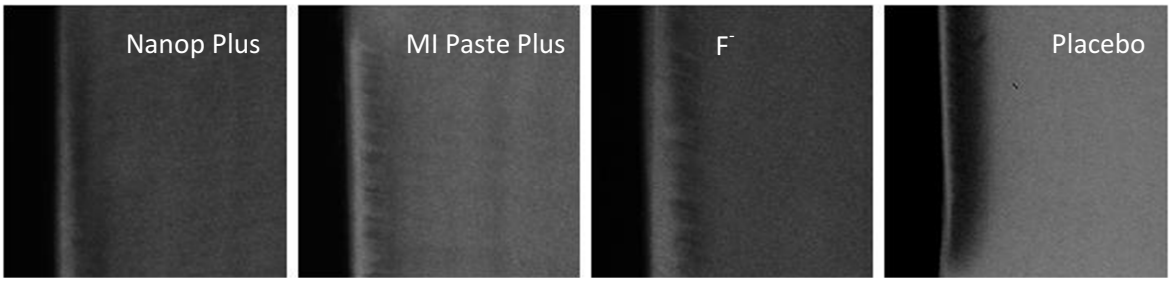


Figure 1. Microradiograph pictures of sound dentin subjected to demineralization and the treatments *in situ*

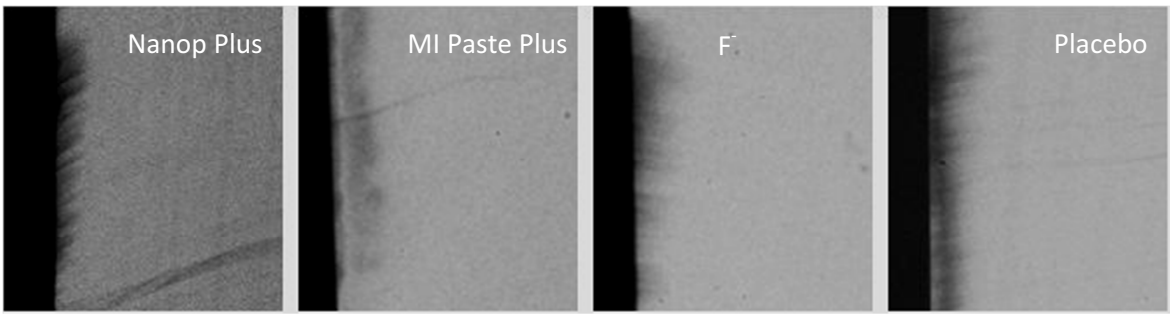


Figure 2. Microradiograph pictures of sound enamel subjected to demineralization and the treatments *in situ*

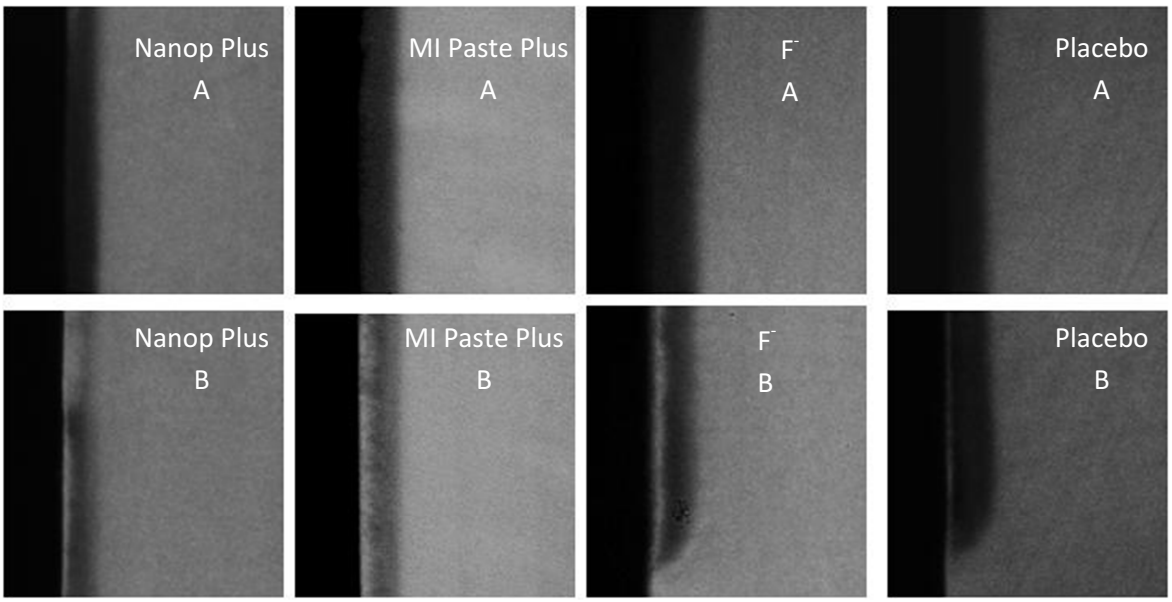


Figure 3. Microradiograph pictures of pre-demineralized dentin subjected to remineralization and the treatments *in situ* (A-demineralized area, B- de-remineralized area)

