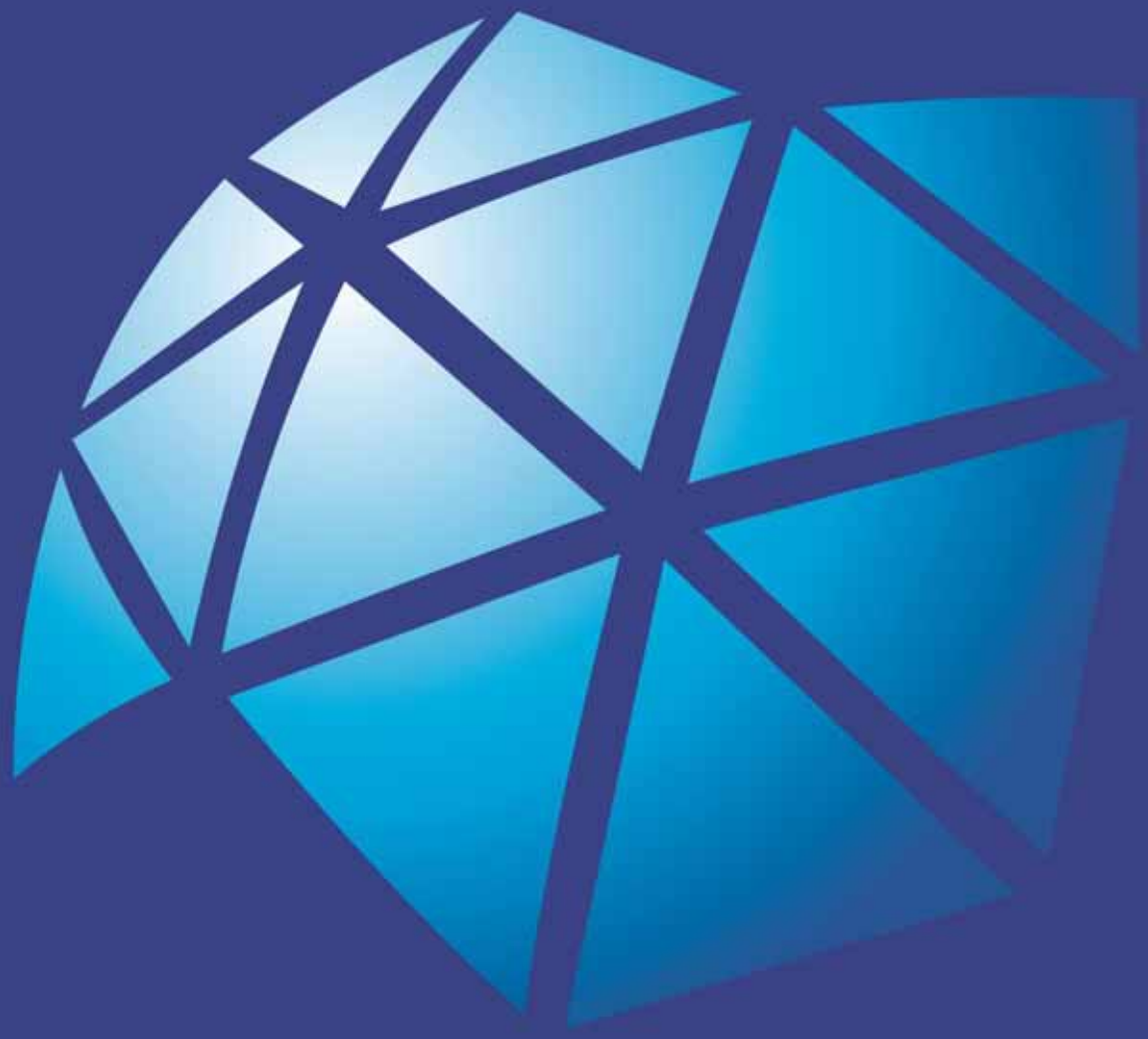
Maria Daniela Basso de Souza

EFEITO DE NANOPARTÍCULAS DE TRIMETAFOSFATO DE SÓDIO ASSOCIADAS A DENTIFRÍCIO COM CONCENTRAÇÃO REDUZIDA DE FLUORETO SOBRE A DESMINERALIZAÇÃO DO ESMALTE BOVINO ESTUDOS *IN VITRO* E *IN SITU*



MARIA DANIELA BASSO DE SOUZA

EFEITO DE NANOPARTÍCULAS DE TRIMETAFOSFATO DE SÓDIO ASSOCIADAS A DENTIFRÍCIO COM CONCENTRAÇÃO REDUZIDA DE FLUORETO SOBRE A DESMINERALIZAÇÃO DO ESMALTE BOVINO ESTUDOS *IN VITRO* E *IN SITU*

Tese Apresentada à Faculdade de Odontologia de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutor em Ciência Odontológica, Área de Saúde Bucal da Criança.

Orientador: Prof. Adj. Alberto C. B. Delbem

Araçatuba

Setembro/ 2014

Catalogação na Publicação (CIP)

Serviço Técnico de Biblioteca e Documentação – FOA / UNESP

Souza, Maria Daniela Basso de.
S729e Efeito de nanopartículas de trimetafosfato de sódio associadas a dentifrício com concentração reduzida de fluoreto sobre a desmineralização do esmalte bovino : estudos in vitro e in situ / Maria Daniela Basso de Souza. - Araçatuba, 2014
125 f. : il. ; tab. + 1 CD-ROM

Tese (Doutorado) – Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba
Orientador: Prof. Alberto Carlos Botazzo Delbem

1. Fosfatos 2. Nanopartículas 3. Fluoretos 4. Dentifrícios 5. Desmineralização I. T.

Black D27
CDD 617.645

COMISSÃO EXAMINADORA

TESE PARA OBTENÇÃO DO GRAU DE DOUTOR

Prof. Dr. Alberto Carlos Botazzo Delbem - Orientador, Professor Adjunto do Departamento de Odontologia Infantil e Social, Disciplina de Odontopediatria da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

Profa. Dra. Débora Barros Barbosa - Professora Assistente Doutora do Departamento de Materiais Odontológicos e Prótese, Disciplina de Prótese Total da Faculdade de Odontologia - Araçatuba, UNESP - Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba.

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

Profa. Dra. Juliana Fraga Soares Bombonatti - Professora Doutora do Departamento de Dentística, Endodontia e Materiais Dentários - Disciplina de Dentística, Faculdade de Odontologia de Bauru / Universidade de São Paulo.

Prof. Dr. Edo Hirata - Professor Assistente Doutor, Curso de Odontologia, Disciplina de Odontopediatria e Clínica Integrada Infantil, Cascavel, UNIOESTE – Universidade Estadual do Oeste do Paraná, Cascavel.

DADOS CURRICULARES

MARIA DANIELA BASSO DE SOUZA

Nascimento	29.10.1971 – Ribeirão Preto – SP
Filiação	Francisco Gilberto Basso Marisa Marquesi Basso
1993/1996	Curso de Graduação em Odontologia pela Faculdade de Odontologia de Araraquara – UNESP.
1999/2000	Curso de Pós Graduação em Odontopediatria, nível de Mestrado, na Faculdade de Odontologia de Araraquara – UNESP.
2001/2004	Professora das Disciplinas de Odontopediatria e Odontologia Preventiva, Social e Ergonomia do Curso de Odontologia da Universidade Paranaense - UNIPAR, Campus de Umuarama e Cascavel – PR.
2001/	Professora Assistente das Disciplinas de Odontopediatria e Estágio Supervisionado em Clínica Integrada Infantil do Curso de Odontologia da Universidade Estadual do Oeste do Paraná - UNIOESTE, Cascavel – PR.
Associações	CROPR - Conselho Regional de Odontologia do Paraná SBPqO - Sociedade Brasileira de Pesquisa Odontológica IADR - International Association for Dental Research

DEDICATÓRIA

Ao meu **Pai Santíssimo, Deus de misericórdia**, que sempre segurou em minha mão e me conduziu nessa jornada. Por me dar a certeza de que tudo nessa vida tem um tempo e uma razão. Vós sois aquele que nos momentos mais difíceis de minha vida soube a maneira certa de me fazer forte, me concedendo a benção de uma gestação e a oportunidade deste Doutorado. O Senhor sabe quem eu sou e do que preciso.

Aos meus pais amados **Francisco** (in memorian) e **Marisa** (in memorian), por tudo de mais lindo, abençoado, eterno e importante que representam em minha existência.

"Nem mesmo o céu nem as estrelas, nem mesmo o mar e o infinito
Não é maior que o meu amor nem mais bonito.
Nunca se esqueça, nem um segundo, que eu tenho o amor maior do mundo
Como é grande o meu amor por você."

Ao meu esposo **Ricardo**, sempre maravilhoso e companheiro. Por ter compreendido minhas necessidades profissionais e apoiado totalmente minhas decisões. Agradeço todo esforço que fez e por ter suportado minha ausência e de nossos filhos durante o período do curso.

"Seus olhos, meu clarão, me guiam dentro da escuridão.
Seus pés me abrem o caminho; eu sigo e nunca me sinto só."

Aos meus amados filhos **Davi** e **Mateus**, vocês são tudo para a mamãe: minha vida, minha luz. Nos momentos de maior dificuldade, o azul dos olhinhos de vocês me deu força para respirar, erguer a cabeça, seguir em frente e não desistir, apesar de tudo.

"Eu não sei se vem de Deus do céu ficar azul
Ou virá dos olhos teus essa cor que azuleja o dia."

Ao meu sogro **Valdir** e minha sogra **Henriqueta** (**Netinha**) (in memorian), por terem alterado sua rotina para nos receber em sua casa por 2 anos. Agradeço todos os dias o amor, o carinho, a paciência e a atenção que tiveram conosco. Sem a ajuda de vocês eu não teria conseguido. O amor e a doação à família fazem realmente coisas incríveis.

"Um oceano de afeto
Quanto mais ama mais tem amor
Quanto mais serve mais tem pra dar."

Á minha irmã **Tatiana** que, no tempo em que estive em Araçatuba, passou horas preciosas ao telefone me ouvindo chorar de saudade de casa e do Ricardo. Pacientemente, me escutou lamentar as imensas dificuldades que tive que enfrentar. Chorou comigo, riu das bobagens que fiz, levantou meu ânimo, me orientou e me mostrou o quanto nossos pais nos fizeram fortes e guerreiras. Você sempre me apoiou, incentivou e me mostrou o imenso amor que nos unirá eternamente. Te amo! Minha vida sem você não teria sentido!

"Moverei montanhas pra te alcançar, e o mar do tempo pra não te deixar,
Quando houver flores no teu jardim, olhe pra luz, lembre de mim."

AGRADECIMENTOS ESPECIAIS

Ao meu irmão Fernando, meus cunhados Marcos e Luciana, meus sobrinhos Pedro, Sofia e Marcelo. Como é bom acordar de manhã e saber que tenho o amor de vocês!

Aos meus cunhados Cibele e Julio e meus sobrinhos Bruno, Maurício, Leonardo e Isadora. Obrigada por terem feito parte dessa etapa da minha vida tão de perto.

Aos meus cunhados Gustavo e Isabela e meus sobrinhos Diego e Caio. Agradeço o apoio e o carinho, e sei que apesar da distância estiveram por perto em pensamento.

Á toda família Souza, pelo apoio, carinho e companhia em Araçatuba.

Aos professores e grandes amigos da Disciplina de Odontopediatria, Ortodontia e Clínica Integrada Infantil da Universidade Estadual do Oeste do Paraná – Cascavel - PR: Prof. Dr. Edo Hirata, Prof. Dr Adriano Tomio Hoshi, Profa. Dra. Denise César de Oliveira Davidoff, Profa. Ms. Maria de Fátima Monteiro Tomasin, Prof. Ms. Amadeu Tomasin Neto, Prof. Dr. Luiz Carlos Marchi, Prof. Ms. Roberto Bombonatti e Prof. Dr. Mauro Carlos Agner Busato, pela amizade de tantos anos, por concordarem que eu me afastasse integralmente de minhas atividades letivas. Muitíssimo obrigada por se esforçarem para suprir minha ausência.

Ao professor e amigo da Disciplina de Endodontia e Clínica Integrada da Universidade Estadual do Oeste do Paraná – Cascavel - PR: Prof. Dr. Christian Giampietro Brandão pela amizade e grande colaboração no transporte de materiais da pesquisa de Araçatuba para Cascavel.

Ao professor e amigo da Disciplina de Endodontia e Clínica Integrada Adulto da Universidade Estadual do Oeste do Paraná – Cascavel - PR: Prof. Ms Eduardo Tanaka de Castro e aos professores da Disciplina de Odontopediatria e Clínica Integrada Infantil Prof. Dr. Edo Hirata e Prof. Dr Adriano Tomio Hoshi pela colaboração para a realização do estudo *in situ*.

A todos os meus amigos de Cascavel, que torceram por mim e me apoiaram. Em especial: Juliana e Rogério, Fabiana e Murilo.

Às estagiárias do curso de Graduação em Odontologia da FOA-Unesp Marcela Lumi e Flavia Castilho, pela ajuda na realização da fase laboratorial do estudo *in vitro*;

À Faculdade de Odontologia de Araçatuba, na pessoa da Profa. Adj. Ana Maria Pires Soubhia, digníssima Diretora e Prof. Tit. Wilson Roberto Poi, digníssimo Vice-Diretor.

Ao Curso de Pós-Graduação em Ciência Odontológica da Faculdade de Odontologia de Araçatuba –UNESP, na pessoa de seu coordenador Prof. Dr. Alberto Carlos Botazzo Delbem.

Aos docentes da Disciplina de Odontopediatria da Faculdade de Odontologia de Araçatuba, UNESP, Prof. Tit. Célio Percinoto, Profª Drª Cristiane Duque, Prof. Dr. Juliano Pelim Pessam, Prof. Adj. Robson Federico Cunha, Profª Dra. Rosângela dos Santos Nery, Profª Adj. Sandra M. H. C. Ávila de Aguiar, exemplos de profissionalismo, pela alegre convivência e conhecimentos transmitidos.

Aos funcionários da Disciplina de Odontopediatria, Maria dos Santos Ferreira Fernandes, Mario Luis da Silva e Maria Bertolina Mesquita de Oliveira. Em especial à Maria dos Santos Ferreira Fernandes que tanto me auxiliou no laboratório. Ao funcionário, Luiz Carlos dos Santos (Luizinho), pela atenção e pelo capricho na limpeza do laboratório.

A todos os colegas do curso de Pós-Graduação em Ciência Odontológica, áreas de Saúde Bucal da Criança da Faculdade de Odontologia de Araçatuba – UNESP pela convivência alegre e por terem me recebido com tanto carinho e atenção. Agradeço imensamente a todos aqueles que me ajudaram e me apoiaram durante a rotina laboratorial, nos momentos de aprendizado e principalmente nos de dúvida, sempre com disposição e uma palavra de incentivo.

Colegas do Mestrado: Nathalia Viana, Carla Favretto, Karina Kondo, Juliana Mendonça, Luciene Castro, José Antônio Souza, Ana Laura Esteves. Colegas

do Doutorado: Dani Câmara, Marcelle Danelon, Dani Oliveira, Jackeline Amaral, Nathalia Manrique, Marcelo Moretto, Michele Manarelli, Natália Colombo, Paulo Duarte e Remberto Valdez. Pós-Doutorado: Ana Elisa Vieira e Carol Lodi

Aos colegas Carol Lodi e José Antônio, que foram fundamentais para a realização do estudo *in situ* na cidade de Cascavel. Sem a ajuda de vocês teria sido impossível a execução do trabalho.

À Valéria, Cristiane e Liliam, da Seção de Pós-Graduação da Faculdade de Odontologia de Araçatuba- UNESP, pelo profissionalismo e atenção.

Ao Frigorífico Friboi, que permitiu a coleta dos dentes bovinos.

A todos aqueles que, de certa forma, contribuíram para a elaboração e conclusão deste trabalho,

Meus sinceros agradecimentos !

AGRADECIMENTOS ESPECIAIS

Ao **Prof. Adjunto Alberto Carlos Botazzo Delbem,** meu orientador a partir do segundo ano do Doutorado, pela imensa paciência frente minha inexperiência em laboratório e pelos conhecimentos compartilhados. Sou muito grata pelas oportunidades.

Á **Profa. Dra. Kikue Takebayashi Sassaki,** minha orientadora no primeiro ano do Doutorado. Exigente, ensinou-me com rigor científico. Nas situações de erro ou dúvida, corrigiu-me, mas sempre com um sorriso calmo e carinhoso acompanhado de palavras consoladoras e de incentivo. Admiro-te! Embora tenha sentido sua falta quando se aposentou, sei que sempre esteve comigo em pensamento, torcendo por mim.

Á **Profa. Dra. Ana Elisa de Mello Vieira,** por ter me recebido com tanto carinho e por ter sido tão verdadeira. Por ter me ensinado toda a rotina de laboratório desde o início, com tanta paciência e excelência. Os conselhos que me deu nos momentos difíceis no início do Doutorado me mantiveram firme.

Aos voluntários da pesquisa *in situ*: Hoje entendo a dificuldade na execução deste tipo de estudo, pois para ser um voluntário é necessário comprometimento, responsabilidade, paciência, e acima de tudo, vontade de ajudar o pesquisador sem receber nada em troca. Por esse motivo, meus sinceros agradecimentos por todo o esforço de vocês.

EPÍGRAFE

"Nas grandes batalhas da vida, o primeiro passo para a vitória é o desejo de vencer."

Mahatma Gandhi

Souza, Maria Daniela Basso de. Efeito de nanopartículas de trimetafosfato de sódio associadas a dentifrício com concentração reduzida de fluoreto sobre a desmineralização do esmalte bovino: estudos in vitro e in situ [tese]. Araçatuba: Universidade Estadual Paulista; 2014.

RESUMO GERAL

O objetivo deste estudo foi avaliar o efeito de dentifrício com concentração reduzida de fluoreto (250 ppm F) contendo trimetafosfato de sódio nanoparticulado (TMPnano) sobre a desmineralização do esmalte bovino (*in vitro* e *in situ*), bem como sua ação no biofilme dentário formado *in situ*. Para o estudo *in vitro*, blocos de esmalte bovino (*n* = 144) foram divididos em 12 grupos de dentifrícios (ph: 7,0): Placebo (sem F ou TMP), 1100 ppm F (1100 - DC – dentifrício convencional), 250 ppm F (250) e, 250 ppm F suplementado com TMP micrométrico (250-TMP) ou TMP nanoparticulado moído por 24h (250-TMPnano24) ou por 48h (250-TMPnano48). O TMP micrométrico e o TMP nanoparticulado foram adicionados à formulação nas concentrações de 0,05%, 0,1% e 0,25%. Os blocos foram submetidos à ciclagem de pH durante cinco dias, sendo o tratamento com suspensões dos respectivos dentifrícios realizados 2x/dia. Para avaliar a perda mineral, a dureza de superfície final (SHf) e a porcentagem de perda de dureza de superfície (%SH) foram determinadas, bem como a concentração de fluoreto (F), cálcio (Ca), fósforo (P) e proporção molar Ca/P no esmalte. Os dados foram submetidos aos testes Kruskal-Wallis e Student-Newman-Keuls ($p<0,05$). Todas as concentrações de TMPnano reduziram a %SH ($p<0,05$). Os dentifrícios 250-TMPnano24 e 250-

TMPnano48 levaram a maior retenção de F, Ca, P e Ca/P no esmalte apenas na concentração de 0,05% ($p<0,05$); mas, apenas o dentifrício 250-0,05TMPnano48 não apresentou diferença estatística quando comparado ao 1100 para a proporção molar Ca/P no esmalte ($p<0,05$). Já para o estudo *in situ*, 19 voluntários utilizaram aparelhos contendo 4 blocos de esmalte bovino e foram aleatoriamente distribuídos quanto ao dentifrício a ser utilizado: placebo (sem F/TMP), 250 ppm F (250), 250 ppm F suplementado com 0,05% de TMP nanoparticulado triturado por 48h (250-TMPnano) e 1100 ppm F (1100 - DC – dentifrício convencional), sob desafio cariogênico (sacarose 30%, 6x/dia), por 7 dias. A dureza de superfície final (SHf), a porcentagem de perda de dureza de superfície (%SH), a perda integrada de dureza (ΔKHN) e as concentrações de F, Ca e P no esmalte foram determinadas. Em acréscimo, o biofilme formado sobre os blocos foi analisado para a concentração de F, Ca, P e polissacarídeo extracelular (EPS). Os dados foram submetidos a ANOVA (1 critério) e teste de Student-Newman-Keuls ($p<0,05$). O dentifrício 250-TMPnano promoveu o menor valor de ΔKHN dentre todos os grupos ($p<0,001$), enquanto que a %SH foi similar ao grupo 1100. Não houve diferença estatística entre os grupos 1100 e 250-TMPnano para a concentração de F, Ca e P no esmalte. A maior concentração de F no biofilme foi observada para o 1100; a concentração de Ca foi similar entre os grupos 1100 e 250-TMPnano e, o P foi similar entre todos os grupos. Valores similares entre os grupos 1100 e 250-TMPnano foram observados com respeito a concentração de EPS ($p< 0,001$), atividade iônica de $CaHPO_4^0$, CaF^+ and HF^0 ($p < 0.05$), grau de saturação da HA e CaF_2 ($p < 0,05$). Concluiu-se que o efeito anticárie do dentifrício com 250 ppm F

suplementado com 0,05% de nanopartículas de TMP submetidas a moagem por 48 h foi superior ao 250 ppm F, mas similar ao dentifrício convencional.

Palavras-Chave: Dentifrícios, Esmalte Dentário, Fluoretos, Nanopartículas, Fosfatos, Desmineralização.

Souza, Maria Daniela Basso de. Effect of nano-sized sodium trimetaphosphate associated to low-fluoride dentifrice on bovine enamel demineralization: *in vitro* and *in situ* studies [tese]. Araçatuba: Universidade Estadual Paulista; 2014.

GENERAL ABSTRACT

The aim of this study was to evaluate the effect of low-fluoride dentifrices (250 ppm F) containing nano-sized sodium trimetaphosphate (TMPnano) on the demineralization of bovine enamel as well their action on the biofilm formed *in situ*. For study 1 (*in vitro*), blocks of bovine enamel (*n*=144) were randomly divided into 12 experimental groups of dentifrices (pH 7.0). Placebo (no F or TMP), 1,100 ppm F (conventional dentifrice), 250 ppm F (250), 250 ppm F plus micrometric TMP (250-TMP) or nano-sized TMP milled for 24h (250-TMPnano24) or 48h (250-TMPnano48). Micrometric and nano-sized TMP were added to the formulations at concentrations of 0.05%, 0.1% and 0.25%. Blocks were subjected to a pH-cycling regimen and treatments with dentifrice slurries during 7 days. To evaluate mineral loss, surface hardness (SH) and the percentage of surface hardness loss (%SH) were analyzed. Fluoride (F), calcium (Ca), phosphorus (P) and Ca/P molar ratio in enamel were also determined. Data were submitted to Kruskal-Wallis test, followed by Student–Newman–Keuls' test ($p<0.05$). All concentrations of TMPnano significantly reduced %SH ($p<0.05$). In addition, the dentifrices 250-TMPnano24 and 250-TMPnano48 led to higher F, Ca, P and Ca/P content in enamel only at 0.05% ($p<0.05$); but only the 250-0.05TMPnano48 did not show significantly statistical difference when compared with 1,100 in respect of Ca/P molar ratio in

enamel ($p<0.05$). For study 2 (*in situ*), nineteen subjects wore palatal appliances containing 4 blocks of bovine enamel, and were randomly assigned to brush their teeth with placebo (no F or TMP), 250 ppm F (250), 250 plus 0.05% nano-sized TMP milled for 48h (250-TMPnano) and 1,100 ppm F (1,100 - conventional dentifrice) dentifrices, under cariogenic challenge (30% sucrose solution, 2 times/day), during 7 days. Post-cariogenic challenge hardness (SHf), the percentage of surface hardness loss (%SH), the integrated loss of subsurface hardness (ΔKHN), as well as fluoride (F), calcium (Ca), and phosphorus (P) concentrations were analyzed. Moreover, the biofilm formed on the blocks were analyzed for F, Ca, P and insoluble extracellular polysaccharide (EPS) concentrations. Data were submitted to one-way ANOVA (repeated measures), followed by Student-Newman-Keuls post-hoc test ($p<0.05$). The 250-TMPnano promoted the lowest ΔKHN among all groups ($p<0.001$), while the % SH loss was similar to the 1,100 group. The 1,100 and 250-TMPnano dentifrices led to similar F, Ca, P concentrations in enamel. The highest F content in biofilm was observed for 1,100; Ca content was similar between 1,100 and 250-TMPnano; and, P content was similar among the groups. Similar values were observed for 250-TMPnano and 1,100 groups regarding EPS ($p<0.001$), ionic activities of $CaHPO_4^0$, CaF^+ and HF^0 ($p<0.05$) and degree of saturation of HA and CaF_2 ($p<0.05$). It was concluded that the anticaries effect of the 250 ppm F dentifrice containing 0.05% nano-sized TMP milled for 48h was greater than the 250 ppm F, and similar to the conventional dentifrice.

Keywords: Dentifrices, Dental Enamel, Fluorides, Phosphates, Nanoparticles, Tooth Demineralization.

SUMÁRIO

INTRODUÇÃO GERAL

INTRODUÇÃO GERAL

A fluorose dentária é um efeito adverso decorrente da ingestão excessiva de fluoreto durante o desenvolvimento da dentição, e a literatura relata que quanto mais precoce o uso de dentifrícios convencionais por crianças, maior a chance de seu desenvolvimento [Wong *et al.*, 2011 [1]]. Com o objetivo de minimizar ou até mesmo prevenir essa alteração, a redução na concentração de fluoreto nos dentifrícios infantis tem sido proposta [Moraes *et al.*, 2007[2]]. Porém, de acordo com metanálise recente, não existem estudos suficientes para se atestar a equivalência entre dentifrícios com concentração reduzida de fluoreto (DCRF) e dentifrícios convencionais [Walsh *et al.*, 2011[3]]. E, estudo clínico demonstrou que um dentifrício contendo 500 ppm F não foi tão efetivo quanto o convencional em crianças com atividade de cárie [Lima *et al.*, 2008[4]].

Apesar de um dentifrício com 250 ppm F não apresentar eficácia no controle da cárie [Walsh *et al.*, 2011[3]], essa concentração reduzida de fluoreto seria relevante considerando o risco de fluorose dentária, especialmente para crianças menores de 6 anos de idade e bebês. Por esta razão, a modificação desta formulação com a adição de trimetafosfato de sódio (TMP) em partículas micrométricas foi testada e promoveu uma eficácia semelhante à de um

[1] Wong MC, Clarkson J, Glenny AM, Lo EC, Marinho VC, Tsang BW, Walsh T, Worthington HV. Cochrane reviews on the benefits/risks of fluoride toothpastes. J Dent Res 2011; 90(5):573-9. doi: 10.1177/0022034510393346.
[2] Moraes SM, Pessan JP, Ramires I, Buzalaf MA. Fluoride intake from regular and low fluoride toothpastes by 2-3-year-old children: influence of the toothpaste flavor. Braz Oral Res 2007; 21(3):234-240.
[3] Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpastes of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev; CD007868, 2011.
[4] Lima TJ, Ribeiro CC, Tenuta LMA, Cury JA. Low-fluoride toothpaste and caries lesion control in children with different caries experience: a randomized clinical trial. Caries Res 2008; 42: 46-50.

dentifrício convencional (DC - 1100 ppm F) em inibir a desmineralização do esmalte [Missel, *et al.*, 2010[5]].

A suplementação de DCRF com TMP parece trazer benefícios, pois além deste sal ser capaz de reduzir a dissolução da hidroxiapatita [McGaughey, Stowell, 1977[6]; Roberts, 1995[7]; Missel *et al.*, 2010[5]; Takeshita *et al.*, 2009[8]; Takeshita *et al.*, 2010[9]; Manarelli et al, 2011[10]; Favretto *et al.*, 2013[11]; Danelon *et al.*, 2013a[12], 2013b[13], 2014[14]], ainda permite a remineralização do corpo da lesão de subsuperfície em profundidade, diferentemente da ação do fluoreto sozinho [Takeshita *et al.*, 2011[15]; Favretto *et al.*, 2013[11]; Danelon *et al.*, 2013a[12],b[13]].

Embora o mecanismo de ação do TMP na dinâmica da cárie ainda não tenha sido completamente elucidado, acredita-se que quando adsorvido à superfície do esmalte [McGaughey; Stowell, 1977[6]; Van Dijk *et al.*, 1980[16]], o que ocorre por por período prolongado [Van Dijk *et al.*, 1980[16]], o TMP leva à formação de uma camada protetora dificultando a difusão ácida [Takeshita *et*

[5] Missel EMC, Delbem ACB, Vieira AEM, Sassaki KT, Cruz NVS, Percinoto C. Avaliação de dentifrícios com concentração reduzida de fluoreto associada ao trimetafosfato de sódio na desmineralização do esmalte 2010. Braz Oral Res 2010;24(Suppl. 1):247-83.
[6] McGaughey C, Stowell EC. Effects of polyphosphates on the solubility and mineralization of HA: relevance to a rationale for anticaries activity. J Dent Res 1977;56(6):579-587.
[7] Roberts J. Role of models in assessing new agents for caries prevention-non-fluoride systems. Adv Dent Res 1995; 9:304-311.
[8] Takeshita EM, Castro LP, Sassaki KT, Delbem AC In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate Caries Res 2009; 43(1) 50-6.
[9] Takeshita E M, Castro L P, Danelon M, Sassaki K T, Delbem AC. Evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate on biofilm and enamel demineralization in an in situ study Caries Res 2010; 44 171-247 (abstract 77).
[10] Manarelli MM, Vieira AE, Matheus AA, Sassaki KT, Delbem AC. Effect of mouth rinses with fluoride and trimetaphosphate on enamel erosion: an in vitro study. Caries Res 2011; 45(6):506-9. doi: 10.1159/000331929.
[11] Favretto CO, Danelon M, Castilho FCN, Vieira AEM, Delbem ACB. In vitro evaluation of the effect of mouth rinse with trimetaphosphate on enamel demineralization. Caries Res 2013; 47(5):532-538.
[12] Danelon M, Takeshita EM, Peixoto LC, Sassaki KT, Delbem AC. Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization. Clin Oral Investig 2013a doi 10.1007/s00784-013-1102-1104.
[13] Danelon M, Castro LP, Souza-neto FN, Camargo ER, Delbem ACB. Avaliação in vitro de dentifrícios suplementados com nanopartículas de trimetafosfato de sódio sobre a desmineralização dentária. Braz Oral Res 2013b;27(Suppl. 1):35-40.
[14] Danelon M, Takeshita EM, Peixoto LC, Sassaki KT, Delbem AC. Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization. Clin Oral Investig 2014; 18(4): 1119-27.
[15] Takeshita EM, Exterkate RA, Delbem AC, ten Cate JM. Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization in vitro. Caries Res 2011; 45(5):494-497.
[16] van Dijk JW, Borggreven JM, Driessens FC. The effect of some phosphates and a phosphonate on the electrochemical properties of bovine enamel. Arch Oral Biol 1980; 25:591–595.

al., 2011[15]; Van Dijk *et al.*, 1980[16]] e reduzindo as trocas minerais entre o meio e o esmalte [McGaughey e Stowell, 1977[6], Van Dijk *et al.*, 1980[16]; Takeshita *et al.*, 2011[15]]. Apesar de reduzir as trocas minerais, o TMP favorece a difusão de íons cálcio [Takeshita *et al.*, 2011[15]; Van Dijk *et al.*, 1980[16], Danelon *et al.*, 2014[14], Manarelli *et al.*, 2014[17]; Pancote *et al.*, 2014[18]] e flúor [Takeshita *et al.*, 2009[8],2011[15]; Van Dijk *et al.*, 1980[16]]. Também se demonstrou que o TMP altera a cariogenicidade do biofilme, em função da melhora no reservatório inorgânico (nesse caso, íons flúor e cálcio) e da redução na concentração de polissacarídeos extracelulares insolúveis [Takeshita *et al.*, 2010[9]] – fatores relacionados ao desenvolvimento da cárie [Cury *et al.*,1997[19], 2000[20]; Tenuta *et al.*, 2003[21], 2006[22], Vale *et al.*, 2007[23]].

A literatura apresenta, também, a suplementação de DCRF com nanopartículas de sais de cálcio e fosfato [Karlinsey *et al.*, 2011[24]; Mensikai *et al.*, 2012[25]]. Estes estudos sintetizaram nanopartículas empregando o método da funcionalização, no qual se promove uma ligação de grupos químicos funcionais a uma superfície [PAS 71:2005[26]] com posterior trituração do composto em um moinho de bolas. Recentemente, aplicou-se o método

[17] Manarelli MM, Delbem ACB, Lima TMT, Castilho FCN, Pessan JP. In vitro remineralizing effect of fluoride varnishes containing sodium trimetaphosphate. Caries Res 2014; 48:299–305.
[18] Pancote LP, Manarelli MM, Danelon M, Delbem ACB. Effect of fluoride gels supplemented with sodium trimetaphosphate on enamel erosion and abrasion: In vitro study. Arch Oral Biol 2014; 5 9:336-340.
[19] Cury JA, Rebello MAB, Del Bel BEL Cury AA. In situ relationship between sucrose exposure and the composition of dental plaque. Caries Re 1997; 31:356-60.
[20] Cury JA, Rebello MAB, Del Bel Cury AA, Derbyshire MTVC, Tabchoury CPM. Biochemical composition and cariogenicity of dental plaque formed in the presence of sucrose or glucose and fructose. Caries Res 2000; 34:491-497.
[21] Tenuta LMA, Lima JE, Cardoso CL, Tabchoury CP, Cury JA. Effect of plaque accumulation and salivary factors on enamel demineralization and plaque composition in situ. Pesqui Odontol Bras 2003; 17(4):326-331.
[22] Tenuta LMA, Del Bel Cury AA, Bortolin MC, Vogel GL, Cury JA. Ca, Pi, and F in the biofilm formed under sucrose. J Dent Res 2006; 85(9):834-838.
[23] Vale GC, Tabchoury CP, Arthur RA, Del Bel Cury AA, Paes Leme AF, Cury JA. Temporal relationship between sucrose-associated changes in dental biofilm composition and enamel demineralization. Caries Res 2007; 41(5): 406-12.
[24] Karlinsey RL, Mackey AC, Walker TJ, Frederick KE, Blanken DD, Flaig SM, Walker ER. In vitro remineralization of human and bovine white-spot lesions by NaF toothpastes: a pilot study. J Dent Oral Hyg 2011; 3:22–29.
[25] Mensinkai PK, Ccahuana-Vasquez RA, Chedjieu I, Amaechi BT, Mackey AC, Walker TJ, Blanken DD, Karlinsey RL. In situ remineralization of white-spot enamel lesions by 500 and 1,100 ppm F toothpastes. Clin Oral Investig 2012; 16(4):1007-14. doi: 10.1007/s00784-011-0591-2.
[26] PAS 71:2005. "Publicly Available Specification", PAS, ISBN 0 580 45925 X. UK Department of Trade and Industry (DTI) and British Standard Institution (BSI). Available at ftp://law.resource.org/uk/ibr/bs.pas.71.2005.pdf. 2013.

denominado “top-down” [PAS 71:2005[26]] ao TMP [Danelon *et al.*, 2013b[13]], pelo qual partículas de TMP de tamanho elevado (“bulk”) foram transformadas em nanopartículas por meio da moagem ou trituração, porém sem funcionalização. Em escala nanométrica, a reatividade química do TMP e sua adsorção ao esmalte foram aumentadas devido ao aumento da área superficial e da maior porcentagem de átomos em sua superfície [Danelon *et al.*, 2013b[13]]. Os autores demonstraram que a adição de nanopartículas de TMP a um DC reduziu a desmineralização (*in vitro*) e promoveu a remineralização (*in situ*) do esmalte, superior a um DC, e em comparação ao TMP micrométrico.

Considerando que um DCRF (250 ppm F) suplementado com TMP micrométrico apresentou eficácia anticárie semelhante a um DC [Missel *et al.*; 2010[5]] e que as nanopartículas de TMP (TMPnano) apresentam maior reatividade e adsorção ao esmalte [Danelon *et al.*, 2013b[13]], levantou-se a hipótese de potencializar o efeito anticárie de um DCRF (250 ppm F) utilizando o TMPnano como agente de suplementação. No entanto, a eficácia da formulação depende de uma proporção molar adequada entre o F e o TMP, pois estes agentes, aparentemente, competem pelos mesmos sítios de ligação na hidroxiapatita [Souza *et al.*, 2013[27]] e a adsorção dos polifosfatos ocorre rapidamente [Anbar *et al.*, 1979[28]]. Uma vez que não há dados suficientes sobre o TMPnano, é importante avaliar a influência do processo de trituração do TMP sobre a reatividade das partículas, bem como o efeito desta reatividade sobre a proporção molar F:TMPnano. Assim, este estudo avaliou o

[27] Souza JA, Amaral JG, Moraes JC, Sassaki KT, Delbem AC. Effect of sodium trimetaphosphate on hydroxyapatite solubility: an in vitro study. Braz Dent J 2013; 24(3):235-40.
[28] Anbar M, Farley EP, Denson DD, Maloney KR. Localized fluoride release from fluorine-carrying polyphosphonates. J Dent Res 1979; 58:1134-1145.

efeito do TMPnano associado a um DCRF (250 ppm F) sobre o processo de desmineralização do esmalte bovino *in vitro* e *in situ*.

O presente estudo foi dividido em dois capítulos:

Capítulo 1: **"Nano-sized sodium trimetaphosphate enhances the anticaries effect of low-fluoride dentifrices *in vitro*"** (artigo redigido segundo as normas do periódico Nanotechnology[a]). Este estudo *in vitro* apresenta a investigação da influência do processo de trituração do TMP sobre a reatividade das partículas, bem como o efeito de diferentes concentrações de TMPnano associadas a um DCRF (250 ppm F) sobre a desmineralização do esmalte bovino. Para tanto, empregou-se um modelo de ciclagem de pH tendo como variáveis de resposta: a porcentagem de perda de dureza de superfície e a concentração de fluoreto (F), cálcio (Ca), fósforo (P) e proporção molar Ca/P no esmalte.

Capítulo 2: **"*In situ* effect of low-F dentifrices containing nano-sized sodium trimetaphosphate on enamel demineralization"** (artigo redigido segundo as normas do periódico Acta Biomaterialia[b]). Este estudo *in situ* apresenta a investigação da efetividade anticárie da formulação experimental que teve o melhor resultado no estudo *in vitro*, empregando-se um modelo *in situ* de desmineralização (4 fases). Dezenove voluntários utilizaram aparelhos contendo blocos de esmalte bovino recobertos por tela plástica para acúmulo de biofilme e foram aleatoriamente distribuídos quanto ao dentifrício a ser utilizado, sob desafio cariogênico (sacarose 30%, 6x/dia), por 7 dias. As

[a] Anexo A
[b] Anexo B

variáveis de resposta no esmalte foram: a porcentagem de perda de dureza de superfície, a perda integrada de dureza e a concentração de F, Ca e P. No biofilme formado sobre os blocos foram analisadas as concentrações de F, Ca, P e polissacarídeo extracelular (EPS).

CAPÍTULO 1

"Nano-sized sodium trimetaphosphate enhances the anticaries effect of low-fluoride dentifrices *in vitro*"

NANO-SIZED sodium trimetaphosphate enhances the anticaries effect of low-fluoride dentifrices *in vitro*

Maria Daniela Basso de Souza[1], Juliano Pelim Pessan[2], Kikue Takebayashi Sassaki[2], Ana Elisa de Mello Vieira[2], Flávia Nantes de Castilho[2], Carolina Simonetti Lodi[2], José Antônio Santos Souza[2], Marcelle Danelon[2], Emerson Rodrigues Camargo[3], Francisco Souza Neto[3] and Alberto Carlos Botazzo Delbem[2,4]

[1]PhD student, Araçatuba Dental School, Univ Estadual Paulista (UNESP), Department of Pediatric Dentistry and Public Health. Assistant Professor at School of Dentistry, UNIOESTE – Univ. Estadual do Oeste do Paraná, Cascavel-PR, Brazil. Rua Universitária, 2069. Cascavel , PR - CEP 85819-110 – Brazil.

[2] Araçatuba Dental School, Univ. Estadual Paulista (UNESP). Department of Pediatric Dentistry and Public Health. Rua José Bonifácio 1193 Araçatuba, SP - Cep 16015-050 – Brazil

[3] LIEC-Deparment of Chemistry, Federal University of São Carlos (UFSCar), 13565-905, Sao Carlos/São Paulo, Brazil

[4]Correponding author: adelbem@foa.unesp.br.

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

1. ABSTRACT

This *in vitro* study evaluated the effect of low-fluoride dentifrices containing different concentrations of micrometric or nano-sized sodium trimetaphsphate (TMP) on enamel demineralization, using a pH cycling model. Bovine enamel blocks were randomly allocated into twelve groups (n = 12/group), according to the dentifrices: placebo (no F or TMP); 1,100 ppm F (conventional dentifrice); 250 ppm F, and 250 ppm F plus micrometric TMP (250-TMP), nano-sized TMP milled for 24h (250-TMPnano24) or nano-sized TMP milled for 48h (250-TMPnano48). Micrometric and nano-sized TMP were added to the formulations at concentrations of 0.05%, 0.1% and 0.25%. Blocks were subjected to pH-cycling and treatment 2x/day with slurries of dentifrices. To evaluate mineral loss, surface hardness (SH) and the percentage of surface hardness loss (%SH) were analyzed. Fluoride (F), calcium (Ca), phosphorus (P) and Ca/P molar ratio concentrations were also determined in enamel. Data were submitted to Kruskal-Wallis test, followed by Student–Newman–Keuls' test ($p<0.05$). All concentrations of TMPnano significantly reduced %SH ($p<0.05$). The TMPnano led to higher F, Ca, P and Ca/P content in enamel only at 0.05% ($p<0.05$). Comparing the 250-0.05TMPnano48 and 250-0.05TMPnano24 dentifrices, only the first did not show significantly statistical difference when compared with 1,100 in respect of Ca/P molar ratio in enamel ($p<0.05$). It was concluded that the anticaries effect of the 250 ppm F dentifrice containing 0.05% nano-sized TMP milled for 48h was greater than the its counterpart without TMP, and similar to the conventional dentifrice. (PACS: 87.85.Rs)

Keywords: Dentifrices, Dental Enamel, Fluorides, Nanoparticles, Phosphates, Demineralization

2. Introduction

Fluoride dentifrices are regarded as effective in preventing caries, at concentrations of 1000 ppm F or above [Walsh *et al.*, 2011]. As dental fluorosis is related to the fluoride intake from this source [Mascarenhas *et al.*, 2000], the use of low-F dentifrices (LFDs) would be safer for children in order to prevent the enamel alteration.