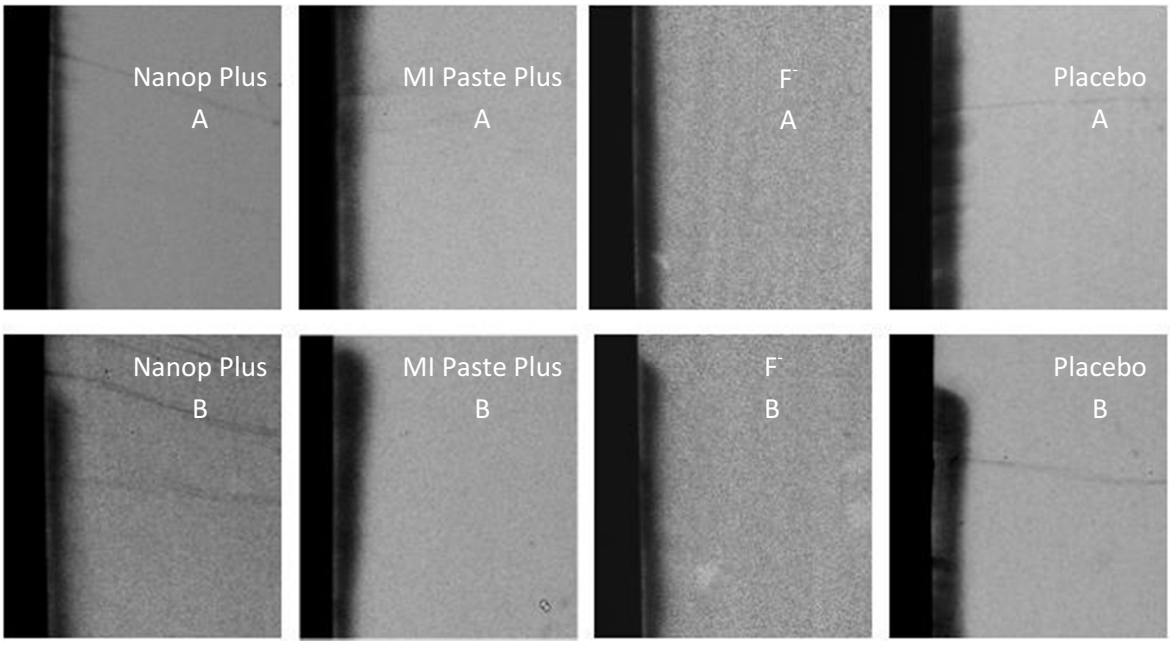
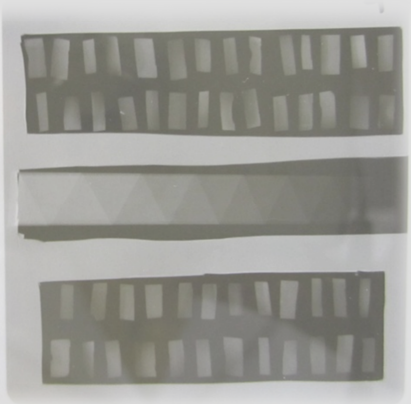
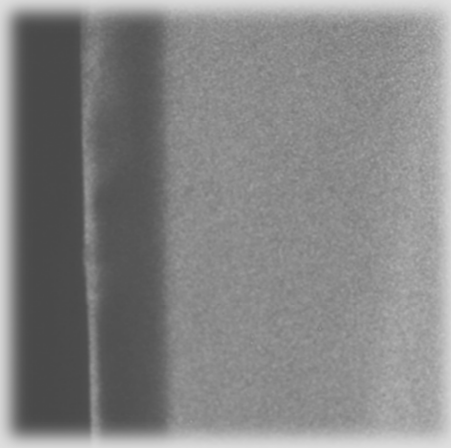


Figure 4. Microradiograph pictures of pre-demineralized enamel subjected to remineralization and the treatments *in situ* (A-demineralized area, B- de-remineralized area)





3 DISCUSSÃO

3 DISCUSSION

Previous studies have shown that nano-HA has some potential in increase enamel remineralization (HUANG; GAO; YU, 2009; HUANG et al., 2010; HUANG et al., 2011; TSCHOPPE et al., 2011; NAJIBFARD et al 2011, PEPLA et al., 2014) However, there are few studies about its effect on dentin remineralization, and most of them analyzed the desensitizing effect or morphological changes (WANG et al., 2012; CAO et al., 2013; ORSINI et al., 2013; WANG et al., 2014).

Products containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) have also shown potential to prevent dental demineralization and hypersensitivity and to increase remineralization *in vitro* (YAMAGUCHI et al., 2007; RAHIOTIS; VOUGIOUKLAKIS, 2007; KUMAR et al., 2008, PULIDO et al., 2008; CAO et al., 2013) as well to repair initial enamel caries lesion *in vivo* (MORGAN et al., 2008; YENGOPAL; MICKENAUTSCH, 2009; BAILEY et al., 2009). However, there is no scientific evidence for the benefit of the application of CPP-ACP by looking at Systematic Reviews (CHEN et al., 2013; LI et al., 2014). Despite this clinical limitation, CPP-ACP is a commercial product whose mechanism of action is similar to Nano-HA. Therefore, we have chosen it as a positive control. The placebo paste was produced without active ingredient, but with the same basic components as the pastes containing Nano-HA and F only. Except the placebo, all pastes have the same fluoride concentration (0.2% NaF, 900 ppm F).

In the present study, the experimental pastes were applied twice a day per 4 minutes, following the company's guidelines, as slurry, to simulate its dilution by saliva. We decide to include the application of dentifrice slurry before the treatment, to simulate the residual effect of fluoride after toothbrushing. It is also important to highlight that the experimental pastes are not dentifrices; therefore, they must be applied after oral hygiene by the patient 2 times a day (BEHNAN et al., 2010; COMAR et al., 2013).

To provoke demineralization, S samples were exposed to 20% sucrose solution per 5 minutes, 4 x day for dentin and 8 x day for enamel, to allow the formation of cariogenic dental biofilm able to cause demineralization within 14 days

(AIRES et al., 2006; TENUTA et al., 2006; CURY et al., 2010; COMAR et al., 2012). For provoke remineralization, the WS samples were not covered with biofilm to simulate a patient who changed his/her caries risk and activity.

While enamel is a highly mineralized tissue containing more than 95% of mineral (hydroxyapatite) organized in nanostructure crystals arranged in parallel arrays-enamel rods (BALDASSARRI, MARGOLIS, BENIASH, 2008), dentin is a calcified collagen matrix, in which hydroxyapatite is classified as intrafibrillar crystallites found within the hole zones and pore spaces of collagen fibrils (CAO et al., 2013).

Due to the morphological characteristic, the diffusion process in dentin is more facilitated than on enamel; larger and more porous lesions can be more easily remineralized than smaller and less porous lesions (STRANG et al., 1987). This might be the reason for a better effect of the Nano-HA on dentin (both sound and pre-demineralized) than on enamel; in which the paste was only effective for demineralized samples due to the presence of large porous.