As a dentifrice containing 500 ppm F has no effect for caries control in caries-active children [Lima *et al*, 2008], researchers have been trying to enhance their efficacy using some salts as supplement. Studies have shown that micrometric particles of sodium trimetaphosphate (TMP) added to 500 ppm F dentifrice promoted protection against caries similar to a conventional dentifrice (1,100 ppm F) [Takeshita *et al.*, 2009, 2010]. TMP was used because this polyphosphate is effective in reducing the dissolution of hydroxyapatite [McGaughey; Stowell, 1977; Roberts 1995; Manarelli *et al*., 2011; Favretto *et al.*, 2013; Danelon *et al.*, 2013a, 2014].

The nanoscience enabled the use of non-F agents in nanoparticles to enhance the performance of LFDs in de-remineralization process [Karlinsey *et al*., 2011; Mensikai *et al.*, 2012]. To the best of our knowledge, nano-sized TMP has only been investigated when added to 1,100 ppm F dentifrices. The addition of nano-sized TMP promoted significant lower enamel demineralization when compared with micrometric TMP, due to the higher reactivity of nanoparticles [Danelon *et al.*, 2013b] differently from the bulk micrometric counterpart (TMP). Given the scarcity of data about dental application of nano-sized TMP in de-remineralization of enamel, it is important to assess the influence of the milling process on the reactivity of the nanoparticles, as well as the molar ratio of nano-sized TMP:F, aiming to prevent and/or control the enamel demineralization by using LFDs containing these compounds. Based on the above, it was hypothesized that nano-sized TMP would be a promising tool to further enhance the protective effect against enamel demineralization of a 250 ppm F dentifrice containing TMP

[Missel *et al.*, 2010]. This is a relevant aspect because this formulation would promote an effect to caries control while the reduced fluoride concentration would make it significantly safer for children under six years old, especially for babies.

Therefore, the aim of this study was to evaluate the ability of LFDs (250 ppm F) supplemented with nano-sized TMP at different milling times and concentrations in inhibiting enamel demineralization, using a pH cycling model. A comparison among the dentifrice 250 ppm F and that supplemented with nano-sized TMP or micrometric TMP was made to validate the results. The null hypothesis was that a) LFDs containing nano-sized TMP presents similar anticaries effect when compared with its counterpart without TMP (250 ppm F) and b) LFDs containing nano-sized TMP presents similar anticaries effect when compared with the conventional dentifrice.

3. Material and Methods

3.1. Experimental design

Enamel blocks (4×4×3 mm, n=144) were obtained from bovine incisors. Enamel surfaces were sequentially polished and the blocks selected by initial surface hardness (SHi) (326 up to 376 KHN; p = 0.403). Blocks were randomly divided into 12 experimental groups (n=12), according dentifrices: Placebo (no F or TMP), 250 ppm F, 250 ppm F plus micrometric TMP or nano-sized TMP milled for 24h and 48h, and 1,100 ppm F (conventional dentifrice). TMP and nano-sized TMP were added at concentrations of 0.05, 0.1 and 0.25%. Blocks were subjected to a pH-cycling regimen and treatments with dentifrice slurries during 5 days. To evaluate mineral loss, surface hardness (SH) was analyzed. Fluoride (F), calcium (Ca), phosphorus (P) and Ca/P molar ratio were also determined in enamel.

3.2. Synthesis and characterization of nano-sized TMP particles

To prepare the nano-sized TMP, 70 g of pure (micrometric) sodium trimetaphosphate (TMP, Sigma™ - Aldrich Co., USA; $Na_3O_9P_3$,purity ≥ 95% CAS 7785-84-4) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol. After 24 h (for TMPnano24) or 48 h (for TMPnano48), the powder was separated from the alcoholic media and ground in a mortar [Danelon *et al.*, 2013b]. The powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC difractometer in the 2θ range from 10 to 80º with a scanning rate of 2º/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation: $L = \frac{K\lambda}{B\cos\theta_B}$

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

3.3. Dentifrice formulation and fluoride assessment

The dentifrices (Table 1) were produced with the following components: carboxymethylcellulose, titanium dioxide, sodium methyl-*p*-hydroxybenzoate, sodium saccharin, peppermint oil, glycerol, hydrated silica, sodium lauryl sulfate and water [Takeshita *et al.*, 2009]. Dentifrices containing 250 ppm F (NaF, Merck, CAS 7681-49-4, Germany) and TMP (Aldrich Chemistry, CAS 7785-84-4, China) were prepared with micrometric TMP or nano-sized TMP milled for 24h and 48h at concentrations of 0.05, 0.1 and 0.25%. Dentifrices without F and TMP (placebo), 250 ppm F and 1,100 ppm F (conventional dentifrice) were also manufactured with the same components described previously to compare and validate the results.

Dentifrice	Group
Placebo	Placebo
250 ppm F	250
250 ppm F + 0.05% micrometric TMP	250-0.05TMP
250 ppm F + 0.05% nano-sized TMP milled for 24h	250-0.05TMPnano24
250 ppm F + 0.05% nano-sized TMP milled for 48h	250-0.05TMPnano48
250 ppm F + 0.1% micrometric TMP	250-0.1TMP
250 ppm F + 0.1% nano-sized TMP milled for 24h	250-0.1TMPnano24
250 ppm F + 0.1% nano-sized TMP milled for 48h	250-0.1TMPnano48
250 ppm F + 0.25% micrometric TMP	250-0.25TMP
250 ppm F + 0.25% nano-sized TMP milled for 24h	250-0.25TMPnano24
250 ppm F + 0.25% nano-sized TMP milled for 48h	250-0.25TMPnano48
1,100 ppm F	1,100

Table 1: Dentifrices and groups.

Total fluoride (TF) and ionic fluoride (IF) were determined using a specific electrode for F (9609 BN, Orion Research Inc., Beverly Inc.) [Delbem *et al.*, 2012]. Mean (SD) TF and IF of the placebo dentifrice were 8.8 (0.5) and 10.7 (2.1) ppm F, respectively. For the dentifrice groups with 250 ppm F were 267.1 (7.3) (ranging from 260.2 to 288.7) and 270.7 (3.7) (ranging from 263.9 to 282.2). For 1,100 ppm F dentifrice were 1,184.2 (41.4) and 1,178.6 (10.3).

3.4. pH-cycling and dentifrice treatment

Blocks were subjected to 5 pH cycles at 37 ºC and kept in fresh remineralizing solution for 2 days afterwards. The treatment was performed under agitation (twice a day/ 1 min) using dentifrice suspension (dentifrice: deionized water, 1:3 – w/w; 2 mL/block) before and after immersion in a demineralizing solution (6 hours—2.0 mmol/L $Ca(NO_3)_24H_2O$ and 2.0 mmol/L $NaH_2PO_4H_2O$ in 0.075 mol/L acetate buffer, 0.05 µg F/ml at pH 4.7—2.2 mL/mm^2). The enamel blocks were kept in a remineralizing solution (1.5 mmol/L $Ca(NO_3)_2$ $4H_2O$, 0.9 mmol/L NaH_2PO_4 H_2O, 0.15 mol/L KCl in 0.02 mol/L cacodylate buffer, 0.05 µg F/mL at pH 7.0—1.1 mL/mm^2) for 18 hours. The blocks were washed with deionized water during 30 seconds, after being removed from de- and remineralizing solutions or dentifrice suspension. [Vieira *et al.*, 2005]

3.5. Hardness measurements

Surface hardness (Knoop) was determined before (SHi) and after (SHf) pH-cycling using a microhardness tester (Micromet 5114, Buehler, Lake Bluff, USA) under a 25-g load for 10 s. Five indentations, spaced 100 µm apart, were made in the center of the enamel blocks [Takeshita *et al.*, 2009]. After pH-cycling, five indentations (SHf), spaced 100 µm from the baseline indentations, were made to calculate the percentage of surface hardness loss (%SH=[(SHf−SHi)/SHi]×100).

3.6. Fluoride, Calcium and Phosphorus analysis in enamel

Enamel blocks (2x2 mm, n=144) obtained from half of the longitudinally sectioned blocks were attached to a mandrel, which was fixed to the top of a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, São Paulo) to measure the depth. One layer (~50 µm) was removed from each enamel block [Alves *et al.*, 2007; Takeshita *et al.*, 2009] using self-adhesive polishing discs (13mm in diameter, 400 grit silicon carbide, Buehler) fixed to the bottom of polystyrene crystal flasks (J-10; Injeplast, São Paulo, Brazil). The F present in enamel was determined according to method described in Weatherell *et al.* [1985] modified by Alves *et al.* [2007]. After adding 0.5 mL of 1.0 mol/L HCl to the enamel powder, the vials were agitated for 1 hour. Then, 0.25 mL of the solution was transferred to flasks and 0.25 mL of 1.0 mol/L NaOH followed by 0.5 mL of TISAB II were then added. For determination of the F content (µg/mm^3), a specific electrode 9409BN (Thermo Scientific) and reference microeletrode (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720Aplus) were used. The electrodes were previously calibrated with standards containing 0.125 to 4.0 µg F/mL in the same conditions of the samples. The measurements were made in duplicate using 50 µL of the samples and same volume of TISAB II. Calcium was measured in a spectrophotometer plate reader (Microplate Spectrophotometer EONC Biotek, Winooski, USA) using a wavelength of 650 nm by adapting the method described by Vogel *et al.* [1983]. An aliquot 5µL of the

standards and 10 µL of samples were used in duplicate, and 50 µL of Arsenazo III and deionized water were added. For calibration, 40 to 200 mg Ca / mL of standards were used. Phosphorus was measured according to Fiske and Subbarow [1925], using 20 µL of the sample and 100 µL of the standards, plus 50 µL of molybdate and 20 µL of reductive reagent at a wavelength of 660 nm. The concentrations of F, Ca and P were determined and the results were expressed in µg/mm^3.

3.7. Statistical analysis

For statistical analysis, SigmaPlot software version 12.0 (SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) was used, and the significance limit was set at 5%. The data did not present normal (Shapiro-Wilk test) and homogenous (Cochran test) distribution, so they were submitted to Kruskal-Wallis test, followed by Student-Newman-Keuls' test.

4. Results and Discussion

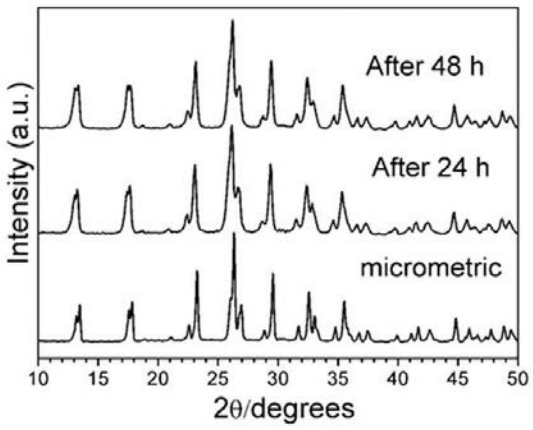


Figure 1. X-ray patterns of the micrometric and of the nano-sized TMP after milling for 24h and 48 h.

The milling processing reduced the particle size of the TMP powders without affecting the crystalline structure of the material. The X-ray diffraction (XRD) pattern of the nano-sized TMP after 24 and 48 h of milling (figure 1) shows broader peaks due the smaller crystallites,

which could be used to estimate an average particle size of 24 nm for the powder milled for 24 h and of 22.7 nm for the powder milled for 48 h.

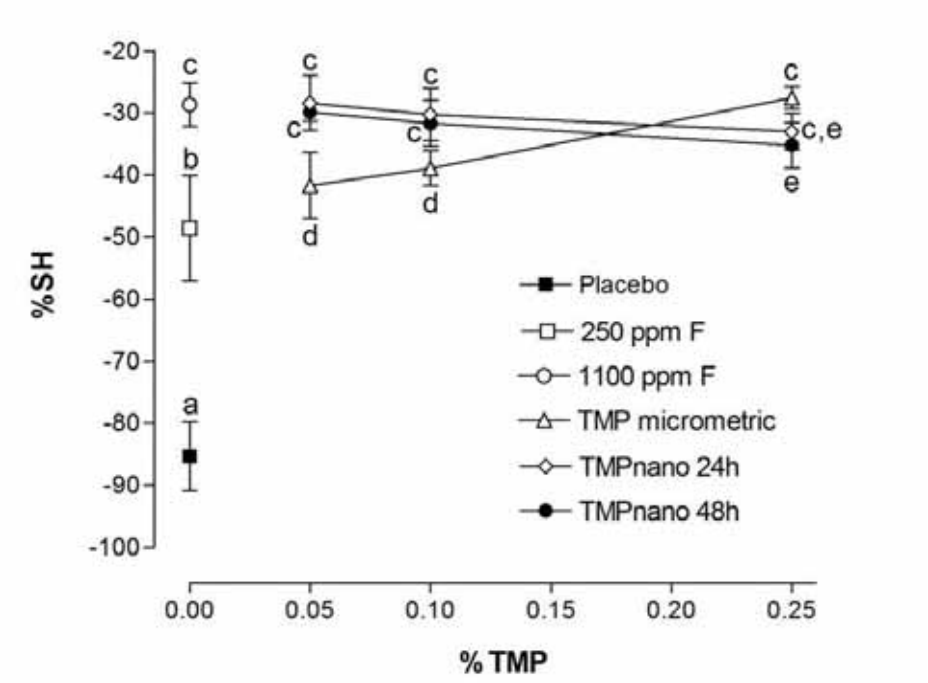


Figure 2 – Graphic representation of mean values of surface hardness loss after pH cycling according fluoride and TMP concentrations in the dentifrices as well as time of milling. Distinct letters shows statistical difference among groups (Kruskal-Wallis, Student-newman-Keuls's test; p <0.05%). Vertical bars indicate the standard deviations of means.

A dose-response relationship was seen between %SH and fluoride concentration in dentifrices without micrometric TMP (Placebo, 250 and 1,100) (Figure 2). The addition of micrometric TMP further reduced mineral loss when compared to their counterpart without TMP, showing a dose-response relationship (figure 2). The maximum effect was observed with 250-0.25TPM dentifrice, being similar to 1,100 ($p<0,05$) (figure 2).

Both 250-TMPnano24 and 250-TMPnano48 were able to reduce the mineral loss at the test concentrations. The maximal effect was achieved at 0.05% and 0.1%, similar to 1,100 dentifrice. Raising the concentration to 0.25%, the 250-TMPnano48 led to a higher mineral loss when compared to 1,100 dentifrice ($p<0,05$) (figure 2).

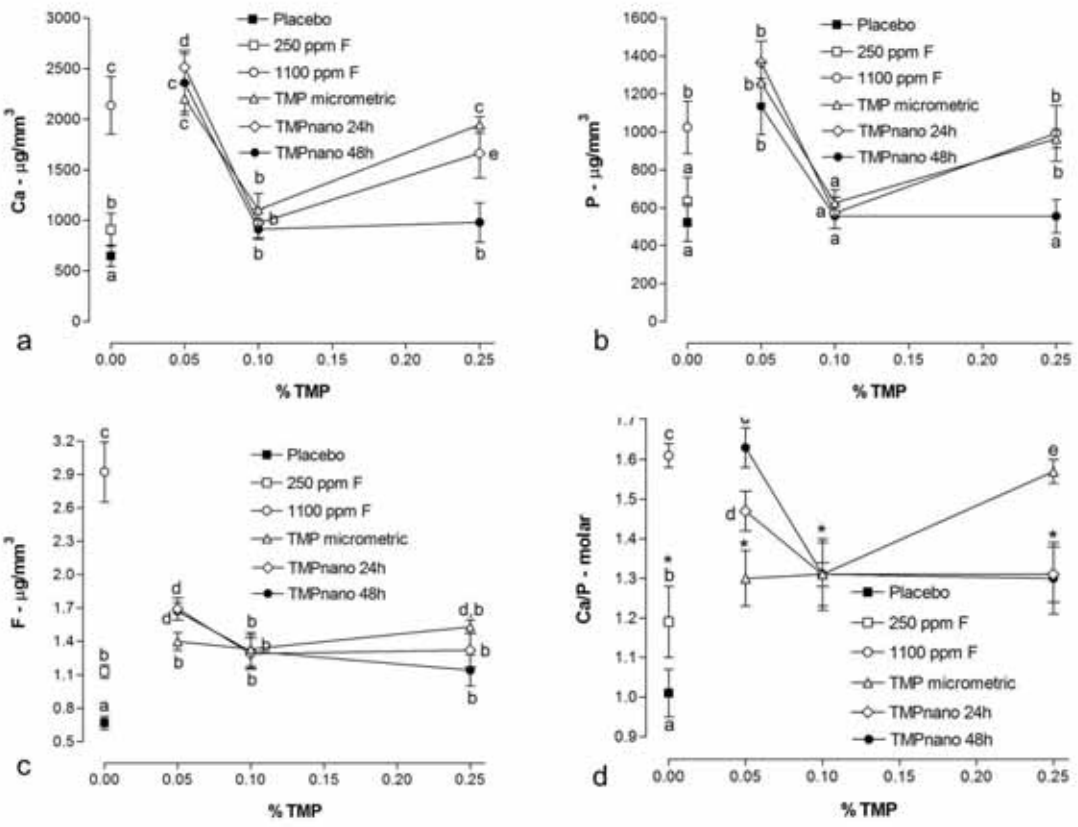


Figure 3 – Graphic representation of mean values of calcium (a), phosphorus (b), fluoride (c) and Ca/P molar ratio (d) present in the enamel after pH cycling according to fluoride and TMP concentrations in the dentifrices as well as time of milling. Distinct letters shows statistical difference among groups (Kruskal-Wallis, Student-newman-Keuls's test; $p < 0.05\%$). Bars indicate the standard error of mean.

Figure 3 shows enamel mineral composition according to the treatment groups. The higher F content in enamel was observed for 1,100 dentifrice (figure 3(c)). Micrometric TMP (0.25%) enhanced fluoride uptake, but at levels significantly lower than that of 1,100 dentifrice ($p<0,05$) (figure 3(c)). Both 250-TMPnano24 and 250-TMPnano48 showed enamel calcium, fluoride and Ca/P values, close to those seen for the 1,100 dentifrice (in comparison to 250 dentifrice), but only at low concentration (0.05%) (figures 3 (a), (c) and (d)). Between the 250-0.05TMPnano48 and 250-0.05TMPnano24 dentifrices, the dentifrice 250-0.05TMPnano48 was the only that did not show significantly statistical difference when compared with 1,100 in respect of Ca/P molar ratio in enamel ($p<0.05$) (figure 3 (d)). Higher nano-sized TMP

concentration compromised calcium and fluoride uptake, as well as Ca/P ratio (figures 3(c), (a) and (d)). The inorganic phosphorus (P) content was increased by nano-sized TMP (0.05%) and micrometric TMP (0.25%) to levels similar to 1,100 dentifrice (figure 3(b)). P concentrations were reduced when higher nano-sized TMP concentrations were used (figure 3(b)).

This study evaluated the ability of 250 ppm F dentifrice supplemented with nano-sized TMP at different milling times and concentrations in inhibiting enamel demineralization, using a pH cycling model. The results showed that the addition of nano-sized TMP to fluoride dentifrice with 250 ppm led to superior anticaries effects when compared to its counterpart without TMP (250). Thus, the first null hypothesis was rejected. Since the the addition of nano-sized TMP to fluoride dentifrice with 250 ppm enable to reach the same level than the conventional dentifrice on the enamel surface, the second null hypothesis was accepted. However, given the *in vitro* model limitations, the results should be futher assessed using *in situ* caries model. Moreover, the mechanism of nano-sized TMP as well as the roles of the inorganic and organic composition of biofilm on enamel demineralization should be investigated.

Among all the micrometric TMP concentrations, only the addition of 0.25% to the 250 ppm F dentifrice resulted in a decrease in %SH similar to that of 1,100, confirming previous findings [Missel *et al.*, 2010]. However, the 250-0.25TMP did not produce a superior effect when compared to 1,100, as observed previously [Missel *et al.*, 2010] and confirmed in the present study. The 250-0.25TMP dentifrice promoted F retention in the enamel as observed in previous study [Missel *et al.*, 2010]. When compared to that of 250 dentifrice, the F retention for 250-0.25TMP was 35% higher. However, compared with 1,100 dentifrice, the F retention for 250-0.25TMP dentifrice was 48% lower. The data showed that TMP effect is mainly related to Ca and P content present in the enamel. The Ca/P molar ratio confirms this hypothesis since lower mineral loss was observed in the cases of higher Ca/P ratio.