Initial caries lesions can be re-hardened by the deposition of hydroxyapatite that first happens near the surface layer, but then it is gradually transferred inward and finally precipitated in the dark zone during the long-term remineralization (HUANG et al., 2010). We expected that Nano-HA acts in depth, because of its size, infiltrating in the pores of lesion (HUANG et al., 2011). In our study, Nano-HA improved in 3-folder and 2-folder dentin and enamel remineralization compared to placebo, respectively.

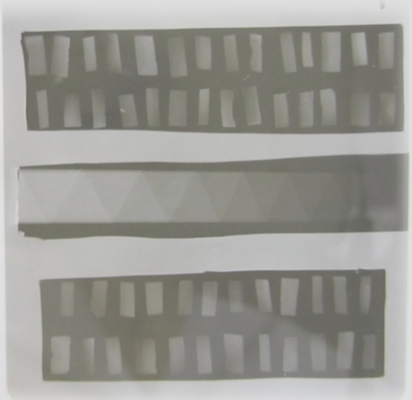
Fluoride, on the other hand, acts mainly in the surface layer, which is incorporated into the crystal structure forming fluorapatite more resistant to acid dissolution (BUZALAF et al., 2011). We have confirmed this statement since Fluoride paste had a significant effect on the surface by the analysis of SL and Zmax.

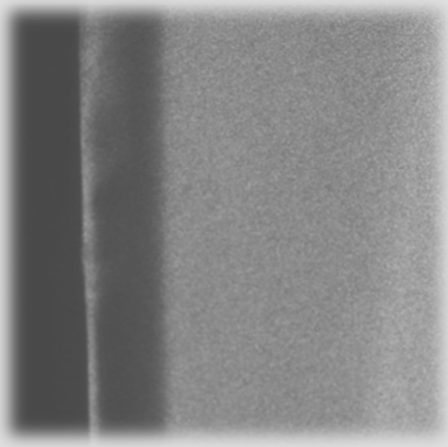
Our *in situ* results were more promising than those found for *in vitro* study (COMAR et al., 2013), as saliva and biofilm may modulate the effect of paste on the tooth. Salivary proteins may stabilize the particles and precipitates on tooth surface *in situ*, while these particles may be lost to de-remineralizing solutions *in vitro* (WANG et al., 2012; COMAR et al., 2013).

Dental biofilm is a diffusion barrier for calcium and phosphate from saliva, but at the same time is a good mineral reservoir. A recent study shows that in the presence of a biofilm the efficacy of Nano-HA was improved, while the opposite happens for Fluoride (ZHANG et al., 2015). In the case of dentin, the % preventive effect was about 33% for Nano-HA compared to placebo in our study.

Vanichvatana and Auychai [2013] in their *in situ* study demonstrated that CPP-ACP plus has remineralizing effect on the enamel lesions as well as fluoride, but no additional benefit of CPP-ACP plus associated with fluoride toothpaste was seen compared to fluoride toothpaste only. Another *in situ* study using CPP-ACP without fluoride showed that this product was less effective than fluoride dentifrice (long-term use) in remineralizing caries lesions (MEYER-LUECKEL et al., 2015). These studies are in agreement with our results, showing limited benefit of the application of CPP-ACP.

In conclusion, Nanop Plus is more effective than MI Paste Plus on the reduction of dentin demineralization and improving of enamel remineralization. No treatments were able to reduce enamel demineralization, while for dentin remineralization all treatments were effective. Additional randomized clinical trials are needed to confirm our findings. If it is proved that Nanop Plus has a good clinical effect, it can be further indicated for patients with high-risk for caries.





REFERÊNCIAS

REFERÊNCIAS

Aires CP, Tabchoury CP, Del Bel Cury AA, Koo H, Cury JA. Effect of sucrose concentration on dental biofilm formed in situ and on enamel demineralization. Caries Res. 2006;40(1):28-32.

Angmar B, Carlstromd D, Glas JE. Studies on the ultrastructure of dental enamel. IV. The mineralization of normal human enamel. J Ultrastruct Res. 1963;8:12-23.

Ammari MM, Soviero VM, da Silva Fidalgo TK, Lenzi M, Ferreira DM, Mattos CT, de Souza IP, Maia LC. Is non-cavitated proximal lesion sealing an effective method for caries control in primary and permanent teeth? A systematic review and meta-analysis. J Dent. 2014;42(10):1217-1227.

Assunção IV, da Costa G de F, Borges BC. Systematic review of noninvasive treatments to arrest dentin non-cavitated caries lesions. World J Clin Cases. 2014;2(5):137-141.

Bailey DL, Adams GG, Tsao CE, Hyslop A, Escobar K, Manton DJ, Reynolds EC, Morgan MV. Regression of post-orthodontic lesions by a remineralizing cream. J Dent Res. 2009;88:1148-1153.

Balasundaram G, Sato M, Webster TJ. Using hydroxyapatite nanoparticles and decreased crystallinity to promote osteoblast adhesion similar to functionalizing with RGD. Biomaterials. 2006;27(14):2798-2805

Baldassarri M, Margolis HC, Beniash E. Compositional determinants of mechanical properties of enamel. J Dent Res. 2008;87(7):645-649.

Behnan SM, Arruda AO, González-Cabezas C, Sohn W, Peters MC. In-vitro evaluation of various treatments to prevent demineralization next to orthodontic brackets. Am J Orthod Dentofacial Orthop. 2010;138(6):712.e1-7.

Boing AF, Bastos JL, Peres KG, Antunes JL, Peres MA. Social determinants of health and dental caries in Brazil: a systematic review of the literature between 1999 and 2010. Rev Bras Epidemiol. 2014;17 Suppl 2:102-115.

Bönecker M, Ardenghi TM, Oliveira LB, Sheiham A, Marcenes W. Trends in dental caries in 1- to 4-year-old children in a Brazilian city between 1997 and 2008. Int J Paediatr Dent. 2010;20(2):125-131.

Buchalla W, Attin T, Roth P, Hellwig E: Influence of olive oil emulsions on dentin demineralization in vitro. Caries Res. 2003;37:100-107.

Buchalla W, Imfeld T, Attin T, Swain MV, Schmidlin PR. Relationship between nanohardness and mineral content of artificial carious enamel lesions. Caries Res. 2008;42(3):157-163.

Buskes JA, Christoffersen J, Arends J. Lesion formation and lesion remineralization in enamel under constant composition conditions. A new technique with applications. Caries Res.1985;19(6):490-496.

Buzalaf MA, Pessan JP, Honório HM, ten Cate JM. Mechanisms of action of fluoride for caries control. Monogr Oral Sci. 2011;22:97-114.

Cao Y, Mei ML, Xu J, Lo EC, Li Q, Chu CH. Biomimetic mineralisation of phosphorylated dentine by CPP-ACP. J Dent. 2013;41(9):818-825.

Cardoso L, Rösing C, Kramer P, Costa CC, Costa Filho LC. Polarização da cárie em município sem água fluoretada. Cad. Saúde Pública. 2003;19:237-243.

Ccahuana-Vasquez RA, Tabchoury CP, Tenuta LM, Del Bel Cury AA, Vale GC, Cury JA. Effect of frequency of sucrose exposure on dental biofilm composition and enamel demineralization in the presence of fluoride. Caries Res 2007;41(1):9-15.

Chen H, Liu X, Dai J, Jiang Z, Guo T, Ding Y. Effect of remineralizing agents on white spot lesions after orthodontic treatment: a systematic review. Am J Orthod Dentofacial Orthop. 2013;143(3):376-382.

Cochrane NJ, Cai F, Huq NL, Burrow MF, Reynolds EC. New approaches to enhanced remineralization of tooth enamel. J Dent Res. 2010;89:1187-1197.

Comar LP, Souza BM, Gracindo LF, Buzalaf MAR, Magalhães AC. The impact of experimental pastes containing hydroxyapatite nanoparticles, with or without fluoride, on the dental demineralization in vitro. Braz Dent J. 2013;24(3):273-278.

Comar LP, Wiegand A, Moron BM, Rios D, Buzalaf MA, Buchalla W, Magalhães AC. In situ effect of sodium fluoride or titanium tetrafluoride varnish and solution on carious demineralization of enamel. Eur J Oral Sci. 2012;120(4):342-348.

Cury JA, do Amaral RC, Tenuta LM, Del Bel Cury AA, Tabchoury CP. Low-fluoride toothpaste and deciduous enamel demineralization under biofilm accumulation and sucrose exposure. Eur J Oral Sci. 2010;118(4):370-375.

Gushi LL, Rihs LB, Soares Mda C, Forni TI, Vieira V, Wada RS, Sousa M da L. Dental caries and treatment needs in adolescents from the state of São Paulo, 1998 and 2002. Rev Saude Publica. 2008;42(3):480-486.

Hoffmann RHS, Cypriano S, Sousa MLR, Wada RS. Dental caries experience in children at public and private schools from a city with fluoridated water. Cad Saude Publica. 2004;20:522-528.

Huang S, Gao S, Cheng L, Yu H. Combined effects of nano hydroxyapatite and Galla chinensis on remineralisation of initial enamel lesion in vitro. J Dent 2010;38:811-819.

Huang S, Gao S, Cheng L, Yu H. Remineralization potential of nano- hydroxyapatite on initial enamel lesions: an in vitro study. Caries Res. 2011;45:460-468.

Huang SB, Gao SS, Yu HY. Effect of nano-hydroxyapatite concentration on remineralization of initial enamel lesion in vitro. Biomed Mater. 2009;4:034104.

Hugo FN, Vale GC, Ccahuana-Vásquez RA, Cypriano S, Sousa MLR. Polarization of dental caries among individuals aged 15 to 18 years. J. Appl. Oral Sci. 2007;15(4):253-258.

Kumar VLN, Itthagarun A, King NM. The effect of casein phosphopeptide-amorphous calcium phosphate on remineralization of artificial caries-like lesions: an in vitro study. Aust Dent J. 2008;53:34-40.

Lemos LV, Myaki SI, Walter LR, Zuanon AC. Oral health promotion in early childhood: age of joining preventive program and behavioral aspects. Einstein(Sao Paulo) 2014;12(1):6-10.

Li J, Xie X, Wang Y, Yin W, Antoun JS, Farella M, Mei L. Long-term remineralizing effect of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on early caries lesions in vivo: a systematic review. J Dent. 2014;42(7):769-777.

Magalhães AC, Moron BM, Comar LP, Wiegand A, Buchalla W, Buzalaf MA. Comparison of cross-sectional hardness and transverse microradiography of artificial

carious enamel lesions induced by different demineralising solutions and gels. Caries Res. 2009;43(6):474-483.

Magalhães AC, Furlani T de A, Italiani F de M, Iano FG, Delbem AC, Buzalaf MA. Effect of calcium pre-rinse and fluoride dentifrice on remineralisation of artificially demineralised enamel and on the composition of the dental biofilm formed in situ. Arch Oral Biol. 2007;52(12):1155-1160.

Martins RJ, Garbin CAS, Garbin AJI, Moimaz SAS, Saliba O. Declínio da cárie em um município da região noroeste do Estado de São Paulo, Brasil, no período de 1998 a 2004. Cad Saúde Pública 2006;22(5):1035-1041.

Meyer-Lueckel H, Wierichs RJ, Schellwien T, Paris S. Remineralizing efficacy of a CPP-ACP cream on enamel caries lesions in situ. Caries Res. 2015;49(1):56-62.

Morgan MV, Adams GG, Bailey DL, Tsao CE, Fischman SL, Reynolds EC. The anticariogenic effect of sugar-free gum containing CPP-ACP nanocomplexes on approximal caries determined using digital bitewing radiography. Caries Res. 2008;42:171-184.

Moron BM, Comar LP, Wiegand A, Buchalla W, Yu H, Buzalaf MA, Magalhães AC. Different protocols to produce artificial dentine carious lesions in vitro and in situ: hardness and mineral content correlation. Caries Res. 2013;47(2):162-170.

Najibfard K, Ramalingam K, Chedjieu I, Amaechi BT. Remineralization of early caries by a nano-hydroxyapatite dentifrice. J Clin Dent. 2011;22(5):139-143.