Regarding nano-sized TMP, the 250-0.25TMPnano did not lead to a superior anti-caries effect when compared to the 1,100 as well. Despite all TMPnano concentration had led high values of %SH (some similar to that of 1,100), data from other variables (F, Ca, P and Ca/P

enamel) showed that the nano-sized TMP improved the effect of the formulation only at the lowest concentration, leading to a more mineralized enamel, significantly higher than its counterpart without TMP, what is important in a clinical approach in future demineralization cycles that enamel may undergo. The results observed when the small concentration of TMPnano (0.05%) was used in the present study is an important finding since it might reduce the side effects [Lanigam, 2001], compared to that 0.25% of micrometric TMP used by Missel *et al.* [2010]. At 0.05%, both 250-TMPnano24 and 250-TMPnano48 were similar to 1,100 dentifrice on enamel surface hardness, and this might be related to the F enamel content (50% higher when compared to 250 dentifrice, although significantly lower than that of 1,100), as well to the Ca and P enamel content. Other studies also showed that TMP increases F and Ca enamel uptake when compared with the counterpart without TMP in dentifrices [Missel *et al.*, 2010; Takeshita *et al.*, 2009, 2010]. However, given the Ca/P molar ratio is an important parameter in terms of enamel solubility, the result observed for the 250-0.05TMPnano48 dentifrice (similar to the 1,100 dentifrice) seems to justify the use of the 48h milling time instead of 24h. The results from mineral content of enamel observed for the 250-0.05TMPnano48 and 250-0.05TMPnano24 suggest that the processing (top-down subtractive processing) [PAS 71:2005] used to synthesize nano-sized TMP from bulk TMP promoted more reactive particles with an increased adsorption to enamel, due to the reduction on the size and the increase on surface area (in proportion to its volume), which led to a high percentage of atoms on the surface [Danelon *et al.*, 2013b].

The mechanism of action of nano-sized TMP seems to be similar to that of TMP. When TMPnano at 0.05% adsorbs on enamel, this interaction might have altered the selective permeability and ion diffusion into the enamel [Takeshita *et al.*, 2009, 2011; Favretto *et al.* 2013; Souza *et al.*, 2013; Van Dijk *et al.*, 1980; Danelon *et al.*, 2014; Manarelli *et al.*, 2014]. However, the data of the present study shows that TMP effect is mainly related to calcium and phosphate enamel content, confirmed by Ca/P molar ratio. Thus, nano-sized TMP possibly retains charged ions of CaF^{+} and Ca^{2+} by replacing Na^{+} from cyclic structure [Manarelli *et al.*, 2013] promoting the reduction on the acid diffusion [Takeshita *et al.*, 2011; Danelon *et al.*,

2014; Manarelli *et al.*, 2014] as well as during the de-remineralization processes, leading to the formation and flux of neutral species ($CaHPO_4^0$ and HF^0) into the enamel. It is an important aspect given the diffusion coefficient of these neutral species in enamel is thousandfold higher than that of their charged counterparts [Cochrane *et al.*, 2008]. Moreover, the high percentage of atoms on the surface of nano-sized TMP at higher concentrations could sequester a greater amount of Ca^{2+} (as well as CaF^+), negatively influencing the incorporation of these ions into the enamel, what might impair the effect of the formulation [Camara *et al.*, 2014], given that the complexing ability of a condensed phosphate is proportional to the total number of phosphorus atoms [van Wazer, Campanella, 1950].

Differently from the functionalized nano-sized non-F agent (in what there is an attachment of organic reagents to the salt surface) used as suplements to LFDs [Karlinsey *et al.*, 2011; Mensikai *et al.*, 2012], the present study showed the nano-sized TMP synthesized by [Danelon *et al.*, 2013b] enhanced the anticaries effect of the 250 ppm F dentifrices, which have significantly lower effect in caries control compared to a conventional (above 1000 ppm F) formulation [Walsh *et al.*, 2011]. The developed experimental formulation promoted an effect in inhibiting enamel demineralization similar to 1,100 dentifrice, and it could be used for people in general. Besides, in a public oral health approach, the 4-fold reduction on fluoride concentration compared to the conventional dentifrices makes the 250-0.05TMPnano48 a safe alternative dentifrice for children under six years old, mainly for babies, whose fluoride intake from this source is, in fact, a problem with respect to dental fluorosis.

5. Conclusion

On the basis of the findings of this *in vitro* study, the addition of 0.05% nano-sized TMP milled for 48h to a 250 ppm F dentifrice promoted greater inhibition of enamel demineralization when compared to its counterpart without micrometric TMP. It also promoted inhibition of enamel demineralization similar to the conventional dentifrice.

Acknowledgements

We thank Ms. Maria Fernandes for her technical laboratorial assistance. The authors also thank LIEC-Deparment of Chemistry, UFSCar, São Carlos, Brazil for the production and characterization of sodium trimetaphosphate nanoparticles. Financial support: National Board of Scientific and Technological Development/CNPq (grant #18263/2011) and Coordination of Improvement of Higher Education Personnel/CAPES (grant #23038.033495/2008-97).

18263

6. References

Walsh T, Worthington H V, Glenny A M, Appelbe P, Marinho V C, Shi X 2011 Fluoride toothpaste of different concentrations for preventing dental caries in children and adolescents *Cochrane Database Syst Rev* **20**: CD007868.

Mascarenhas A K 2000 Risk factors for dental fluorosis: a review of the recent literature *Pediatr Dent* **22**:269–277.

Lima T J, Ribeiro C C, Tenuta L M A, Cury J A 2008 Low-fluoride toothpaste and caries lesion control in children with different caries experience: a randomized clinical trial *Caries Res.* **42** 46-50.

Takeshita E M, Castro L P, Sassaki K T, Delbem A C 2009 In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate *Caries Res.* **43**(1) 50-6.

Takeshita E M, Castro L P, Danelon M, Sassaki K T, Delbem A C 2010 Evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate on biofilm and enamel demineralization in an in situ study *Caries Res.* **44** 171-247 (abstract 77). (DOI:10.1159/000315447)

McGaughey C, Stowell E C 1977 Effects of polyphosphates on the solubility and mineralization of HA: relevance to a rationale for anticaries activity *J. Dent. Res.* **56**(6) 579-87.

Roberts J 1995 Role of models in assessing new agents for caries prevention-non-fluoride systems *Adv. Dent. Res.* **9** 304-11.

Manarelli M M, Vieira A E, Matheus A A, Sassaki K T, Delbem A C 2011 Effect of mouth rinses with fluoride and trimetaphosphate on enamel erosion: an in vitro study *Caries Res.***45**(6) 506-9. doi: 10.1159/000331929.

Favretto C O, Danelon M, Castilho F C N, Vieira A E M, Delbem A C B 2013 In vitro evaluation of the effect of mouth rinse with trimetaphosphate on enamel demineralization *Caries Res*. **47**(5) 532-38.

Danelon M, Takeshita E M, Peixoto L C, Sassaki K T, Delbem A C 2013a Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization C*lin. Oral Investig.* DOI 10.1007/s00784-013-1102-1104.

Danelon M, Takeshita E M, Peixoto L C, Sassaki K T, Delbem A C 2014 Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization *Clin. Oral Investig.* **18**(4) 1119-27.

Karlinsey R L, Mackey A C, Walker T J, Frederick K E, Blanken D D, Flaig S M, Walker E R 2011 In vitro remineralization of human and bovine white-spot lesions by NaF toothpastes: a pilot study *J. Dent. Oral Hyg.* **3** 22–9.

Mensinkai P K, Ccahuana-Vasquez R A, Chedjieu I, Amaechi B T, Mackey A C, Walker T J, Blanken D D, Karlinsey R L 2012 In situ remineralization of white-spot enamel lesions by 500 and 1,100 ppm F toothpastes *Clin. Oral Investig.* **16**(4) 1007-14. doi: 10.1007/s00784-011-0591-2.

Danelon M, Castro L P, Souza-Neto F N, Camargo E R, Delbem A C B 2013b Avaliação in vitro de dentifrícios suplementados com nanopartículas de trimetafosfato de sódio sobre a desmineralização dentária *Braz. Oral Res.* **27**(Suppl. 1) 35-40.

Missel E M C, Delbem A C B, Vieira A E M, Sassaki K T, Cruz N V S, Percinoto C 2010 Avaliação de dentifrícios com concentração reduzida de fluoreto associada ao trimetafosfato de sódio na desmineralização do esmalte *Braz. Oral Res.* **24** (Suppl. 1) 247-83.

Delbem A C, Bergamaschi M, Rodrigues E, Sassaki K T, Vieira A E, Missel E M C 2012 Anticaries effect of toothpastes with calcium citrate and sodium trimetaphosphate *J. Appl. Oral Sci.* **20** 94–8.

Vieira A E, Delbem A C, Sassaki K T, Rodrigues E, Cury J A, Cunha R F 2005 Fluoride dose response in pH-cycling models using bovine enamel *Caries Res*. **39** 514-20.

Alves K M, Pessan J P, Brighenti F L, Franco K S, Oliveira F A, Buzalaf M A, Sassaki K T, Delbem A C 2007 In vitro evaluation of the effectiveness of acidic fluoride toothpastes *Caries Res.*;**41**(4) 263-68.

Weatherell J A, Robinson C, Strong M, Nakagaki H 1985 Micro-sampling by abrasion *Caries Res*. **19** 97-102.

Vogel G L, Chow L C, Brown W E 1983 A microanalytical procedure for the determination of calcium, phosphate and fluoride in enamel biopsy samples *Caries Res*. **17** 23-31.

Fiske C H, Subbarow Y 1925 The colorimetric determination of phosphorus *J. Biol. Chem.* **66** 375-400.

Lanigan R S 2001 Final report on the safety assessment of sodium metaphosphate, sodium trimetaphosphate, and sodium hexametaphosphate *Int J Toxicol* **20**: 75–89. doi: 10.1080/10915810152630756

PAS 71:2005. “Publicly Available Specification”, PAS, ISBN 0 580 45925 X. UK Department of Trade and Industry (DTI) and British Standard Institution (BSI). Available at ftp://law.resource.org/uk/ibr/bs.pas.71.2005.pdf. 2013

Takeshita E M, Exterkate R A, Delbem A C, ten Cate J M 2011 Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization in vitro *Caries Res*. **45**(5) 494-97.

Souza J A, Amaral J G, Moraes J C, Sassaki K T, Delbem A C 2013 Effect of sodium trimetaphosphate on hydroxyapatite solubility: an in vitro study *Braz. Dent. J.* **24**(3) 235-40.

van Dijk J W, Borggreven J M, Driessens F C 1980 The effect of some phosphates and a phosphonate on the electrochemical properties of bovine enamel *Arch. Oral Biol.* **25** 591–5.

Manarelli M M, Delbem A C B, Lima T M T, Castilho F C N, Pessan J P 2014 In vitro remineralizing effect of fluoride varnishes containing sodium trimetaphosphate *Caries Res.* **48** 299–305.

Manarelli M M, Delbem A C B, Lima T M T, Castilho F C N, Pessan J P 2013 Effect of fluoride varnish supplemented with sodium trimetaphosphate on enamel erosion and abrasion *Am. J. Dent.* **26**(6) 307-12.

Cochrane H J, Saranathan S, Cai F, Cross K J, Reynolds E C 2008 Enamel subsurface lesion remineralisation with casein phosphopeptide stabilised solutions of calcium, phosphate and fluoride *Caries Res.* **42**(2) 88-97.

Camara D M, Miyasaki M, Danelon M, Sassaki K T, Delbem A C 2014 Effect of low-fluoride toothpastes combined with hexametaphosphate on in vitro enamel demineralization *J. Dent.* **42**(3) 256-62.

van Wazer J R, Campanella D A 1950 Structure and properties of the condensed phosphates. IV. Complex ion formation in polyphosphate solutions *J. American Chemical Society* **72**:665-63.

CAPÍTULO 2

“In situ effect of low-F dentifrices containing nano-sized sodium trimetaphosphate on enamel demineralization”

***In situ* effect of low-F dentifrices containing nano-sized sodium trimetaphosphate on enamel demineralization**

Souza M. D. B. [a], Pessan J. P.[b], Lodi C.S.[b], Souza J.A.S. [b], Camargo E.R. [c], Souza Neto F. N.[c], Delbem A. C. B.[b]

[a]PhD student, Araçatuba Dental School, Univ. Estadual Paulista (UNESP), Department of Pediatric Dentistry and Public Health. Assistant Professor at School of Dentistry, UNIOESTE – Univ. Estadual do Oeste do Paraná, Cascavel-PR, Brazil. Rua Universitária, 2069. Cascavel , PR - CEP 85819-110 – Brazil.

[b] Araçatuba Dental School, Univ. Estadual Paulista (UNESP). Department of Pediatric Dentistry and Public Health. Rua José Bonifácio 1193 Araçatuba, SP - Cep 16015-050 – Brazil

[c] LIEC-Deparment of Chemistry, Federal University of São Carlos (UFSCar), 13565-905, Sao Carlos/São Paulo, Brazil

Alberto Carlos Botazzo Delbem

São Paulo State University – UNESP, Department of Pediatric Dentistry. Rua José Bonifácio 1193. Araçatuba, SP - Cep 16015-050. Brazil. Tel. +55 18 3636 3235 - Fax +55 18 3636 3332
Email: adelbem@foa.unesp.br

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

ABSTRACT

This crossover study evaluated the effect of low-fluoride dentifrices containing nano-sized trimetaphosphate TMP (TMPnano) on the enamel demineralization *in situ*. Nineteen subjects wore palatal appliances containing 4 blocks of bovine enamel, and randomly assigned to brush their teeth with dentifrices placebo (without F/TMP), 250 ppm F, 250 ppm F plus 0.05% TMPnano and 1,100 ppm F (conventional dentifrice), under cariogenic challenge, during 7 days. Enamel surface hardness (SH) and cross-sectional hardness (ΔKHN), as well as fluoride (F), calcium (Ca), and phosphorus (P) concentrations were analyzed. Biofilm formed on the blocks were analyzed for F, Ca, P and insoluble extracellular polysaccharide (EPS) concentrations. Data were submitted to one-way ANOVA and Student-Newman-Keuls post-hoc test ($p<0.05$). The 250-TMPnano dentifrice promoted the lowest ΔKHN among all groups ($p<0.001$), while the percentage of SH loss was similar to the 1,100 ppm F group. The 1,100 and 250-TMPnano dentifrices led to similar F, Ca, P concentrations in enamel. In biofilm, the highest F content was observed for 1,100; Ca content was similar between 1,100 and 250 TMPnano; and, P content was similar among the groups. Similar values were observed for 250 TMPnano and 1,100 groups regarding EPS ($p<0.001$), ionic activity of $CaHPO_4^0$, CaF^+ and HF^0 ($p<0.05$) and degree of saturation of HA and CaF_2 ($p<0.05$). It was concluded that the anticaries effect of the 250 ppm F dentifrice containing nano-sized TMP was similar to a conventional dentifrice for most of the variables studied, while the effect of the TMP-containing formulation on the subsurface lesion was significantly higher among all groups.

Keywords: Tooth Demineralization, Phosphates, Dentifrices, Nanoparticles, Dental Plaque

1. Introduction

Low-fluoride dentifrices (LFDs) have been indicated to children to reduce fluoride intake and, therefore, to minimize the risk of dental fluorosis, given that the use of fluoridated dentifrices by young children has been associated with dental fluorosis in the permanent dentition [Wong *et al.*, 2011]. However, the use of these formulations should be carefully considered because there is evidence the LFDs (500 ppm F) are not as effective as a conventional formulation (1,100 ppm F) in caries-active children [Lima *et al.*, 2008].

In order to increase the anticaries effect of LFDs, the addition of phosphate salts, such as micrometric sodium trimetaphosphate (TMP) have been proposed [Missel *et al.*, 2010; Takeshita *et al.*, 2009, 2010; Delbem *et al.*, 2012; Hirata *et al.*, 2013] since TMP has been shown to reduce the dissolution of hydroxyapatite [McGaughey; Stowell, 1977; Roberts 1995; Manarelli *et al.*, 2011; Favretto *et al.*, 2013; Danelon *et al.*, 2013a]. When added in dentifrices containing 500 ppm F, the association between TMP and fluoride (F) at an adequate molar ratio promoted protection against caries similar to a 1,100 ppm F dentifrice, without compromise F bioavailability in the TMP-containing formulations [Takeshita *et al.*, 2009, 2010]. Moreover, the above mentioned formulation was also able to increase the reservoirs of F and calcium (Ca) in biofilm formed on enamel surface as well as to decrease the formation of extracellular insoluble polysaccharide (EPS) in the biofilms [Takeshita *et al.*, 2010]. Interestingly, a synergistic effect of F and TMP has also been demonstrated in dentifrices with even lower fluoride content. The addition of TMP to a 250 ppm F dentifrice promoted a similiar protective effect against enamel demineralization when compared with a 1,100 ppm F, despite the over

4-fold difference in fluoride content between the formulations [Missel *et al.*, 2010].

The use of nanotechnology has been proposed as an attempt to further improve the anticaries and anti-erosive potential of TMP-F containing dentifrices (due to the more reactivity of nano-sized TMP) [Danelon *et al.*, 2013b]. Considering that the addition of micrometric particles of TMP to a 250 ppm F dentifrice significantly improved the anticaries effect of this formulation [Missel *et al*., 2010], it is possible that the use of nano-sized TMP might further improve the anticaries potential of this dentifrice. Unpublished *in vitro* data from our group showed that the association between nano-sized TMP and 250 ppm F led to a similar inhibition of enamel demineralization when compared with a 1,100 ppm F dentifrice. Based on that, it was hypothesize that such experimental formulation would inhibit enamel demineralization similarly as 1,100 ppm F, by an *in situ* model. Besides the direct effects on enamel mineral content, it could be expected that this formulation might render a less cariogenic biofilm through enhancing the ion reservoirs and reducing the EPS concentration [Takeshita *et al.*, 2010].

Based on the above, the present study evaluated the ability of LFDs (250 ppm F) supplemented with nano-sized TMP in inhibiting enamel demineralization, and its effect on the mineral composition of enamel and biofilm as well as the EPS concentration in this biofilm. The null hypothesis was that LFDs containing nano-sized TMP presents similar anticaries effect when compared to its counterpart without TMP (250 ppm F) and to conventional dentifrice (1,100 ppm F).

2. Material and Methods

2.1. Experimental design

The procedures adopted in this study were approved by the Human Ethics Committee of Araçatuba Dental School, São Paulo State University, Brazil (Protocol: 16348414.4.0000.5420) and the subjects provided signed informed consent prior to beginning of the study. The cross-over *in situ* study was performed in 4 phases of 7 days each. A sample size of nineteen volunteers was calculated considering minimum detectable difference in mean hardness of 20.0, standard deviation of 18.0, α-error level of 5%, β-error level of 20% (SigmaPLot 12.0 software; Afonso *et al.*, 2013). Healthy subjects [Zero, 1995] wore palatal appliances containing 4 enamel bovine blocks selected by surface hardness (SH). Volunteers were randomly assigned into the following dentifrices (pH 7,0) treatment groups: placebo (no F or nano-sized TMP), 250 ppm F, 250 ppm F supplemented with 0.05% nano-sized TMP (250-TMPnano) and 1,100 ppm F (conventional dentifrice). During the experimental periods, the volunteers dripped a 30% sucrose solution on the enamel blocks 6 times a day. For the treatments, slurries of test dentifrices were applied on the blocks twice daily. After each experimental period, the biofilm was collected for F, calcium (Ca), phosphorus (P) and EPS analysis. The ionic activity (IA) of the various species and the degree of saturation (DS) of the solid phases of hidroxiapatite (HA) and CaF_2 were calculated from the concentrations (mmol/kg) of Ca, F and P on the biofilm for each dentifrice. Final surface hardness (SHf), %SH, integrated loss of subsurface hardness (ΔKHN) and enamel F, Ca and P concentrations were also determined.

2.2. Synthesis and characterization of nano-sized TMP particles

To prepare the nano-sized TMP, 70 g of pure (micrometric) sodium trimetaphosphate ($Na_3O_9P_3$, Aldrich Chemistry, China, purity ≥ 95% CAS 7785-84-4) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol. After 48 h the powder was separated from the alcoholic media and ground in a mortar [Danelon *et al.*, 2013b]. The powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC difractometer in the 2θ range from 10 to 80º with a scanning rate of 2º/min. The coherent crystalline domains (crystallite size) were estimated using the Scherrer equation:

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

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

2.3. Dentifrices formulation and fluoride assessment

The experimental dentifrices were produced with the following components: carboxymethylcellulose, titanium dioxide, sodium methyl-*p*-hydroxybenzoate, sodium saccharin, peppermint oil, glycerol, hydrated silica, sodium lauryl sulfate and water [Takeshita *et al.*, 2009]. A dentifrice containing 250 ppm F (NaF, Merck, CAS 7681-49-4, Germany) and 0.05% nano-sized TMP (Aldrich Chemistry, CAS 7785-84-4, China) milled for 48h was prepared (250-

TMPnano). Dentifrices placebo (no F or nano-sized TMP), 250 ppm F (250) and 1,100 ppm F (1,100 - conventional dentifrice) were produced with the same basic components described previously to compare and validate the results.