Narvai PC, Frazão P, Roncalli AG, Antunes JFL. Cárie dentária no Brasil: declínio, polarização, iniqüidade e exclusão social. Rev Panam Salud Publica. 2006;19:385–393.

Nongonierma AB, Fitzgerald RJ. Biofunctional properties of caseinophosphopeptides in the oral cavity. Caries Res. 2012;46(3):234-267.

Orsini G, Procaccini M, Manzoli L, Sparabombe S, Tiriduzzi P, Bambini F, Putignano A. A 3-day randomized clinical trial to investigate the desensitizing properties of three dentifrices. J Periodontol. 2013;84(11):e65-73.

Pepla E, Besharat LK, Palaia G, Tenore G, Migliau G. Nano-hydroxyapatite and its applications in preventive, restorative and regenerative dentistry: a review of literature. Ann Stomatol (Roma). 2014;5(3):108-114.

Pulido MT, Wefel JS, Hernandez MM, Denehy GE, Guzman-Armstrong S, Chalmers JM, Qian F. The inhibitory effect of MI paste, fluoride and a combination of both on the progression of artificial caries-like lesions in enamel. Oper Dent. 2008;33:550-555.

Rahiotis C, Vougiouklakis G. Effect of a CPP-ACP agent on the demineralization and remineralization of dentine in vitro. J Dent. 2007;35(8):695-698.

Sales-Peres SHC, Carvalho FS, Carvalho CP, Bastos JRM, Lauris JRP. Polarization of dental caries in teen-agers in the southwest of the State of São Paulo, Brazil. Cienc. Saúde Coletiva. 2008;13(2):2155-2162.

Schirrmeister JF, Seger RK, Altenburger MJ, Lussi A, Hellwig E. Effects of various forms of calcium added to chewing gum on initial enamel carious lesions *in situ*. Caries Res. 2007:41(2):108-114. Erratum in: Caries Res. 2007;41(3):243.

Schlueter N, Klimek J, Ganss C. Efficacy of an experimental tin-F-containing solution in erosive tissue loss in enamel and dentine in situ. Caries Res. 2009;43(6):415-21.

Shen P, Manton DJ, Cochrane NJ, Walker GD, Yuan Y, Reynolds C, Reynolds EC.Effect of added calcium phosphate on enamel remineralization by fluoride in a randomized controlled in situ trial. J Dent 2011;39(7):518-525.

Strang R, Damato FA, Creanor SL, Stephen KW. The effect of baseline lesion mineral loss on in situ remineralization. J Dent Res. 1987;66(11):1644-1646.

Takahashi N, Nyvad B. The role of bacteria in the caries process: ecological perspectives. J Dent Res. 2011;90(3):294-303.

Ten Cate JM, Larsen MJ, Pearc EIF, Fejerskov O. Chemical interactions between the tooth and oral fluids. In Fejerskov O, Kidd EAM (eds): Dental Caries: The Disease and it's clinical management. Oxford; Blackwell Munksgaard, 2003:49-69.

Tenuta LM, Ricomini Filho AP, Del Bel Cury AA, Cury JA. Effect of sucrose on the selection of mutans streptococci and lactobacilli in dental biofilm formed in situ. Caries Res. 2006;40(6):546-549.

Tschoppe P, Zandim DL, Martus P, Kielbassa AM. Enamel and dentine remineralization by nano-hydroxyapatite toothpastes. J Dent. 2011;39(6):430-437.

Vanichvatana S, Auychai P. Efficacy of two calcium phosphate pastes on the remineralization of artificial caries: a randomized controlled double-blind in situ study. Int J Oral Sci. 2013;5(4):224-228.

Wang L, Magalhães AC, Araujo D, Francisconi LF, Calabria MP, Buzalaf MA, Lauris, JR, Pereira JC. Clinical Performance of new agents on reduction of dentinal hypersensitity. In: 90th General Session and Exhibition of the IADR, 2012, Foz do Iguaçu. J Dent Res. 2012;91B:1384.

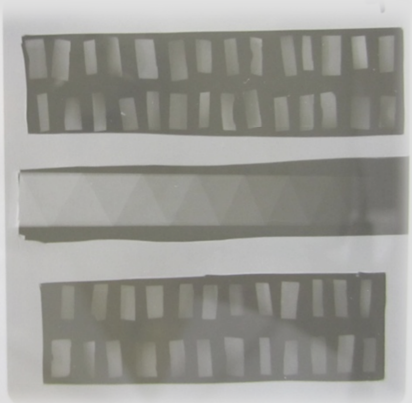
Wang R, Wang Q, Wang X, Tian L, Liu H, Zhao M, Peng C, Cai Q, Shi Y. Enhancement of nano-hydroxyapatite bonding to dentin through a collagen/calcium dual-affinitive peptide for dentinal tubule occlusion. J Biomater Appl. 2014;29(2):268-277.

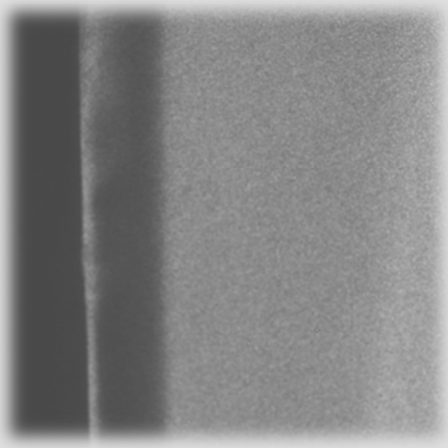
Yamaguchi K, Miyazaki M, Takamizawa T, Inage H, Kurokawa H. Ultrasonic determination of the effect of casein phosphopeptide-amorphous calcium phosphate paste on the demineralization of bovine dentin. Caries Res. 2007;41(3):204-207.

Yengopal V, Mickenautsch S. Caries preventive effect of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP): a meta-analysis. Acta Odontol Scand 2009;67:321-332.