Total fluoride (TF) and ionic fluoride (IF) were determined using a specific electrode for F (9609 BN, Orion Research Inc., Beverly Inc.) and calibrated with standards containing 0.125 to 2.000 µg F/g [Delbem *et al.*, 2012]. Mean (SD) TF and FI (n = 3) for the placebo, 250 ppm F, 250-TMPnano and 1,100 ppm F dentifrices were 8.4 (0.6) and 9.3 (0.3); 274.7 (5.2) and 274.5 (5.5); 262.1 (7.0) and 261.0 (5.9); and 1,184.2 (41.4) and 1,137.3 (10.3), respectively.

2.4. Preparation of enamel blocks and palatal appliances

Enamel blocks measuring 4 × 4 mm were obtained from bovine incisors previously stored in 2% formaldehyde solution (pH 7.0) for 1 month. They were sequentially polished and selected by surface hardness test (330.0–380.0 KHN) as previously described [Takeshita *et al.*, 2009]. Blocks were then randomly allocated into four groups of 76 teeth each [Amaral *et al.*, 2013]. Four enamel blocks were fixed in the palatal appliance in each phase. A 4.0-mm deep space was created in the appliances, leaving approximately the space of 1.0-mm for biofilm accumulation on the enamel blocks. Biofilms were protected from mechanical forces by a plastic mesh fixed with wax on the acrylic surface appliance [Amaral *et al.*, 2013].

2.5. Intraoral procedures

Fresh 30% sucrose solutions were prepared every 48 hours as the cariogenic challenge. The volunteers were instructed to remove the appliances

from the oral cavity and drip 2 drops of this solution (enough to fill the 1.0 mm space) onto each enamel block 6 times a day at predetermined times (8:00 am, 11:00 am, 2:00 pm, 5:00 pm, 7:00 pm, and 9:00 pm). After dripping, the appliances were left to rest for 5 minutes before being reinserted into the mouth. The treatment with the dentifrice slurries was performed twice daily for 7 days (at 7:30 a.m. and 9:30 p.m.) by dripping a slurry (dentifrice/deionized water suspension 1:3, weight:weight) on each enamel block, and after that reinserted in the mouth. The volunteers brushed their natural teeth three times a day (after the main meals) with the same dentifrice that had been using during the experimental period. They used a non-fluoride dentifrice during the 7-day pre-experimental and 7-day washout periods. Diet restrictions were not imposed on the volunteers, but they were instructed to remove the appliance only during meals and when practicing oral hygiene. They were restricted from using any antimicrobial or fluoride product during the experiment all phases [Amaral *et al.*, 2013].

2.6. Dental biofilm analysis

In the morning of the eighth day of each phase, the biofilm formed on the enamel blocks was collected and weighed in pre-weighed microcentrifuge cap tubes. The biofilms were then frozen for later analysis. After that, the biofilm samples were dried in vacuum with phosphorus pentoxide (Vetec Quimica Fina Ltda., Duque de Caxias, RJ, Brazil) for 12 h at room temperature. Hydrochloric acid (0.5 mol/L) was added to the tubes in the proportion of 250 µL/mg biofilm wet weight. After extraction for 3 h at room temperature under constant

agitation, the same volume of NaOH 0.5 mol.L-1 was added. The samples were then centrifuged (11,000 x *g*) for 1 min [Nobre dos Santos *et al.*, 2002] and the supernatant retained for determination of F [Amaral *et al.*, 2013] Ca [Vogel *et al.*, 1983] and P [Fiske, Subbarow, 1925]. Insoluble polyssaccharides (EPS) were extracted by adding NaOH 1.0 mol.L-1 (500 µL/mg dry weight) in the biofilm. The samples were vortexed for 1 min and after 3 h under agitation at room temperature, and centrifuged (1 min, 11,000 x *g* at room temperature) [Nobre dos Santos *et al.*, 2002]. Supernatants were precipitated with 75% ethanol overnight, centrifuged and re-suspended in 1.0 mol.L-1 NaOH [Ccahuana-Vasquez *et al.*, 2007]. Carbohydrate analysis was carried out by the phenol-sulfuric acid procedure [Dubois *et al.*, 1956]. The results were expressed as µg/mg dry weight.

The ionic activity (IA) of the various species (Ca^{2+}, $CaPO_4^-$, $CaHPO_4^0$, $CaH_2PO_4^+$, PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^-$, CaF^+, HF^0, and F^-) and the degree of saturation (DS) of the solid phases of HA and CaF_2 were calculated from the concentrations (mmol/kg) of calcium, fluoride, and phosphorus on the biofilm for each dentifrice [Amaral *et al.*, 2013]. All calculations were performed for conditions at 37°C, pH 7, and density of 1.0 g/cm^3 by the PHREEQC Interactivef (version 2.18.3) speciation program.

2.7. Enamel hardness analysis

The post-cariogenic challenge (SH*f*), the percentage of surface hardness loss (%SH), the cross-sectional hardness measurements and the integrated loss of subsurface hardness (ΔKHN) was performed and calculated as described by Takeshita *et al.* [2009].

2.8. Fluoride, Calcium and Phosphorus analysis in enamel

Enamel blocks (2x2 mm) obtained from half of the longitudinally sectioned blocks were attached to a mandrel which was fixed to the top of a modified microscope with a micrometer (Micrometer 733 MEXFLZ-50, Starret, São Paulo) to measure the depth of the wear of the enamel. One layer (~50 µm) was removed from each enamel block [Takeshita *et al.*, 2009] using self-adhesive polishing discs (13mm in diameter, 400 grit silicon carbide, Buehler) fixed to the bottom of polystyrene crystal flasks (J-10; Injeplast, São Paulo, Brazil). The F present in the enamel was determined according to the method described by Weatherell *et al*. [1985], as modified by Alves *et al.* [2007]. After adding 0.5 mL HCl 1.0 mol/L to the enamel powder, the vials were agitated for 1 hour. Then, 0.25 mL of the solution was transferred to flasks and 0.25 mL NaOH 1.0 mol/L followed by 0.5 mL TISAB II was then added. For determination of the F content (µg/mm^3), a specific electrode 9409BN (Thermo Scientific) and reference microelectrode (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A^{plus}) were utilized. The electrodes were previously calibrated with standards containing 0.125 to 4.0 µg F/mL in the same conditions of the samples. The measures were made, in duplicate, using 50 µL of the samples and same volume of TISAB II. Calcium was measured in a spectrophotometer plate reader (Microplate Spectrophotometer EONC Biotek, Winooski, USA) using a wavelength of 650 nm by adapting the method described by Vogel *et al.* [1983]. An aliquot 5µL of the standards and 10 µL of samples were used in duplicate, and 50 µL of Arsenazo III and deionized water were added to them. For calibration, 40 to 200 mg Ca / mL of standards were used. Phosphorus was measured according to the Fiske, Subbarow [1925], by

20 µL of the sample and 100 µL of the standards, plus 50 µL of molybdate and 20 µL of reductive reagent at a wavelength of 660 nm. The concentrations of F, Ca and P were determined and the results were expressed in µg/mm^3.

2.9. Statistical analysis

Statistical analysis was performed using SigmaPlot 12.0 software at a significance level of 5%. Statistical analysis was applied considering the dentifrices as variation factors. The statistical power calculated was 100% considering all the differences among groups of each main outcome response (%SH and ΔKHN). The variables were tested for normality and homogeneity (Kolmogorov-Smirnov and Cochran, respectively). The data from biofilm analysis as well as the enamel analysis were submitted to one-way ANOVA followed by Student-Newman-Keuls post-hoc testing.

3. Results

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

Table 1 shows an inverse relationship between F content in the dentifrices without TMP and the percentage of surface hardness loss, with significant difference among the dentifrices. Values seen for the group 250-TMPnano were the lowest among all the test dentifrices, but not significantly different than those of 1,100 ppm F. The integrated subsurface hardness loss (ΔKHN) in the

placebo group (Table 1) was higher than that in the other groups ($p<0.001$). The lowest ΔKHN value was observed for 250-TMPnano, which yielded statistical difference when compared with all groups ($p<0.001$). Fluoride, calcium, and phosphorus values in enamel were similar among 250-TMPnano and 1,100 dentifrices, and higher than those seen for the 250 group.

The values of F, Ca and P in biofilm according to dentifrices are shown in Table 1. The highest F content was observed for 1,100, followed by 250-TMPnano, 250 and placebo groups. Calcium content was similar between 1,100 and 250-TMPnano, and higher than placebo and 250 dentifrices, while P was similar among all the dentifrices groups. The following variables were similar between 250-TMPnano and 1,100 dentifrice groups: alkali-soluble EPS concentration ($p<0.001$), ionic activities of $CaHPO_4^0$, CaF^+ and HF^0 ($p<0.05$) and the degree of saturation of HA and CaF_2 ($p<0.05$) (table 2).

4. Discussion

There is a substantial body of evidence showing that the early use of fluoride dentifrice by young children can be considered as a risk factor of dental fluorosis [Wong *et al.*, 2011], since fluoride intake from this source is influenced by the age of the child, the amount of dentifrice used and dentifrice flavour [Moraes *et al.*, 2007; Kobayashi *et al.*, 2011]. This has led some professionals to indicate the use of LFDs to children at age risk for developing dental fluorosis [Pessan *et al.*, 2010, 2011]. A recent meta-analysis, however, showed that the anticaries effect of dentifrices containing 250 ppm F is significantly lower in comparison with a conventional (above 1,000 ppm F) formulation [Walsh *et al.*, 2011], what has prompted to the study of alternatives to increase the anticaries

effect of low-F formulations. The results showed that the addition of nano-sized TMP to fluoride dentifrice with 250 ppm led to superior anticaries effects when compared to a 250 ppm F dentifrice without TMP, reaching levels significantly higher than the conventional dentifrice on the lesion body. Thus, the null hypothesis was rejected.

The addition of 0.05% nano-sized TMP to the 250 ppm F dentifrice resulted in a decrease in %SH and ΔKHN when compared to 250 ppm F without TMP. Although 250-TMPnano and 1,100 dentifrices were shown to produce similar on enamel surface hardness, for 250-TMPnano the integrated subsurface hardness loss was significantly lower than that of 1,100. Considering that the addition of micrometric TMP to a 250 ppm F dentifrice increases its anticaries effect to levels comparable with a conventional formulation at both enamel surface (SH) and subsurface (ΔKHN) [Missel *et al.*, 2010], the present ΔKHN findings seem to justify the use of nanoparticles instead of micrometric TMP. The more pronounced effect of nano-sized TMP on ΔKHN suggested that, under clinical conditions, a subsurface lesion would take more time to develop when compared with a conventional dentifrice. This is extremely desirable from a clinical perspective, considering that a cavity could take a longer time to occur – or might not occur at all - when using the formulation tested in the present study. The analysis of inorganic enamel composition showed that nano-sized TMP had an effect on calcium and phosphate contents, confirming previous *in vitro* study conducted by our group with the same toothpaste (unpublished data). Despite the over 4-fold difference in F content between F the 250-TMPnano and 1,100 dentifrices, similar enamel F, Ca and P concentrations were observed in enamel treated with these dentifrices. In this respect, other

studies also showed that TMP increases F and Ca enamel uptake when compared with the counterpart without TMP [Missel *et al.*, Takeshita *et al.*, 2009, 2010]. The increased concentration of those minerals indicate that enamel treated with the TMP-containing dentifrice is significantly more mineralized than its counterpart without TMP, what is important in future demineralization cycles that enamel may undergo.

The biofilm organic and inorganic composition and the ionic activity of some compounds were also influenced by nano-sized TMP. A comparison between 250 and 250-TMPnano showed nano-sized TMP was able to significantly increase the F and Ca concentration in biofilm. A similar ionic activity of charged (CaF^+) and neutral ($CaHPO_4^0$ and HF^0) species observed for 250-TMPnano and 1,100 dentifrices showed that nano-sized TMP had a great impact on the biochemical properties of the biofilm formed as well. It can be hypothesized that such species might have contributed to reduce enamel demineralization in a surface and mainly on the body of lesion. Moreover, the degree of saturation of HA and CaF_2 was similar between the groups 1,100 and 250-TMPnano, variables which have a direct impact on enamel demineralization and remineralization. On the other hand, nano-sized TMP did not increase P levels in the biofilm, confirming that TMP cannot be regarded as a source of free P [Takeshita *et al.*, 2009].

Regarding EPS analysis, the high concentration observed for the placebo is in line with previous reports [Vale *et al.*, 2007; Takeshita *et al.*, 2010; Amaral *et al.*, 2013]. The calcium values in biofilm for 250-TMPnano and 1,100 (similar each other) can also be related to the lowest amount of EPS in biofilm for those groups. Thus, both TMP [Takeshita *et al.*, 2010] and nano-sized TMP have a

direct effect on EPS formation. It has been previously suggested that nano-sized TMP would only led to a bacterial alteration due to the F and Ca ions interference on biofilm [Takeshita *et al.*, 2010]. However, no assessment for acid production as well as colony forming unit counts was done in the present study, so this question remains unanswered.

Nano-sized TMP particles were synthesized by ball milling mechanical process (top-down subtractive processing) from bulk TMP [PAS 71:2005], reducing the particle size. This increased surface area (in proportion to its volume) with higher percentage of atoms on the surface leads to the more reactivity of TMPnano [Danelon *et al.*, 2013b]. Therefore, when adsorbed to enamel in the present study, TMPnano might have altered the selective permeability and ion diffusion into the lesion [van Dijck *et al.*, 1980; Takeshita *et al.*, 2010], which led to a lower mineral loss in enamel (ΔKHN), compared with 1,100 dentifrice. Conventional dentifrices (1,100 ppm F) have been shown a more pronounced action on the surface of the lesion. The resulting hypermineralized surface limits the penetration of ions into the lesion (subsurface), what can be seen clinically as an inactive white spot lesion.

Differently, instead of acting only in the enamel surface, TMP enables remineralization throughout the body of the lesion, mainly in depth [Takeshita *et al.*, 2011; Favretto *et al.*, 2013; Danelon *et al.*, 2013a,b; Manarelli *et al.*, 2014]. The present study demonstrated that nano-sized TMP acts in a similar way as micrometric TMP. Biofilm data indicate that 0.05%nano-sized TMP possibly leads to the retention of charged ions of CaF^{+} and Ca^{2+} by replacing Na^{+} from its cyclic structure [Manarelli *et al.*, 2013]. As the biofilm was under acidic conditions (due to the sucrose challenge), CaF^{+} and Ca^{2+} species might have

reacted with $H_2PO_4^-$, increasing the ionic activity of neutral ions $CaHPO_4^0$ and HF^0. As their diffusion coefficients into enamel lesions is thousand fold higher than their charged counterparts, they would not be impeded by the charged enamel surface [Cochrane *et al.*, 2008]. However, Ca^{2+} has thousand fold lower diffusion coefficient through enamel than $CaHPO_4^0$ [Cochrane *et al.*, 2008]. Analysis from biofilm shows that nano-sized TMP, in fact, increases the concentration of those reactive compounds which might act in the inner part of the subsurface lesion, thus enhancing enamel remineralization [Cochrane *et al.*, 2008], reducing the demineralization [Missel *et al.*, 2010; Takeshita *et al.*, 2009], as well as reducing the acid diffusion [Takeshita *et al.*, 2011; Danelon *et al.*, 2014; Manarelli *et al.*, 2014].

Although 250-TMPnano had increased the amount of most of the ions analyzed in the biofilm, the mechanisms proposed above need to be considered with caution given that data from ionic activity was calculated based on the total ion concentration from the whole biofilm. In this sense, the study of the inorganic composition of saliva and biofilm fluid could greatly contribute to understand the mechanism of nano-sized TMP on enamel demineralization. Besides, due to the limitation of the present *in situ* protocol, the effect of nano-sized TMP plus 250 ppm F dentifrice should be further performed using *in situ* remineralization protocol and clinical studies.

On the basis of the findings of this *in situ* study, the addition of nano-sized TMP to a 250 ppm F dentifrice promoted greater inhibition of enamel demineralization when compared to its counterpart without TMP (250 ppm F) and similar to a conventional dentifrice. The experimental dentifrice could be an alternative to conventional dentifrices for people in general. However,

considering the risk-benefit relationship between fluorosis and dental caries, this formulation would be more attractive for children under 6 years of age, especially for babies.

Acknowledgments

We thank Ms. Maria Fernandes for her technical laboratorial assistance. The authors also thank LIEC-Deparment of Chemistry, UFSCar, São Carlos, Brazil for the production and characterization of sodium trimetaphosphate nanoparticles. Financial support: Coordination of Improvement of Higher Education Personnel/CAPES (grant #23038.033495/2008-97).

5. References

[1] Wong MC, Clarkson J, Glenny AM, Lo EC, Marinho VC, Tsang BW, Walsh T, Worthington HV. Cochrane reviews on the benefits/risks of fluoride toothpastes. J Dent Res 2011; 90(5):573-9. doi: 10.1177/0022034510393346.

[2] Lima TJ, Ribeiro CC, Tenuta LMA, Cury JA. Low-fluoride toothpaste and caries lesion control in children with different caries experience: a randomized clinical trial. Caries Res 2008; 42: 46-50.

[3] Missel EMC, Delbem ACB, Vieira AEM, Sassaki KT, Cruz NVS, Percinoto C. Avaliação de dentifrícios com concentração reduzida de fluoreto associada ao trimetafosfato de sódio na desmineralização do esmalte 2010. Braz Oral Res 2010 ;24(Suppl. 1):247-83.

[4] Takeshita EM, Castro LP, Sassaki KT, Delbem AC. In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate. Caries Res 2009;43(1): 50-56.

[5] Takeshita EM, Castro LP, Danelon M, Sassaki KT, Delbem AC. Evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate on biofilm and enamel demineralization in an in situ study. Caries Res 2010;44:171-247 (abstract 77). (DOI:10.1159/000315447)

[6] Danelon M, Takeshita EM, Peixoto LC, Sassaki KT, Delbem AC. Effect of fluoride gels supplemented with sodium trimetaphosphate in reducing demineralization. Clin Oral Investig 2013a. DOI 10.1007/s00784-013-1102-1104.

[7] Delbem ACB, Bergamaschi M, Rodrigues E, Sassaki KT, Vieira AEM, Missel EMC. Anticaries effect of toothpastes with calcium citrate and sodium trimetaphosphate. J Appl Oral Sci 2012;20:94-98.

[8] Hirata E, Danelon M, Freire IR, Delbem ACB. In vitro enamel remineralization by low-fluoride toothpaste with calcium citrate and sodium trimetaphosphate. Braz Dent J 2013;24(3):253-257. http://dx.doi.org/10.1590/0103-6440201302116.

[9] McGaughey C, Stowell EC. Effects of polyphosphates on the solubility and mineralization of HA: relevance to a rationale for anticaries activity. J Dent Res 1977;56(6):579-587.

[10] Roberts J. Role of models in assessing new agents for caries prevention-non-fluoride systems. Adv Dent Res 1995;9:304-311.

[11] Manarelli MM, Vieira AE, Matheus AA, Sassaki KT, Delbem AC. Effect of mouth rinses with fluoride and trimetaphosphate on enamel erosion: an in vitro study. Caries Res 2011;45(6):506-9. doi: 10.1159/000331929.

[12] Favretto CO, Danelon M, Castilho FCN, Vieira AEM, Delbem ACB. In vitro evaluation of the effect of mouth rinse with trimetaphosphate on enamel demineralization. Caries Res 2013;47(5):532-38.

[13] Danelon M, Castro LP, Souza-neto FN, Camargo ER, Delbem ACB. Avaliação in vitro de dentifrícios suplementados com nanopartículas de trimetafosfato de sódio sobre a desmineralização dentária. Braz Oral Res 2013b;27(Suppl. 1):35-40.

[14] Afonso RL, Pessan JP, Igreja BB, Cantagallo CF, Danelon M, Delbem AC. In situ protocol for the determination of dose-response effect of low-fluoride dentifrices on enamel remineralization. J Appl Oral Sci 2013; 21(6):525-32.

[15] Zero DT. In situ caries models. Adv Dent Res 1995; 9(3): 214-30.

[16] Amaral JG, Martinhon CC, Delbem AC Effect of low-fluoride toothpastes supplemented with calcium glycerophosphate on enamel demineralization in situ.Am J Dent 2013; 26(2):75-80.

[17] Nobre dos Santos M. Relationship among dental plaque composition, daily sugar exposure and caries in the primary dentition. Caries Res 2002; 36:347-352.

[18] Vogel G L, Chow L C, Brown W E A microanalytical procedure for the determination of calcium, phosphate and fluoride in enamel biopsy samples Caries Res 1983; 17 23-31.

[19] Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. J Biol Chem 1925;66:375-400.

[20] Ccahuana-Vásquez RA, Tabchoury CPM, Tenuta LMA, Del Bel Cury AA, Vale GC. Effect of frequency of sucrose exposure on dental biofilm composition

and enamel demineralization in the presence of fluoride. Caries Res 2007;41:9–15. DOI:10.1159/000096100

[21] Dubois M, Grilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350-356.

[22] Alves KM, Pessan JP, Brighenti FL, Franco KS, Oliveira FA, Buzalaf MA, Sassaki KT, Delbem ACB. In vitro evaluation of the effectiveness of acidic fluoride toothpastes Caries Res 2007;41(4) 263-68.