Zhang M, He LB, Exterkate RA, Cheng L, Li JY, Ten Cate JM, Crielaard W, Deng DM. Biofilm Layers Affect the Treatment Outcomes of NaF and Nano-hydroxyapatite. J Dent Res. 2015;94:602-607.

Zhou C, Zhang D, Bai Y, Li S. Casein phosphopeptide-amorphous calcium phosphate remineralization of primary teeth early enamel lesions. J Dent 2014;42(1):21-29.





APÊNDICES

APÊNDICE A- **TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Nome do participante da pesquisa_____

1 - Título do Trabalho Experimental

Avaliação do efeito de uma pasta experimental com nanopartículas de hidroxiapatita e fluoreto sobre a desmineralização e remineralização dentária *in situ*

2 - Objetivo

O objetivo deste estudo é avaliar o efeito de um novo tratamento para cárie: pasta experimental com nanopartículas de hidroxiapatita 10% e 900 ppm de flúor (NOVO), pasta comercial MI Paste Plus (fosfato de cálcio amorfo+ caseína+ 900 ppm de flúor, CONTROLE POSITIVO), pasta contendo 900 ppm de flúor e pasta placebo (sem princípios ativos) (CONTROLE NEGATIVO).

3 - Procedimentos da Fase Experimental

Você utilizará, durante quatro períodos de 14 dias de duração cada um, com um período de descanso de 5 dias entre eles, um aparelho de acrílico no céu da boca, no qual serão fixados 4 blocos de esmalte bovino e 4 blocos de dentina bovina previamente desinfetados. Uma semana antes do início de cada período, você deverá utilizar pasta de dente, escova e fio dental fornecidos pela pesquisadora para a higiene bucal. O aparelho será instalado um dia antes do início de cada período, à noite, após a última higiene bucal.

Durante o período experimental, você só poderá remover o aparelho para a realização das 4 principais refeições (1h no máximo cada uma, seguida pela higienização bucal), sendo que neste período o aparelho deverá ficar envolvido em gaze umedecida por água de abastecimento. Em todas as fases do experimento, você deverá aplicar 1 gota de SACAROSE sobre cada bloco **COM** tela plástica; para o esmalte (cor verde) 8 vezes ao dia e para dentina (cor azul) 4 vezes aos dia. A sacarose deverá permanecer durante 5 minutos sobre os blocos, com o aparelho fora da boca. O intervalo entre as exposições à sacarose deve ser de 30 minutos. Nas amostras sem tela plástica, a sacarose não deverá ser aplicada. Adicionalmente, você deverá pingar uma gota de pasta fluoretada em TODAS as amostras (1gota/amostra COM e SEM tela) por 1 minuto, 2 vezes ao dia, e enxaguar. Na sequência, você deverá aplicar a solução de TRATAMENTO em TODAS as amostras (1 gota/amostra 2 vezes ao dia), durante 4 minutos cada, sem enxaguar. A exposição à pasta fluoretada e ao tratamento deverá ser feita no começo da manhã e outra a noite (ambas após a higienização), com o aparelho fora da boca. Na sequência, o aparelho deverá ser recolocado na boca. Estes procedimentos serão repetidos em todas as fases diariamente durante os 14 dias. Ao final das fases experimentais, os aparelhos deverão ser devolvidos aos pesquisadores.

4 - Benefícios do Experimento

O uso da pasta experimental e da pasta MI Paste Plus vem de encontro com a necessidade de proteção do dente contra cárie dentária. Dessa forma, o entendimento mais detalhado do papel do fluoreto, e das novas tecnologias, como as nanopartículas de hidroxiapatita, na prevenção e tratamento da cárie dentária, tem suma importância para reduzir a progressão da lesão e a necessidade de tratamentos mais invasivos. Não há benefício direto para o participante dessa pesquisa.

5 - Riscos do Experimento

Não há riscos aos participantes pelo uso dos aparelhos palatinos com dentes desinfetados, procedimento comumente realizado em estudos desta natureza. Alguns participantes podem eventualmente apresentar enjoos ou dor de garganta. Nestes casos, o participante irá comunicar o responsável pela pesquisa, que irá acompanhá-lo até um médico. E então será liberado da participação na pesquisa, sem penalização alguma. Os gastos que forem gerados por este trabalho ficará a cargo da responsável pelo projeto. Importante ressaltar que não está sendo considerado nenhum pagamento ou recompensa material pela participação do sujeito neste estudo. Você terá garantido o direito à indenização compensatória caso fique comprovado que a sua participação acarretou algum problema a você.

Fica claro que o você poderá, a qualquer momento, retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar do estudo alvo da pesquisa e ciente que todo trabalho realizado torna-se informação confidencial guardada por força do sigilo profissional (Art. 9º do Código de Ética Odontológica).

Qualquer dúvida ou maiores esclarecimentos o participante da pesquisa poderá recorrer a qualquer um dos membros da equipe do projeto (Laboratório de Bioquímica 14-3235-8247) ou a pesquisadora responsável Beatriz Martines de Souza (telefone 14 981548927/ 98168-3023, e-mail beatriz.martines.souza@usp.br). Caso queira apresentar reclamações em relação a sua participação na pesquisa, poderá entrar em contato com o Comitê de Ética em Pesquisa em Seres Humanos, da FOB-USP, pelo endereço da Al. Dr. Octávio Pinheiro Brisolla, 9-75 (sala no prédio da Pós Graduação FOB/USP) ou pelo telefone (14)3235-8356, ou por e-mail (cep@fob.usp.br).

Pelo presente instrumento que atende às exigências legais, o Sr. (a) _____, portador da cédula de identidade _____, após leitura minuciosa das informações constantes neste TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO, devidamente explicada pelos profissionais em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e explicado, DECLARA e FIRMA seu CONSENTIMENTO LIVRE E ESCLARECIDO concordando em participar da pesquisa proposta. Fica claro que o participante da pesquisa, pode a qualquer momento retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO e deixar de participar desta pesquisa e ciente de que todas as informações prestadas tornar-se-

ão confidenciais e guardadas por força de sigilo profissional (Art. 9º do Código de Ética Odontológica).

Por fim, como pesquisador(a) responsável pela pesquisa, DECLARO o cumprimento do disposto na Resolução CNS nº 466 de 2012, contidos nos itens IV.3, item IV.5.a e na íntegra com a resolução CNS nº 466 de dezembro de 2012.

Por estarmos de acordo com o presente termo o firmamos em duas vias igualmente válidas (uma via para o sujeito da pesquisa e outra para o pesquisador) que serão rubricadas em todas as suas páginas e assinadas ao seu término, conforme o disposto pela Resolução CNS nº 466 de 2012, itens IV.3.f e IV.5.d.

Bauru, SP, _____ de _____ de _____.

_____ _____

Assinatura do Participante da Pesquisa Beatriz Martines de Souza

O **Comitê de Ética em Pesquisa – CEP**, organizado e criado pela **FOB-USP**, em 29/06/98 (**Portaria GD/0698/FOB**), previsto no item VII da Resolução nº 466/12 do Conselho Nacional de Saúde do Ministério da Saúde (publicada no DOU de 13/06/2013), é um Colegiado interdisciplinar e independente, de relevância pública, de caráter consultivo, deliberativo e educativo, criado para defender os interesses dos participantes da pesquisa em sua integridade e dignidade e para contribuir no desenvolvimento da pesquisa dentro de padrões éticos.
Qualquer denúncia e/ou reclamação sobre sua participação na pesquisa poderá ser reportada a este CEP:

Horário e local de funcionamento:

Comitê de Ética em Pesquisa
Faculdade de Odontologia de Bauru-USP - Prédio da Pós-Graduação (bloco E - pavimento superior), de segunda à sexta-feira, no horário das **13h30 às 17 horas**, em dias úteis.
Alameda Dr. Octávio Pinheiro Brisolla, 9-75
Vila Universitária – Bauru – SP – CEP 17012-901
Telefone/FAX(14)3235-8356
e-mail: cep@fob.usp.br

APÊNDICE B – Orientações entregue aos voluntários da pesquisa

ORIENTAÇÕES AO VOLUNTÁRIO

Você, voluntario, irá dormir com o aparelho na boca na noite do dia _____(_____). Irá colocar o aparelho após a higiene bucal noturna e não ingerir nenhum alimento após este procedimento.

O experimento iniciará no dia ____/___, _____, com duração de 14 dias. Portanto, terminará no dia _____/____ (_____). O voluntário deverá dormir com o aparelho e na manhã do dia ____/_____ (_____), o voluntario, ao acordar, deverá retirar o aparelho e enrolar em gaze umedecida com água e guardar na caixa e trazer para o responsável da pesquisa (não realizar nenhum tratamento no aparelho neste dia da entrega)

Entregar o aparelho no dia ____/____ (_____) no laboratório de bioquímica em um dos seguintes horários. (**9h30-10h** ou **13h-14h30** ou **17h30-18h30)**

Qualquer dúvida, entrar em contato com a pesquisadora responsável Beatriz Martines de Souza (contato: 14 981548927 (OI) / 98168-3023 (TIM) / 14-3235-8247 (Laboratório de Bioquímica) ou pelo e-mail beatriz.martines.souza@usp.br)

1. ORIENTAÇÕES GERAIS:

- O aparelho deverá permanecer na boca durante todo o período experimental.
- Remover durante as 4 refeições diárias (1 h de duração cada NO MÁXIMO, tempo total por dia:4h). Durante o período de refeições, o aparelho deverá ser armazenado em gaze umedecida com água. O intervalo entre as refeições deverá ser de 2-3h.
- Após as refeições, realizar higiene bucal com dentifrício fluoretado 5 minutos antes de o aparelho ser recolocado na boca.
- Não usar produtos fluoretados ou anti-placa durante o experimento. Caso algum dos materiais esteja acabando, por favor entrar em contato imediato com a pesquisadora.

2. ORIENTAÇÕES ESPECÍFICAS:

2.1 APÓS O CAFÉ DA MANHÃ E REALIZAÇÃO DA HIGIENE BUCAL:

◊ Mexer o pote com as soluções para homogeneização antes das aplicações;

◊ Aplicar 1 gota de **solução fluoretada (pasta de dente)** em cada amostra durante 1 minuto, em TODAS AS AMOSTRAS (N=8). Decorrido o 1 minuto de tratamento, enxaguar as amostras, para remoção do excesso da pasta.

◊ Aplicar 1 gota de **solução de tratamento** em TODAS AS AMOSTRAS (N=8) durante 4 minutos. Decorridos os 4 minutos de tratamento, **NÃO LAVAR**. Recolocar o aparelho na boca e não retirá–lo nos próximos 30 minutos para alimentação ou aplicação de sacarose.