[23] Weatherell JA, Robinson C, Strong M, Nakagaki H. Micro-sampling by abrasion. Caries Res 1985;19:97-102.

[24] Moraes SM, Pessan JP, Ramires I, Buzalaf MA. Fluoride intake from regular and low fluoride toothpastes by 2-3-year-old children: influence of the toothpaste flavor. Braz. Oral Res 2007; 21(3):234-40.

[25] Kobayashi CA, Belini MR, Italiani F de M, Pauleto AR, Araújo JJ, Tessarolli V, Grizzo LT, Pessan JP, Machado MA, Buzalaf MA, Whitford GM, Wasdin JL, Shaffer TE, Adair SM. Plaque fluoride concentrations are dependent on plaque calcium concentrations. Caries Res 2002; 36:256-265.

[26] Pessan JP, Alves KMRP, Ramires I, Taga MFL, Sampaio FC, Whitford GM, Buzalaf MAR. Effects of regular and low-fluoride dentifrices on plaque fluoride. J Dent Res 2010;89:1106-1110.

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

[28] Walsh T, Worthington HV, Glenny AM, Appelbe P, Marinho VC, Shi X. Fluoride toothpaste of different concentrations for preventing dental caries in children and adolescents. Cochrane Database Syst Rev 2011; 20: CD007868.

[29] Vale GC, Tabchoury CP, Arthur RA, Del Bel Cury AA, Paes Leme AF, Cury JA. Temporal relationship between sucrose-associated changes in dental biofilm composition and enamel demineralization. Caries Res 2007; 41(5): 406-12.

[30] PAS 71:2005. “Publicly Available Specification”, PAS, ISBN 0 580 45925 X. UK Department of Trade and Industry (DTI) and British Standard Institution (BSI). Available at ftp://law.resource.org/uk/ibr/bs.pas.71.2005.pdf. 2013

[31] van Dijk JW, Borggreven JM, Driessens FC. The effect of some phosphates and a phosphonate on the electrochemical properties of bovine enamel. Arch Oral Biol 1980; 25:591–595.

[32] Takeshita EM, Exterkate RA, Delbem AC, ten Cate JM. Evaluation of different fluoride concentrations supplemented with trimetaphosphate on enamel de- and remineralization in vitro. Caries Res 2011; 45(5):494-497.

[33] Manarelli MM, Delbem ACB, Lima TMT, Castilho FCN, Pessan JP. In vitro remineralizing effect of fluoride varnishes containing sodium trimetaphosphate. Caries Res 2014; 48:299–305.

[34] Manarelli MM, Delbem ACB, Lima TMT, Castilho FCN, Pessan JP. Effect of fluoride varnish supplemented with sodium trimetaphosphate on enamel erosion and abrasion. Am J Dent 2013; 26(6):307-312.

[35] Cochrane HJ, Saranathan S, Cai F, Cross KJ, Reynolds EC. Enamel subsurface lesion remineralisation with casein phosphopeptide stabilised solutions of calcium, phosphate and fluoride. Caries Res 2008; 42:(2) 88-97.

Figures and Tables

Figure legend

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

Table legend

Table 1 – Mean (SD) of variables analyzed according to the dentifrices used

Table 2 - Ionic activity of ions species and phase saturation from biofilms treated with different dentifrices

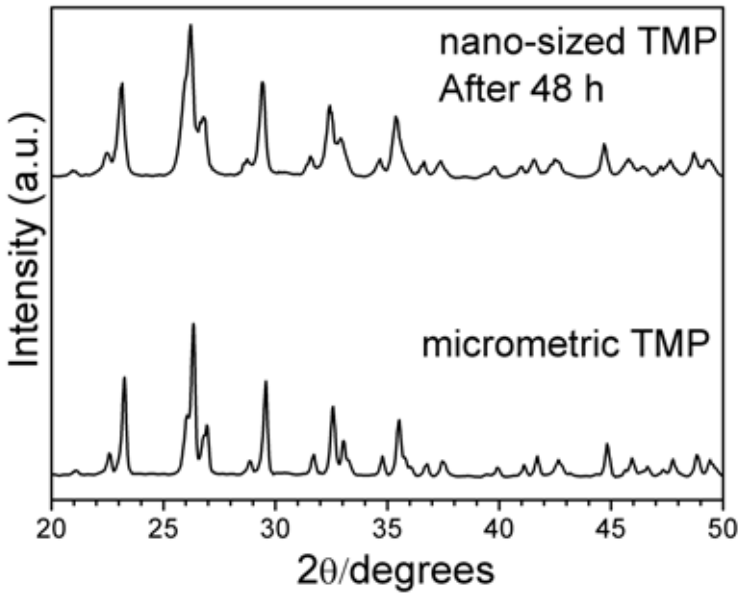


Figure 1. X-ray patterns of the nano-sized TMP after milling for 48 h.

Table 1 – Mean (SD) of variables analyzed according to the dentifrices treatments

Analysis	Dentifrices			
	Placebo	**250**	**1100**	**250-TMPnano**
%SH (kgf/mm^2)	-21.8^{a} (6.4)	-12.3^{b} (1.3)	-3.1^{c} (0.9)	-2.6^{c} (0.8)
ΔKHN (Kgf/mm^2 x µm)	$6,234.4^{a}$ (480.7)	$4,700.2^{b}$ (344.3)	$1,925.1^{c}$ (309.3)	$1,585.0^{d}$ (416.2)
Fluoride - enamel ($µg/mm^3$)	0.33^{a} (0.08)	0.45^{a} (0.14)	0.71^{b} (0.31)	0.70^{b} (0.17)
Calcium - enamel ($µg/mm^3$)	899.3^{a} (155.6)	$1,236.0^{b}$ (410.8)	$1,657.7^{c}$ (587.7)	$1,573.3^{c}$ (435.9)
Phosphorus - enamel ($µg/mm^3$)	532.2^{a} (148.9)	$631.5^{a,b}$ (165.9)	771.8^{b} (323.0)	744.6^{b} (257.4)
Fluoride - biofilm (mol/kg)	$5.10E-04^{a}$ (2.77E-04)	$1.60E-03^{b}$ (1.62E-03)	$9.07E-03^{c}$ (6.60E-03)	$4.52E-03^{d}$ (3.35E-03)
Calcium - biofilm (mol/kg)	$5.22E-02^{a}$ (2.03E-02)	$7.08E-02^{a}$ (3.94E-02)	$1.55E-01^{b}$ (7.71E-02)	$1.55E-01^{b}$ (8.58E-02)
Phosphorus - biofilm (mol/kg)	$1.54E-01^{a}$ (7.62E-02)	$1.15E-01^{a}$ (6.70E-02)	$1.76E-01^{a}$ (1.18E-01)	$1.75E-01^{a}$ (9.57E-02)
EPS - biofilm (µg/g)	10.6^{a} (2.2)	10.7^{a} (1.9)	8.2^{b} (1.5)	8.2^{b} (1.3)

n=19. Distinct superscript letters indicate statistical significance among the dentifrices in each analysis (Student-Newman-Keuls's test; $p < 0.001$). Values between parentheses indicate the standard deviation of the mean.

Table 2 - Ionic activity of ions species and phase saturation from biofilms treated with different dentifrices

Ion activity, mol/Kg	**Dentifrices**			
	Placebo	**250**	**1100**	**250-TMPnano**
Ca^{2+}	4.35E-03[a] (4.40E-03)	6.77E-03[b] (5.92E-03)	1.86E-02[c] (1.65E-02)	1.58E-02[c] (1.30E-02)
$CaPO_4^-$	9.95E-04[a] (3.77E-04)	1.24E-03[a] (6.96E-04)	2.76E-03[b] (1.81E-03)	2.58E-03[b] (1.40E-03)
$CaHPO_4^0$	3.38E-02[a] (1.28E-02)	4.21E-02[a] (2.37E-02)	9.39E-02[b] (6.15E-02)	8.78E-02[b] (4.75E-02)
$CaH_2PO_4^+$	2.40E-03[a] (9.09E-04)	2.98E-03[a] (1.68E-03)	6.66E-03[b] (4.36E-03)	6.23E-03[b] (3.37E-03)
PO_4^{3-}	1.02E-07[a] (5.32E-08)	6.89E-08[a] (3.84E-08)	6.72E-08[a] (4.21E-08)	7.03E-08[a] (5.28E-08)
HPO_4^{2-}	1.80E-02[a] (9.38E-03)	1.21E-02[a] (6.76E-03)	1.18E-02[a] (7.42E-03)	1.24E-02[a] (9.31E-03)
$H_2PO_4^-$	2.72E-02[a] (1.42E-02)	1.83-02[a] (1.02E-02)	1.79E-02[a] (1.12E-02)	1.87E-02[a] (1.41E-02)
CaF^+	1.55E-05[a] (1.45E-05)	5.84E-05[b] (5.45E-05)	8.67E-04[c] (6.13E-04)	5.51E-04[c] (5.82E-04)
HF^0	6.41E-08[a] (3.33E-08)	1.62E-07[b] (1.60E-07)	1.05E-06[c] (8.22E-07)	5.94E-07[c] (4.34E-07)
F^-	3.43E-04[a] (1.78E-04)	8.68E-04[b] (8.57E-04)	5.60E-03[c] (4.40E-03)	3.17E-03[c] (2.32E-03)
Degree of Saturation				
HA	14.43[a] (0.64)	15.13[b] (0.87)	16.82[c] (1.08)	16.80[c] (1.01)
CaF_2	0.99[a] (0.40)	1.76[b] (0.79)	3.37[c] (0.63)	3.73[c] (0.85)

Distinct superscript letters indicate statistical significance among the dentifrices for each ions species or solid phase (Student-Newman-Keuls's test; $p < 0.05$). Values between parentheses indicate the standard deviation of the mean.

ANEXOS

Anexo A: Instruções aos autores do Periódico Nanotechonology

How to prepare and submit an article for publication in an IOP journal using Microsoft Word

Author(s) name(s)

Author Affiliation(s)

E-mail: corresponding author e-mail address

Abstract. The abstract text should be formatted using 10 point Times (or Times Roman, or Times New Roman) and indented by about 25 mm from the left margin. Leave about 10 mm space after the abstract before you begin the main text of your article. The abstract follows the addresses.

1. Introduction: file preparation and submission

These guidelines give some suggestions on how to prepare and format your manuscript using Microsoft Word. It is *not* necessary to try to produce pages that look like published journal pages, the detailed design (typesetting) work will be undertaken by IOP as part of the production process.

1.1. What you will need to supply

Full details on how to submit files to a particular IOP journal are contained in the document *Guidelines for authors* which is discussed in section 1.4. Here we mention the key points you need to consider when preparing your files for submission. You will need to prepare and supply the following:

- **Text.** The text (Word .doc file) of your article with all the figures *embedded*. Refer to appendix A for more information about embedding graphics in a Word document. You can supply a PDF file of your paper but you must also supply the original Word file with embedded graphics *and* separate graphics files where possible.

- **Figures.** In addition to the figures being embedded in the text (Word file) of your article, you are asked, where possible, to supply all figures as separate graphics files in any of the following file formats:

    - EPS, PDF, WMF, TIFF, GIF, JPEG and BMP.

  Please name all figure files using the guidelines in section 1.2.1. For more details on preparing figures please refer to IOP's graphics guidelines, *Preparing graphics for IOP journals*, which can be downloaded from authors.iop.org.

- **Details of any copyright permissions.** If you wish to illustrate your article using material for which you do not own the copyright then you **must** seek permission from the copyright holder, usually both the author and the publisher. It is the author's responsibility to obtain copyright permissions and this should be done prior to submitting your article. If you have obtained permission, please provide full details of the permission granted—for example, copies of the text of any e-mails or a copy of any letters you may have received. Figure captions must include an acknowledgment of the original source of the material even when permission to use has been obtained. Full details on copyright can be found in IOP's *Guidelines for authors*.

1.2. Naming your files

Please name all your files, both figures and text as follows.

- use only characters from the set a to z, A to Z, 0 to 9 and underscore (_);
- do not use spaces in file names;
- include an extension to indicate the file type (e.g., .doc, .txt, .eps, etc);
- do not use any accented characters; for example, à, ê, ñ, ö, ý, etc because these can cause difficulties when processing your files.

1.2.1. Naming your figure files. In addition to the above points, please give each figure file a name which is the same as the figure it contains; for example, figure1.eps, figure2.tif, figure2a.gif etc. If the figure file contains a figure with multiple parts, for example figure 2(a) to 2(e), give it a name such as figure2a_2e.jpg, and so forth.

1.3. Submitting your article files to an IOP journal

For most IOP journals you send your files directly to us by web, e-mail or FTP; however, there are a number of exceptions to this. For certain IOP journals you need to send your files to a different institution or to an external editor. How to find the submission address for a particular journal is discussed in section 1.4, below.

1.4. Where to send your files

Full details of how to submit files to a particular IOP journal are contained in the document *Guidelines for authors* which can be accessed online by going to authors.iop.org. From this document's table of contents, select the 'How to submit' link to access full details of the submission address for a particular journal.

2. The title, author list and abstract

2.1. The title

The first letter of the title should be capitalized with the rest in lower case. The title should be formatted using 17 point Times Bold, flush left and unjustified, and you should leave 28 mm of space above, and 10 mm below, the title. Many database systems used in literature searches rely heavily on the content of titles and abstracts to identify relevant articles so great care should be taken in constructing both.

2.2. Running heads

The title of your article should be used as the running head of your article. If the title is too long, please use a short version of the title that can also be used in the final print version of your article.

2.3. Author list

Include all authors in a single list. The style for the names is: initials (without full stops) or forenames and family name, each author's name separated with a comma, precede the final name with 'and' (see the example in section 2.4). Chinese-style names should be typed as the author wishes his/her name to appear in print.

2.4. Addresses and footnotes

The addresses of the authors' affiliations follow the list of authors. If the authors are at different addresses, numbered superscripts should be used after each family name to indicate his/her address. The numbered superscripts should *not* be inserted using Word's footnote command because this will place the reference in the wrong place—at the bottom of the page (or end of the document) rather than next to the address. A footnote, linked to the author, should be used to indicate an alternate address or the author to whom correspondence should be addressed. Ensure that numbered superscripts used to link author names and addresses start at 1 and continue on to the number of affiliations. Do not add any footnotes until all the author names are linked to the addresses. For example, to format

G M Douglas[1,3], J E Thomas[1,4] and A J Cox[2,5]

where there are three addresses, you should insert superscripts 1, 2 and 3 to link family names to addresses and then insert *footnotes* 4 and 5. Note that the first footnote in the main text will now be number 6.

2.5. E-mail addresses

These may be added after the authors' addresses.

2.6. The abstract

The abstract follows the addresses and should give readers concise information about the content of the article and indicate the main results obtained and conclusions drawn. It should be self-contained with no reference to figures, tables, equations or bibliographic references and should not normally exceed 200 words. The abstract should normally be restricted to a single paragraph.

2.7. Keywords

Keywords should be provided for submissions to *Measurement Science and Technology*, *Physical Biology*, *Physiological Measurement* and both parts of *Journal of Optics*. Add these as a new paragraph after the abstract.

2.8. Subject classification numbers

Physics and Astronomy Classification System (PACS) codes or Mathematics Subject Classification (MSC) scheme numbers should come immediately after the abstract. Classification codes can greatly help in the choice of suitable referees and allocation of articles to subject areas. For *Inverse Problems* and *Nonlinearit*y you may use *either* PACS *or* MSC codes.

2.8.1. Information on PACS and MSC. For more information on PACS and MSC see

- **MSC:** *http://www.ams.org/msc*
- **PACS:** *http://www.aip.org/pacs*

2.8.2. Submitted to. The full title of the journal submitted to can be inserted on a new line after any classification numbers; although this information is helpful, it is not essential.

3. Formatting the text

The text may be divided into sections, subsections and, where necessary, subsubsections.

3.1. Fonts

Please format the text of your article using a 'Times' font at a size of 11 points. Note that you may need to use 'Times Roman' or 'Times New Roman' depending on the fonts installed on your computer.

3.2. Formatting sections, subsections and subsubsections

The first section is normally an introduction, which should state clearly the object of the work, its scope and the main advances reported, with brief references to relevant results by other workers.

3.2.1. Style, spacing and numbering. Table 1 shows our preferred format for section headings.

Table 1. Formatting sections, subsections and subsubsections.

	Numbering	Font	Spacing
Section	1., 2., 3., etc	11 point **Times bold**	1 line space before a section No additional space after a section heading
Subsection	2.1., 2.2., 2.3., etc	11 point *Times Italic*	1 line space before a subsection No space after a subsubsection heading
Subsubsection	2.3.1., 2.3.2., etc	11 point *Times Italic*	Subsubsections should end with a full stop (period) and run into the text of the paragraph

3.3. Acknowledgments

If you wish to acknowledge assistance or encouragement from colleagues, special work by technical staff or financial support from organizations you should do so in an unnumbered 'Acknowledgments' section immediately following the last numbered section of the article.

3.4. Appendices

Technical detail that it is necessary to include, but that interrupts the flow of the article, may be included as an appendix. Appendices should be included at the end of the main text of the article, after the acknowledgments but before the reference list. If there are two or more appendices they should be called Appendix A, Appendix B, etc. Numbered equations will be in the form (A.1), (A.2), etc, figures will appear as figure A1, figure B1, etc and tables as table A1, table B1, etc.

3.5. Some style points

It will help readers if your article is written in a clear, consistent and concise manner. The Production Department at IOP will try to make sure that your work is presented to readers in the best possible way without sacrificing the individuality of your writing. Some recommended points to note, however, are the following.

- Please use 'ize' endings rather than 'ise' (diagonalize, renormalization, minimization, etc), however, there are some common exceptions to this, for example: devise, promise and advise.
- English spellings are mandatory (colour, flavour, behaviour, tunnelling, artefact, focused, focusing, fibre, etc) *except* in the following journals where *either* American *or* English spellings are acceptable: *Physical Biology*, *Smart Materials and Structures* and *Journal of Micromechanics and Microengineering*.
- The words table and figure should be written in full and **not** abbreviated.

Please check your article carefully for accuracy, consistency and clarity before submission. Remember that your article will be read by many people whose native language is not English and who may not therefore be aware of many of the subtle meanings of words or idiomatic phases present in the English language. It therefore helps if you try to keep sentences as short and simple as possible.

3.6. Footnotes

Footnotes should be only be used when essential, and if required should be used only for brief notes that do not fit conveniently into the text.

4. Figures

Each figure should have a brief caption describing it and, if necessary, a key to interpret the various lines and symbols on the figure. Aim to keep the lettering on figures to a minimum and include as much detail as necessary in the captions.

4.1. IOP graphics guidelines

Separate guidelines on preparing figure files, *Preparing graphics for IOP journals*, can be downloaded from authors.iop.org.

4.2. Figure captions/numbering

Captions should be placed below (or next to) the figure and should finish with a full stop (period). Figures should be numbered sequentially—'Figure 1', 'Figure 2', and should be cited in the text as 'figure 1', 'figure 2'.

4.3. Supplying figure files

Please note that all figures must be embedded within the text (Word document) of your article *and* supplied as separate figure files in any one of the acceptable file formats listed in section 1.1 (you can, of course, use any combination of the supported formats). See appendix A and IOP's graphics guidelines, *Preparing graphics for IOP journals*, for detailed instructions.

4.4. Text in figures

Do not put a title or caption detail in the figure file; any description should be placed in the figure caption. Please refer to IOP's graphics guidelines, *Preparing graphics for IOP journals*, for more specific information about fonts/text in EPS figure files.

4.5. Scaling of line widths and text

You should note that as part of the production and typesetting processes, figures may be resized to fit the design of the journal. Scaling of graphics will, of course, affect line thickness and text size in the figures. To achieve the best results you are advised to prepare your figures at approximately the size they will be reproduced in the journal. Refer to recent printed or electronic copies of the appropriate journal to determine the size at which figures are typically reproduced.

4.6. Naming your graphics files

Please follow the file naming guidelines in section 1.2.1 and give each graphics file a name which *easily identifies the content*. For example: Figure1.eps, Figure2a.tif, Figure2b.tif rather than long descriptive names such as deltacurvevariation.eps.

4.7. Colour illustrations

Use of colour in the *online* version of your article is free and you are strongly encouraged to make good use of colour where it will help readers of your article. The use of colour in figure files is discussed in IOP's graphics guidelines, *Preparing graphics for IOP journals*, but please remember that *colour in the print version usually has to be paid for*.

4.8. Positioning figures

Individual figures should normally be centred. It is also more convenient for referees of your article if figures are placed as close as possible, and ideally after, the point where they are first mentioned in the text. If necessary, figures and their captions can be grouped together at the end of the article.

4.9. Figures in parts

If a figure has parts these should be clearly labelled as (a), (b), (c) etc on the figure. Parts should not have separate captions, but the caption should describe the different parts.

5. Supplementary data

All of our journals encourage authors to submit supplementary data attachments to enhance the online versions of published articles. Supplementary data enhancements typically consist of video clips, animations or data files, tables of extra information or extra figures. They can add to the reader's understanding and present results in attractive ways that go beyond what can be presented in the print version of the journal. The printed journal remains the archival version, and supplementary data items are supplements which enhance a reader's understanding of the article but are not essential to that understanding. For electronic-only journals, supplementary data attachments may be used to convey essential information. Further guidelines on supplementary data are contained in IOP's *Guidelines for authors* which can be accessed online by going to authors.iop.org and selecting the 'Supplementary data' link in the table of contents.

6. Tables

6.1. Positioning tables

Tables should be centred unless they occupy the full width of the page.

6.2. Table captions/numbering

Captions should be placed at the top of the table and should finish with a full stop (period). Narrow captions should be centred, longer captions simply typed as a paragraph. Tables should be numbered sequentially—'Table 1', 'Table 2', and should be cited in the text as 'table 1', 'table 2'.

6.3. Rules in tables

Tables should have only horizontal rules and no vertical ones. Generally, only three rules should be used: one at the top of the table, one at the bottom, and one to separate the entries from the column headings.

6.4. Example

Because tables can take so many forms, it is difficult to provide detailed guidelines; however, the following example (and other tables in these guidelines) demonstrates our preferred style.

Table 2. A simple table.

Distance (m)	Velocity (ms^{-1})
100	23.56
150	34.64
200	23.76

6.5. Notes to tables

If you wish to format a table so that it contains notes (table footnotes) to the entries within the body of the table these notes should be formatted using alphabetic superscripts i.e. [a], [b], [c]. Notes should be placed at the bottom of the table; one convenient method is to create an empty row at the bottom of the table to contain them. Merge the cells to give you a single cell the width of the table. Table notes should be 10 point Times Roman. Each note should be on a separate line.

Table 3. A table with headings spanning two columns and containing notes[a].

	Thickness		Separation energies	
Nucleus	(mg cm^{-2})	Composition	γ, n (MeV)	γ, 2n (MeV)
^{181}Ta	19.3±0.1[b]	Natural	7.6	14.2
^{208}Pb	3.8±0.8[c]	99% enriched	7.4	14.1
^{209}Bi	2.6±0.01[c]	Natural	7.5	14.4

[a]Notes are referenced using alphabetic superscripts.
[b]Self-supporting.
[c]Deposited over Al backing.

7. Equations and mathematics

Mathematics should be prepared using Word's built-in 'Equation Editor' or the full MathType product.

Avoid 'Insert Symbol'

Please *avoid* using Word's 'Insert Symbol' command. Symbols inserted in this way may not appear in the PostScript and PDF file of your article—they often 'drop out'. Instead, for example, to type Greek characters, type the corresponding Latin character (e.g., 'a' for α) and manually change the font to Symbol using *Format* ⇨ *Font* and then select 'Symbol' font.

7.1. Fonts in Equation Editor (or MathType)

Make sure that your Equation Editor or MathType fonts, including sizes, are set up to match the text of your document. Please refer to appendix B for more information.

7.2. Points of style

7.2.1. Vectors. Bold italic characters is our preferred style but you may use any standard notation; for example, any of these styles for vectors is acceptable:

'the vector cross product of ***a*** and ***b*** is given by ***a b***…', or 'the vector cross product of **a** and **b** is given by **a b**…', or 'the vector cross product of *a* and *b* is given by *a b* …'.

7.2.2. Roman and italic in mathematics.

- Use a Roman d for a differential d, for example, $\tan\theta = \mathrm{d}y/\mathrm{d}x$.
- Use a Roman e for an exponential e; for example, $y = \mathrm{e}^{x}$.
- Use a Roman i for the square root of –1; e.g., $\mathrm{i} = \sqrt{-1}$.
- Certain other common mathematical functions, such as cos, sin, det and ker, should appear in Roman type.
- Subscripts and superscripts should be in Roman type if they are labels and italic if they are variables or characters that take values. For example in the equation $\varepsilon_m = -g\mu_n Bm$, *m*, the *z* component of the nuclear spin, is italic because it can have different values whereas n is Roman because it is a label meaning nuclear.

7.3. Alignment of mathematics

If your article is accepted for publication the mathematical content will be carefully typeset during the production process to the standard and style of IOP journals. Therefore, for the initial submission, you may simply centre all equations. Long equations that need to be continued on subsequent lines,

should start flush left, continuation lines should be indented by about 25 mm. Equations should be split at mathematically sound points, immediately before =, + or – signs or between terms multiplied together. The connecting signs are not repeated and appear only at the beginning of the turned-over line. A multiplication sign should be added to the start of turned-over lines where the break is between two multiplied terms.

7.3.1. Displayed equations: examples.

$$_{k}(\ )r \quad 2^{2\ 2/3}\exp ik\ r \tag{1}$$

$$C\ 12 \quad x \quad x\ r$$

$$r^{2\ L}x\ x\mathrm{d}$$

$$1\ \mathrm{const}\ \_\_\ \_\_\_{}^{2\ 2} \tag{2}$$

$$L\ _{r}x$$

$$1\ \mathrm{const}\ r_{\overline{2}\ 2}\ln\ \frac{L}{r} \quad .$$

$$L$$

However, if equations will fit on one line, do so; for example, (2) may also be formatted as:

$$C\ 12 \quad x \quad x\ r \quad 1\ \mathrm{const}\ \_\_\ \_\_\_{}^{r}{}_{L^{2}{}_{2}}{}^{L}{}_{r}{}^{x\ x\ \mathrm{d}}{}_{x^{\ 2}}\ 1\ \mathrm{const}\ r_{\overline{L^{2}{}_{2}}}\ln\ \frac{L}{r} \tag{3}$$

7.4. Miscellaneous points

- Except for simple examples, exponential expressions, especially those containing subscripts or superscripts, are clearer if the notation exp is used. For instance, exp i $kx\ t$ and exp z^2 are preferred to $e^{i\ kx\ t}$ and $e^{,z_2}$ but e is acceptable. Similarly, the square root sign $\sqrt{}^{2}$ should only be used with relatively simple expressions, e.g. $\sqrt{\quad}$ 2 and $a^2 + b^2$, cases the power 1 2should be used.
- A two-line / solidus should be avoided where possible; for example, use

  $$1 \quad \_\_\_{}^{\left|S_{0}\right|^{2\ 1}}{}_{0}\mathrm{d} \quad \text{instead} \quad \frac{1}{M_a}\Big/{}_{0}\mathrm{d}\ \frac{\left|S_0\right|^2}{N} \quad \text{of}$$

  $$M_a \qquad N$$

- It is important to distinguish between ln log and $_{e}$ lg log . $_{10}$
- Braces, brackets and parentheses should be used in the following order: {[()]}. The same ordering of brackets should be used within each size.

However, this ordering can be ignored if the brackets have a special meaning (e.g. if they denote an average or a function).

- Decimal fractions should always be preceded by a zero: for example 0.123 *not* .123 (note, do not use commas, use decimal points).
- Equations that are referred to in the text should be numbered with the number on the right-hand side.

7.5. Equation numbering

Equations should be numbered sequentially throughout the text (i.e., (1), (2), (3),...) or numbered by section (i.e., (1.1), (1.2), (2.1), ...) depending on your preference. In articles with several appendices equation numbering by section is useful in the appendices even when sequential numbering has been used throughout the main body of the text: for example, A.1, A.2,…,B.1, B.2,…. When referring to an equation in the text:

• always put the equation number in brackets—e.g. ‘as in (2)’ or ‘as in (2.1)’; • it is not normally necessary to include the word ‘equation’ before the number.

8. References

8.1. General

As part of the production system for IOP journals, online versions of references will, wherever possible, be linked electronically using IOP’s HyperCite™ technology. What this means is that readers of your article will be able to link from the references to the abstract or full text of the cited articles. Whether a particular reference can be linked will depend on what type of publication it is (journal articles are more likely to be linked than books and reports) and when it was published. In order to link as many of your references as possible, it is important that your references are accurate, complete and formatted using the guidelines below. The time and effort spent in preparing your reference list is extremely worthwhile and is one way to enhance your article.

8.2. What should a reference contain?

A complete reference should provide the reader with enough information to locate the article concerned, whether published in print or electronic form, and should, depending on the type of reference, consist of:

- family name(s) and initials of all authors;
- titles of journal articles (optional);
- year of publication;
- title of journal, book or other publication;
- volume number;
- editor(s), if any;
- town of publication and publisher for *books*;
- page numbers.

8.3. Types of reference

The structure of a particular reference depends on whether it is:

- to a printed journal article;
- to an electronic journal article;
- to a preprint (e.g., ArXiv);
- to a book, conference proceeding or report.

8.4. Reference lists

The reference list comes at the end of an article and consists of an *unnumbered* 'References' section containing references sorted according to the Harvard (alphabetic) or Vancouver (numeric) referencing style.

- All author's names should be given except where there are more than ten when only the first should be given followed by *et al*.
- Names of collaborations should be included, e.g. Thomas J E *et al* (for the FFAST Collaboration).
- If you are unsure of the correct abbreviation for a journal title it is best to leave the title in full.

The terms *loc. cit.* and *ibid* should not be used.

- Unpublished conferences and reports should generally not be included in the reference list if they are in a published form elsewhere.
- Articles in the course of publication should have an article title, the journal submitted to or a preprint number.
- A thesis submitted for a higher degree (giving the name of the institution where the work was done) may be included in the reference list if it has not been superseded by a published article and is available through a library.

8.5. Harvard and Vancouver reference styles

Two different styles of referencing are in common use: the Harvard (alphabetic) system and the Vancouver (numeric) system. All IOP journals allow the use of the Harvard *or* Vancouver system (you can use the style you prefer), **apart from** the following journals:

• Physics in Medicine and Biology and • Physiological Measurement

for which the Harvard system must be used.

8.5.1. Harvard system. Using the Harvard system, the name of the author appears in the text together with the year of publication. Either the year or the name and year are in parentheses, depending on the context. Some points of style are

- Where there are only two authors both names should be given in the text; if there are more than two authors only the first name should appear followed by *et al*.
- When two or more references to work by one author or the same group of authors occur for the same year they should be identified by including a, b, etc after the year (e.g. 1986a).
- If several references to different pages of the same article occur the appropriate page number may be given in the text, for example Kitchen (1982, p 39).

Examples:

Binzoni T, Leung T S, Boggett D and Delpy D T 2002 A new near infrared laser-Doppler flowmeter for deep tissue perfusion monitoring *MAGMA* **14** 74–5

Binzoni T, Leung T S, Boggett D and Delpy D T 2003 Non-invasive laser Doppler perfusion measurements of large tissue volumes and human skeletal muscle blood RMS velocity *Phys. Med. Biol.* **48** 2527–49

Kendall M A F and Quinlan N J 2004 Intradermal ballistic delivery of micro-particles into excised human skin for drug and vaccine applications *J. Biomech.* **37** 1733–41

Kendall M A F, Quinlan N J, Thorpe S J, Ainsworth R W and Bellhouse B J 2004a Measurements of the gas and particle flow within a converging-diverging nozzle for high speed powdered vaccine and drug delivery *Exp.Fluids* **37** 128–36

Kendall M A F, Rishworth S, Carter F and Mitchell T 2004b Effects of relative humidity and temperature on the ballistic delivery of micro-particles to excised porcine skin *J. Invest. Dermatol.* **122** 739–46

The above references might be cited in the text as

- Binzoni *et al (*2002) showed that ...
- … may depend on the initial conditions (Binzoni *et al* 2003, Kendall and Quinlan 2004)
- postulated by Kendall *et al (*2004a, b)

8.5.2. Vancouver system. In this system the reference list gives the references in the numerical order in which they are cited in the text. To cite a reference using this system the reference list number is given within square brackets, like this [2], or for two or more entries, [2,5,7–9].

Examples:

[1] Strite S and Morkoc H 1992 *J. Vac. Sci. Technol.* B **10** 1237

[2] Jain S C, Willander M, Narayan J and van Overstraeten R 2000 *J. Appl. Phys.* **87** 965
Kendall M A F and Quinlan N J 2004 Intradermal ballistic delivery of micro-particles into excised human skin for drug and vaccine applications *J. Biomech.* **37** 1733-41

[3] Nakamura S, Senoh M, Nagahama S, Iwase N, Yamada T, Matsushita T, Kiyoku H and Sugimoto Y 1996 *Japan. J. Appl. Phys.* **35** L74

POINTS TO NOTE

- Apart from the numbering of references, the structure of individual entries in a Vancouver list is identical to that in a Harvard list.

- Reference [2], above, contains two discrete references. This is permissible, **but** each discrete reference should start on a new line (never just separate references by a period or semicolon) and should contain full article information (author(s), year, title of journal, volume, pages).

8.6. Formatting references

The detailed structure of references, i.e., the formatting rules discussed below, *are the same for both the Vancouver and Harvard systems.*

8.6.1. References to printed journal articles. A normal reference to a journal article contains three different fonts as listed in table 4.

Table 4. Font styles for a reference to a journal article.

Element	Style
Authors	Roman type
Year	Roman type
Article title (optional)	Roman type
Journal title	Italic type
Volume number	Bold type
Page numbers	Roman type

POINTS TO NOTE

- The authors should be in the form: family name (with only the first letter capitalized) followed by the initials with no periods after the initials. Authors should be separated by a comma except for the last two which should be separated by ‘and’ with no comma preceding it.
- The article title (if given) should be in lower case letters, except for an initial capital, and should follow the year.
- The journal title should be abbreviated and in italic. If a journal has several parts denoted by different letters the part letter should be inserted after the journal in Roman type, e.g. *Phys*. *Rev*. A.
- Both the initial and final page numbers should be given where possible. The final page number should be in the shortest possible form and separated from the initial page number by dash, e.g. 1203–14, i.e. the numbers ‘12’ are not repeated.

8.6.2. References to preprints. For preprints there are two distinct cases. Where the article has been published in a journal and the preprint is supplementary information it should be given as in [1], below, when the only reference available is the preprint it should be given as in [2]:

[1] Kunze K 2003 T-duality and Penrose limits of spatially homogeneous and inhomogeneous cosmologies *Phys. Rev.* D **68** 063517 (*Preprint* gr-qc/0303038)

[2] Milson R, Coley A, Pravda V and Pravdova A 2004 Alignment and algebraically special tensors *Preprint* gr-qc/0401010

8.6.3. References to electronic-only journals. Article numbers are usually given with no page ranges as most electronic-only journals start each article on page 1. For New Journal of Physics (article number may have from one to three digits) see [1], for SISSA journals the volume is divided into monthly issues and these form part of the article number, as in [2]and [3]

[1] Fischer R 2004 Bayesian group analysis of plasma-enhanced chemical vapour deposition data *New. J. Phys.* **6** 25
[2] Horowitz G T and Maldacena J 2004 The black hole final state *J. High Energy Phys.* JHEP02(2004)008
[3] Bentivegna E, Bonanno A and Reuter M 2004 Confronting the IR fixed point cosmology with high-redshift observations *J. Cosmol. Astropart. Phys.* JCAP01(2004)001.

8.6.4. References to books, conference proceedings and reports. These are similar to journal references, but have only two different fonts (see table 5).

Table 5. Font styles for references to books, conference proceedings and reports.

Style	Element
Authors	Roman type
Year	Roman type
Book title	Italic type
Editors	Roman type
Place (city, town etc) of publication	Roman type
Publisher	Roman type
Volume	Roman type
Page number(s)	Roman type

Points to note

- Book titles should have initial capital letters for all except minor words. Words such as Proceedings, Symposium, International, Conference, Second, etc should be abbreviated to *Proc*., *Symp*., *Int*., *Conf*., *2nd*, respectively, but the rest of the title should be given in full, followed by the date (normally the year is sufficient) of the conference and the town or city where the conference was held. For Laboratory Reports the Laboratory should be given wherever possible, e.g. *Argonne National Laboratory Report*.
- Some books are volumes within series (see examples [4] below). In these cases the series information should come immediately after the title, in parentheses, with the series title in italic, but the volume in Roman.
- The volume number, for example vol 2, should be followed by the editors, if any, in a form such as ‘ed A J Smith and P R Jones’. Use *et al* if there are more than two editors. Next comes the town of publication and publisher, within brackets and separated by a colon, and finally the page numbers preceded by p if only one number is given or pp if several numbers are given.

Examples:

[1] Kurata M 1982 *Numerical Analysis for Semiconductor Devices* (Lexington, MA: Heath)
[2] Caplar R and Kulisic P 1973 *Proc. Int. Conf. on Nuclear Physics (Munich)* vol 1 (Amsterdam: NorthHolland/American Elsevier) p 517
[3] Szytula A and Leciejewicz J 1989 *Handbook on the Physics and Chemistry of Rare Earths* vol 12, ed K A Gschneidner Jr and L Erwin (Amsterdam: Elsevier) p 133
[4] Kuhn T, Binder E, Rossi F, Lohner A, Rick K, Leisching P, Leitenstorfer A, Elsaesser T and Stolz W 1994 Coherent excitonic and free-carrier dynamics in bulk GaAs and heterostructures *Coherent Optical Interactions in Semiconductors: Proc. NATO Advanced Research Workgroup (Cambridge, UK, 11–14 August 1993) (NATO Advanced Study Institute, Series B: Physics* vol 330*)* ed R T Phillips (New York: Plenum) pp 33–62

9. Cross referencing

You should not use Word's built-in cross-referencing facilities. Instead, you should type the number of the figure, table, reference, section etc that you wish to cross-reference.

Appendix A. Embedding graphics in a Microsoft Word document

A.1. How to embed graphics into a Word document

- Create a graphic in one of the formats Word is able to import (for example, TIFF, JPG, EPS etc).
- From the *Insert* menu, select *Picture* ⇗ *From File…* (see figure A1).
- When the 'Insert Picture' dialog box is displayed, click on the *Insert* button on the bottom right corner of the dialog box (see figure A2).
- Select the *Insert* option (see figure A2). This will make sure that the graphic is saved with (embedded into) the Word document.

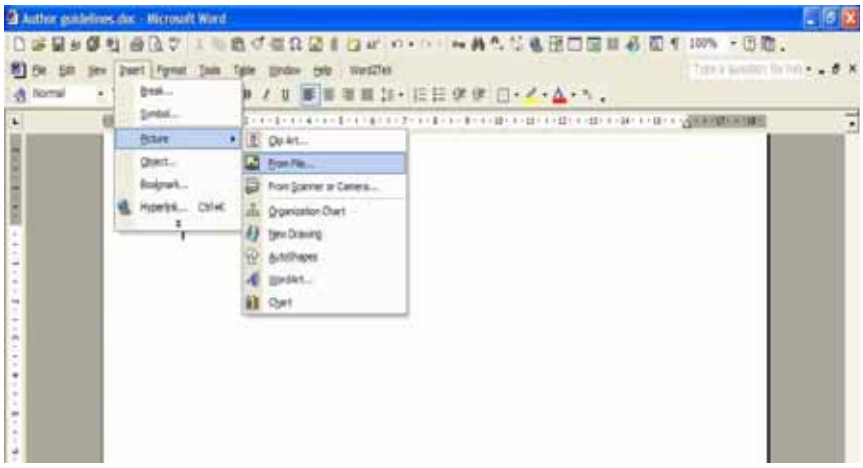


Figure A1. Inserting a graphic into a Word document.

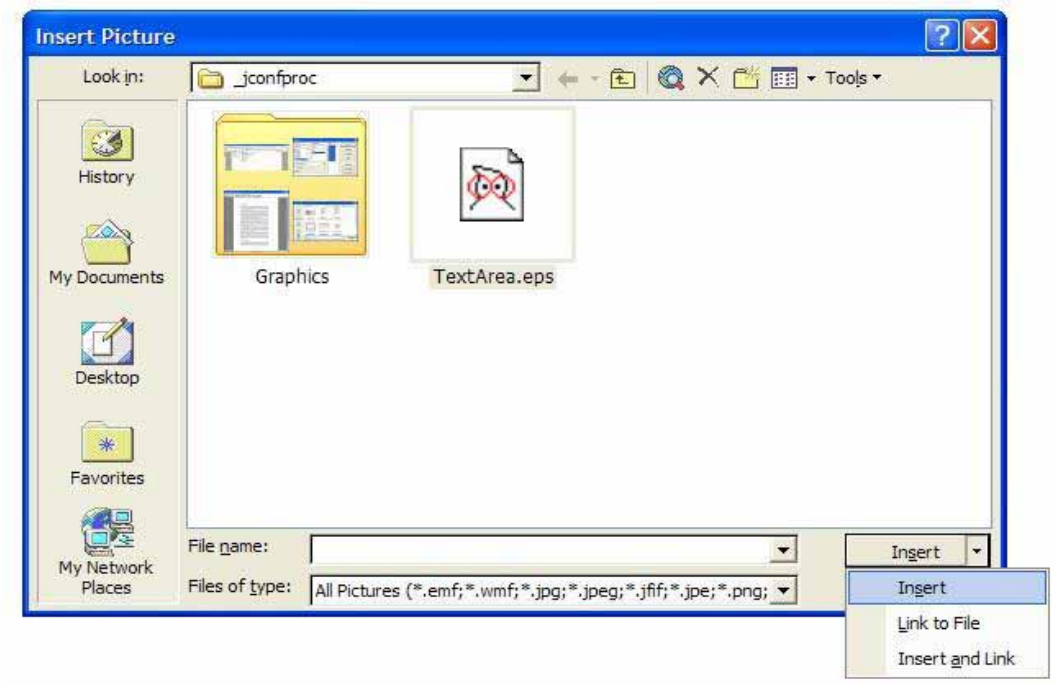


Figure A2. Embedding a graphic into a Word document.