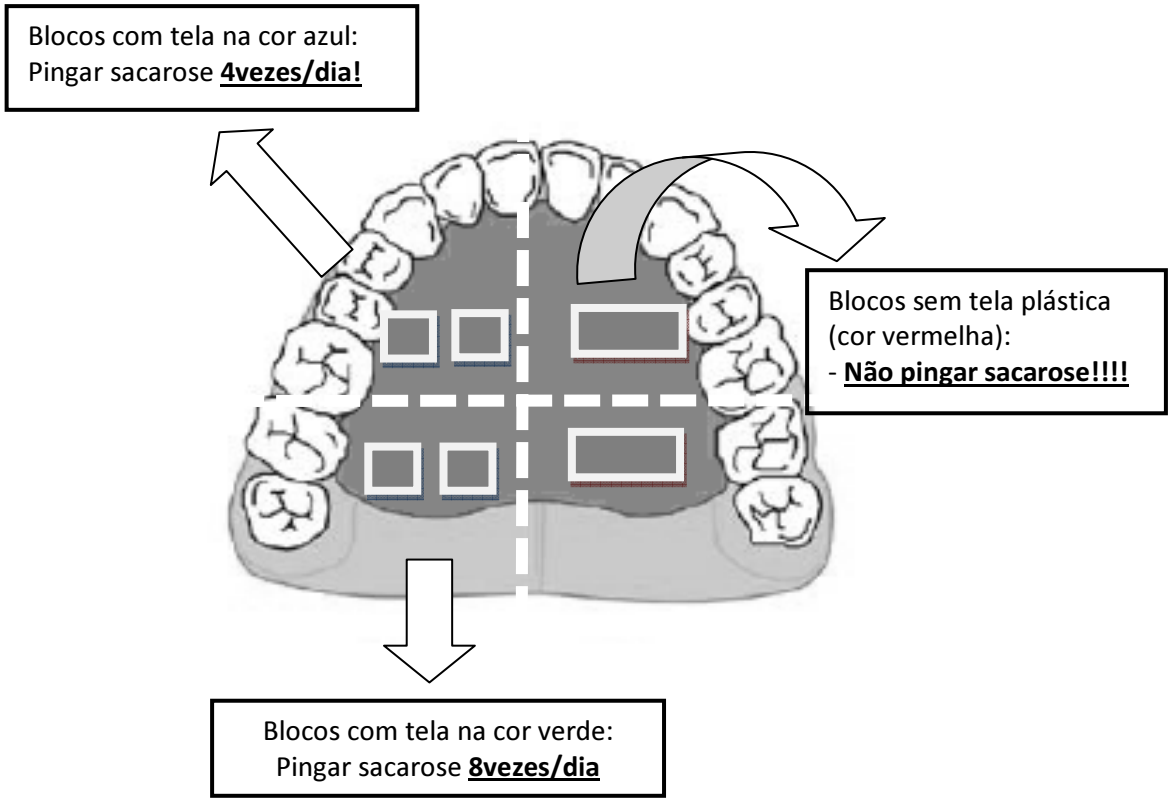
2.2 APÓS DECORRIDOS OS 30 MINUTOS da aplicação da pasta de tratamento, pode-se iniciar a aplicação de sacarose.

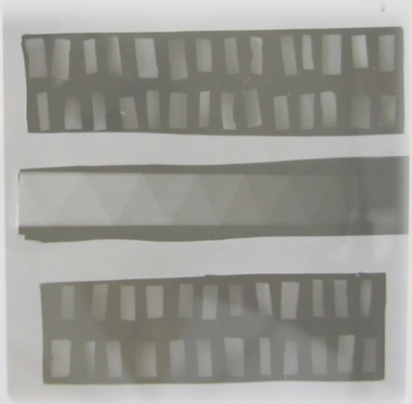
- Pingar 1 gota de sacarose **8 x/dia** em cada um dos **dois blocos verdes**. Aguardar 5 minutos, e recolocar na boca (Tomar cuidado para a solução de sacarose não ir para bloco do lado)
- Pingar 1 gota de sacarose **4 x/dia** em cada um dos **dois blocos azuis**. Aguardar 5 minutos, e recolocar na boca. (Tomar cuidado para a solução de sacarose não ir para bloco do lado)

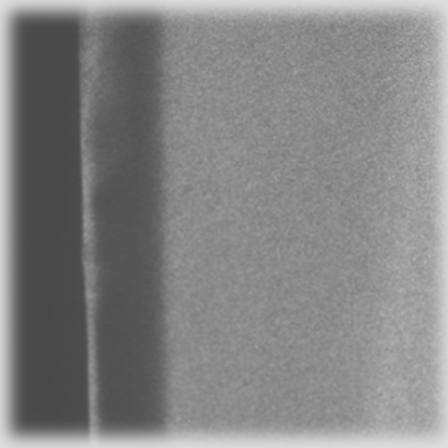
O Intervalo entre as aplicações de sacarose deve ser de **no mínimo 1 h.** Agitar o pote antes da aplicação e anotar os horários de aplicação. Após a aplicação permanecer pelo menos 30 minutos com o aparelho. Não lavar o aparelho após a aplicação da sacarose. **Não aplicar sacarose sobre os blocos VERMELHOS!**

2.3 ANTES DE DORMIR:

- ◊ Mexer o pote com as soluções para homogeneização antes das aplicações;
- ◊ Realizar higiene bucal e repetir o mesmo procedimento descrito no item 2.1. O desafio com sacarose deverá ter terminado antes do último tratamento.
- ◊ Aplicar 1 gota de **solução fluoretada (pasta de dente)** em cada amostra durante 1 minuto, em TODAS AS AMOSTRAS (N=8). Decorrido o 1 minuto de tratamento, enxaguar as amostras, para remoção do excesso da pasta.
- ◊ Aplicar 1 gota de **solução de tratamento** em TODAS AS AMOSTRAS (N=8) durante 4 minutos. Decorridos os 4 minutos de tratamento, **NÃO LAVAR**. Recolocar o aparelho na boca e não retirá–lo nos próximos 30 minutos.







ANEXO

ANEXO A – Parecer Consubstanciado do CEP

FACULDADE DE ODONTOLOGIA DE BAURU-USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AVALIAÇÃO DO EFEITO DE UMA PASTA EXPERIMENTAL COM NANOPARTÍCULA DE HIDROXIAPATITA E FLUORETO SOBRE A DESMINERALIZAÇÃO E REMINERALIZAÇÃO DENTÁRIA IN SITU

Pesquisador: Beatriz Martines de Souza

Área Temática:

Versão: 3

CAAE: 15272613.6.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 718.208

Data da Relatoria: 25/06/2014

Apresentação do Projeto:

Projeto aprovado em maio/13.

Em abril/14, autoras apresentam emenda ao projeto, modificando o título e acrescentando procedimentos à metodologia. Tais alterações se justificam pela necessidade de adequar o estudo às solicitações da FAPESP, que concedeu bolsa de mestrado à pesquisadora.

Objetivo da Pesquisa:

A proposta deste projeto será avaliar o potencial de uma formulação experimental bioativa baseada na inovadora tecnologia de nanopartículas de hidroxiapatita, em minimizar a desmineralização e aumentar a remineralização dentária in situ.

Avaliação dos Riscos e Benefícios:

Riscos:

Não há riscos aos voluntários pelo uso dos aparelhos palatinos com dentes desinfetados, procedimento comumente realizado em estudos desta natureza. Alguns voluntários eventualmente podem apresentar enjôos ou dor de garganta. Nestes casos, os voluntários serão excluídos do experimento.

Benefícios:

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br