A.2. Copy and pasting graphics into a Word document

- If the application you are using to prepare your illustrations does not export or save the graphics in a format that Word is able to import, or you have difficulty exporting the graphic, you should copy and paste the graphic into the Word document. **Note** that you may need to use the *Edit ⇨ Paste Special…* option to correctly paste the graphic into your Word document.

Appendix B. Math fonts

B.1. Math fonts and Microsoft Word

Greek mathematical symbols should be formatted using the 'Symbol' font. Text and non-Greek characters/variables should be formatted using 'Times' (sometimes called 'Times Roman' or 'Times New Roman' depending your computer setup). Sections B.1.1. and B.1.2. show how to set up the font sizes and styles in Word's 'Equation Editor'.

Avoid 'Insert Symbol'

Please *avoid* using Word's 'Insert Symbol' command. Symbols inserted in this way may not appear in the PostScript and PDF file of your article—they often 'drop out'. Instead, for example, to type Greek characters, type the corresponding Latin character (e.g., 'a' for α) and manually change the font to Symbol using *Format ⇨ Font* and then select 'Symbol' font.

B.1.1 To set font sizes in Equation Editor

- Create a new document and insert an equation (*Insert* ⇗ *Object*).
- Double-click the equation to bring up the equation toolbar (see figure B1).

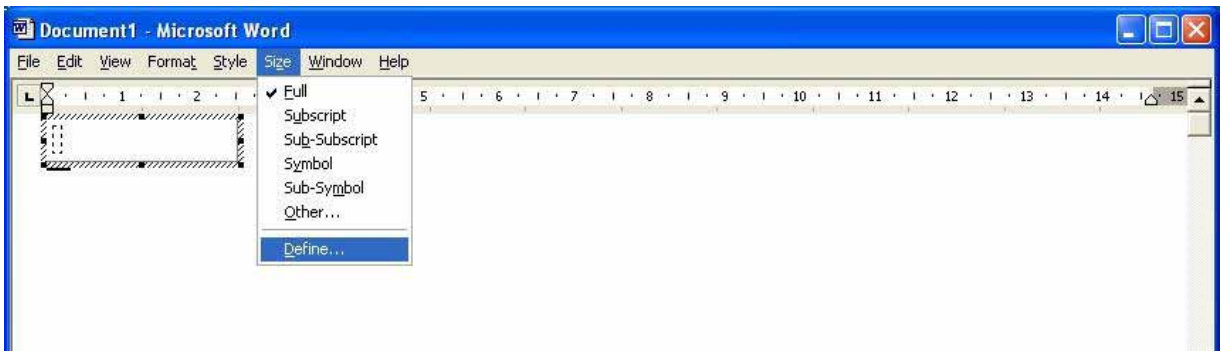


Figure B1. The equation toolbar.

- Select *Size* ⇗ *Define* to display the 'Sizes' dialog box (see figure B2). Type in the following values:

Full:	11pt
Subscript/Superscript:	8pt
Sub-Subscript/Superscript:	6pt

The remaining options can be set as follows:

Symbol:	15pt (or other value if you prefer)
Sub-symbol:	10pt (or other value if you prefer)

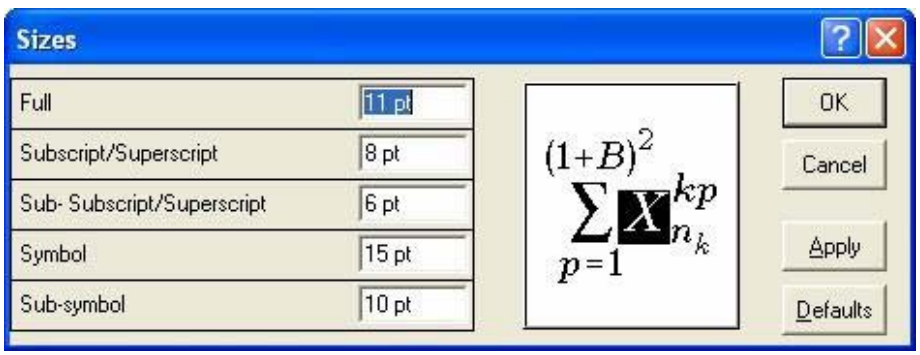


Figure B2. Setting font sizes in Equation Editor.

B.1.2. To set font styles in Equation Editor

- Create a new document and insert an equation.
- Double-click the equation to bring up the equation toolbar (see figure B1).
- Select *Style* ⇨ *Define* to display the 'Styles' dialog box (see figure B3).
- Set the equation styles as shown in figure B3. Note that you may see 'Times' or 'Times Roman' on your computer rather than the 'Times New Roman' shown in figure B3. This will depend on your computer's configuration. **Make sure you use the same 'version' of 'Times' that you used for the text of your article**.

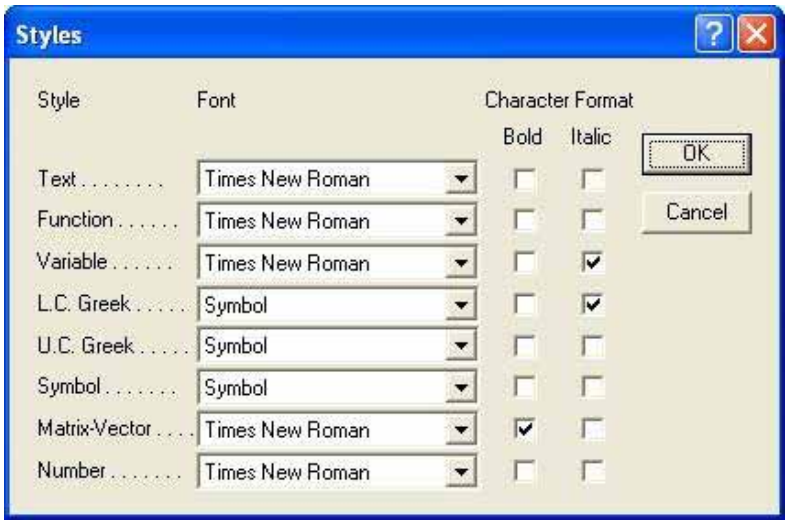
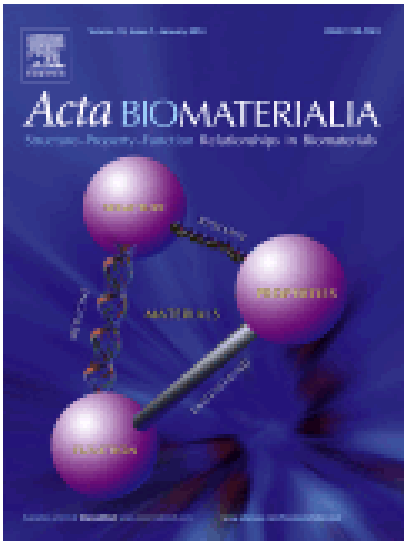


Figure B3. C

Anexo B: Instruções aos autores do Periódico Acta Biomaterialia



Guide for Authors

Article structure

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

Page numbering

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

Subdivision - numbered sections

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

Introduction

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

Materials and methods

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

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

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

Theory/calculation

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

Results

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

Statistics

Careful statistical analysis must be performed and reported to support any statements regarding the existence of differences in study groups. Statistical support should underlie hypothesis testing. Error bars are required on all experimental and calculated data points with an explanation in the text as to how the errors were determined.

Discussion

This should explore the significance of the results of the work, not repeat them. Discussion should be reported independently from Results. Avoid extensive citations and discussion of published literature.

Conclusions

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

Appendices

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

Essential title page information

Title Page

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

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

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

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

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

Abstract

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

Graphical abstract

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

Keywords

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

Abbreviations

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

Acknowledgements

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

Math formulae

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

Footnotes

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

Table footnotes

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

Artwork

Electronic artwork

General points

• Make sure you use uniform lettering and sizing of your original artwork.

• Embed the used fonts if the application provides that option.

• Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.

• Number the illustrations according to their sequence in the text.

• Use a logical naming convention for your artwork files.

• Provide captions to illustrations separately.

• Please assure scale bars are present and legible. In SEM images, please remove small automated scale bars and replace with new clear bars.

• Size the illustrations close to the desired dimensions of the printed version.

• Submit each illustration as a separate file.

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

A detailed guide on electronic artwork is available on our website:

http://www.elsevier.com/artworkinstructions

You are urged to visit this site; some excerpts from the detailed information are given here.

Formats

If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format.

Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

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

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;

• Supply files that are too low in resolution;

• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color on the Web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article.** Please indicate your preference for color: in print or on the Web only. For further information on the preparation of electronic artwork, please see http://www.elsevier.com/artworkinstructions.
Please note: Because of technical complications which can arise by converting color figures to 'gray scale' (for the printed version should you not opt for color in print) please submit in addition usable black and white versions of all the color illustrations.

Figure captions

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

Tables

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

References

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

Citation in text

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

Web references

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

References in a special issue

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

Reference management software

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

Reference style

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

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

List

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

Examples:

Reference to a journal publication:

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

Reference to a book:

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

Journal abbreviations source

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

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

Video data

Elsevier accepts video material and animation sequences to support and enhance your scientific research. Authors who have video or animation files that they wish to submit with their article are strongly encouraged to include links to these within the body of the article. This can be done in the same way as a figure or table by referring to the video or animation content and noting in the body text where it should be placed. All submitted files should be properly labeled so that they directly relate to the video file's content. In order to ensure that your video or animation material is directly usable, please provide the files in one of our recommended file formats with a preferred maximum size of 50 MB. Video and animation files supplied will be published online in the electronic version of your article in Elsevier Web products, including ScienceDirect: http://www.sciencedirect.com. Please supply 'stills' with your files: you can choose any frame from the video or animation or make a separate image. These will be used instead of standard icons and will personalize the link to your video data. For more detailed instructions please visit our video instruction pages at http://www.elsevier.com/artworkinstructions. Note: since video and animation cannot be embedded in the print version of the journal, please provide text for both the electronic and the print version for the portions of the article that refer to this content.

AudioSlides

The journal encourages authors to create an AudioSlides presentation with their published article. AudioSlides are brief, webinar-style presentations that are shown next to the online article on ScienceDirect. This gives authors the opportunity to summarize their research in their own words and to help readers understand what the paper is about. More information and examples are available at http://www.elsevier.com/audioslides. Authors of this journal will automatically receive an invitation e-mail to create an AudioSlides presentation after acceptance of their paper.

Supplementary data

Elsevier accepts electronic supplementary material to support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Supplementary files supplied will be published online alongside the electronic version of your article in Elsevier Web products, including ScienceDirect:http://www.sciencedirect.com. In order to ensure that your submitted material is directly usable, please provide the data in one of our recommended file formats. Authors should submit the material in electronic format together with the article and supply a concise and descriptive caption for each file. For more detailed instructions please visit our artwork instruction pages at http://www.elsevier.com/artworkinstructions.

Submission checklist

The following list will be useful during the final checking of an article prior to sending it to the journal for review. Please consult this Guide for Authors for further details of any item.

Ensure that the following items are present:

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

• E-mail address

• Full postal address

• Phone numbers

All necessary files have been uploaded, and contain:

• Keywords

• All figure captions

• All tables (including title, description, footnotes)

Further considerations

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

• References are in the correct format for this journal

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

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

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

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

For any further information please visit our customer support site at http://support.elsevier.com.

Anexo C: Parecer do Comitê de Ética – Capítulo 2

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: AÇÃO DO TMP NANOPARTICULADO ASSOCIADO A DENTIFRÍCIO DE BAIXA CONCENTRAÇÃO DE FLUORETO SOBRE A DESMINERALIZAÇÃO DO ESMALTE BOVINO. ESTUDO IN SITU.

Pesquisador: Alberto Carlos Botazzo Delbem

Área Temática:

Versão: 1

CAAE: 16348414.4.0000.5420

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

Patrocinador Principal: FUND COORD DE APERFEICOAMENTO DE PESSOAL DE NIVEL SUP

DADOS DO PARECER

Número do Parecer: 612.685

Data da Relatoria: 28/03/2014

Apresentação do Projeto:

O projeto apresenta as características da população a estudar, incluindo o tamanho da amostra e metodologia a ser desenvolvida. A pesquisa tem como finalidade avaliar in situ a ação do TMP nanoparticulado ao F (250 ¿g/g) em dentifrícios sobre a inibição da desmineralização do esmalte bovino e no biofilme dentário.

Objetivo da Pesquisa:

O projeto apresenta objetivos definidos e está bem estruturado. Tem como objetivo analisar a ação do TMP nanoparticulado (0,05% - 48 horas de moagem) associado ao F (250 ¿g F/g na forma do NaF) em formulações dentifrícias sobre a desmineralização do esmalte de dente bovino e no biofilme dentário. Para tanto, serão determinadas após cada fase experimental: a)dureza de superfície do esmalte antes e pós-desafio cariogênico; b)dureza do esmalte em secção longitudinal e a perda integrada de dureza subsuperficial; c)as concentrações de F, Ca e P presentes no esmalte e biofilme; d)o conteúdo de polissacarídeos extracelulares insolúveis no biofilme.

Avaliação dos Riscos e Benefícios:

Esta pesquisa apresenta risco mínimo aos voluntários, uma vez que os mesmos utilizarão

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONCA **CEP:** 16.015-050
UF: SP **Município:** ARACATUBA
Telefone: (18)3636-3200 **Fax:** (18)3636-3232 **E-mail:** [illegible]

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

dispositivos palatinos e escovação com dentifrícios experimentais e placebo. O placebo, embora não tenha fluor em sua formulação, bem como o dentifrício de 250 ¿g F/g (baixa concentração de flúor), associado ou não ao TMP nanoparticulado a 0,05%, sendo usados por curtos períodos e o voluntário mantendo a boa higiene bucal não trará risco à cárie. A utilização do TMP a 0,05% na formulação de um dos dentifrícios experimentais é seguro, pois somente usado em concentrações acima de 10% causam efeitos locais. Além disso, todos os voluntários são alunos, funcionários do curso de Odontologia e possuem instruções adequadas de higiene bucal. O gotejamento da solução de sacarose sobre os blocos será feita com o dispositivo palatino fora da cavidade bucal, sem risco de potencial cariogênico para os outros dentes. O dispositivo palatino será ajustado à cavidade bucal, mas caso gere algum desconforto, será realizado o reajuste. Como benefício, os
participantes serão beneficiados com o exame clínico e atendimento preventivo (limpeza profissional).

Comentários e Considerações sobre a Pesquisa:

A metodologia proposta é abrangente procurando responder o objetivo da pesquisa. Devemos apenas ressaltar que no item que cita as intervenções a serem realizadas faltou incluir o grupo do dentifrício com 250 ¿g F/g.

Considerações sobre os Termos de apresentação obrigatória:

Foram apresentados, de modo adequado, os termos necessários para a apreciação do projeto.

Recomendações:

Nada a acrescentar.

Conclusões ou Pendências e Lista de Inadequações:

O projeto de pesquisa apresenta objetivos definidos, antecedentes científicos e bibliografia que justifiquem a pesquisa, com uma amostragem de 20 indivíduos. O risco é considerado mínimo, uma vez uma vez que os mesmos utilizarão dispositivos palatinos e escovação com dentifrícios experimentais e placebo. Os documentos apresentados estão devidamente preenchidos. Por não haver pendências propõe-se a aprovação do referido projeto.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O projeto de pesquisa apresenta objetivo definido em avaliar in situ a ação do TMP

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONCA CEP: 16.015-050
UF: SP Município: ARACATUBA
Telefone: (18)3636-3200 Fax: (18)3636-3332 E-mail: anacmen@foa.unesp.br

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

Plataforma Brasil

Confirmação do Parecer: 512.685

nanoparticulado ao F (250 ¿g/g) em dentifrícios sobre a inibição da desmineralização do esmalte bovino e no biofilme dentário. Os antecedentes científicos e a bibliografia justificam a pesquisa e há um delineamento metodológico, com uma amostragem de 20 indivíduos. O risco é considerado mínimo, uma vez que os voluntários utilizarão dispositivos palatinos e escovação com dentifrícios experimentais e placebo. Os documentos apresentados estão devidamente preenchidos. Não havendo pendências, o CEP propõe a aprovação do projeto de pesquisa.

O CEP informa que deverão ser apresentados relatórios semestrais, a partir da data da aprovação.

ARAÇATUBA, 11 de Abril de 2014

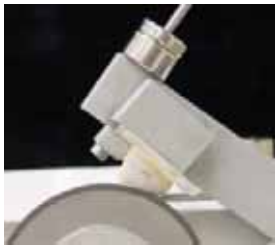
Assinador por:
Ana Claudia de Melo Stevanato Nakamune
(Coordenador)

Endereço: JOSE BONIFACIO 1193
Bairro: VILA MENDONCA CEP: 16.015-050
UF: SP Município: ARACATUBA
Telefone: (18)3636-3300 Fax: (18)3636-3332 E-mail: anacmen@foa.unesp.br

Página 03 de 03

Anexo D: Confecção dos blocos de esmalte bovino (capítulos 1 e 2).

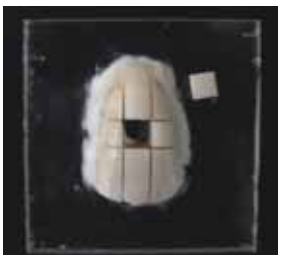
Coroa do dente bovino incisivo central inferior, separada da raiz por meio de disco diamantado de duas faces (KG Sorensen D 91), montado em motor de bancada (Nevoni), mantido sob refrigeração (água destilada/deionizada).



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

Face vestibular fixada na placa de acrílico.

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



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

Anexo E: Planificação da dentina e polimento do esmalte (capítulos 1 e 2).

Bloco de esmalte fixado em disco de resina acrílica pré-fabricada (± 3 cm de diâmetro por ± 11 mm de espessura), com auxílio de cera pegajosa (Kota Ind. e Com. LTDA), com a superfície dentinária voltada para cima.

Ajuste da dentina para obtenção de superfícies paralelas entre esmalte e dentina, utilizando Politriz Beta – Grinder – Polisher e Vector Power Head (Buehler, Lake Bluff, IL, USA) e lixas de granulação 320 (Carbimet Paper Discs, 30-5108-320, Buehler),por 30 segundos sob baixa rotação e refrigeração. Blocos para estudo *in situ:* espessura 2 mm.

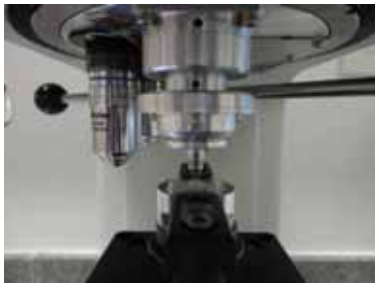
Blocos fixados com a superfície do esmalte voltada para cima para serem polidos.

Sequência do polimento de esmalte:

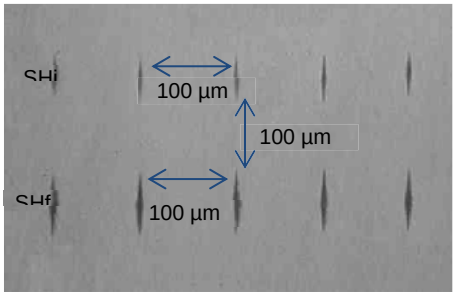
- ✓ Profilaxia com pedra-pomes, água deionizada e taça de borracha montada em contra-ângulo em baixa-rotação.
- ✓ Polimento empregando lixas de granulação 600 (20 segundos), 800 (30 segundos) e 1200 (30 segundos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/ deionizada por 2 minutos, entre cada lixa;
- ✓ Acabamento final com disco de papel feltro TEXMET 1000 (Buehler Polishing Cloth) durante 1 minuto com suspensão de diamante 1 micron base-água (Buehler);
- ✓ Lavagem durante 30 segundos com jato de água deionizada;
- ✓ Limpeza em lavadora ultrassônica Modelo 2110 (Branson, Danbury CT,USA) com água destilada/ deionizada (2minutos).

Anexo F: Análise da dureza de superfície do esmalte (capítulos 1 e 2).

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



Bloco de esmalte sendo submetido à leitura no microdurômetro, penetrador Knoop, carga estática de 25 gramas e tempo de 10 segundos.



Fotomicrografia das impressões para análise de dureza inicial (SHi) e final (SHf) do esmalte (Aumento: 100x).

A dureza de superfície foi determinada segundo Takeshita et al. (2009)[a]. Utilizou-se o microdurômetro Micromet 5114 Hardness Tester (Buehler, Lake Bluff, USA e Mitutoyo Corporation, Kanagawa, Japan), com penetrador (Knoop) (carga estática de 25 gramas e tempo de 10 segundos), acoplado ao Software para análise de imagem Buehler OminMet (Buehler, Lake Bluff, USA). Cinco impressões, separadas entre si por uma distância de 100 µm, foram realizadas na região central de cada bloco (SHi). Após a ciclagem de pH (Capítulo 1) ou após desafio cariogênico (Capítulo 2), realizaram-se outras cinco indentações (SHf) distantes a 100 µm das impressões de SHi.

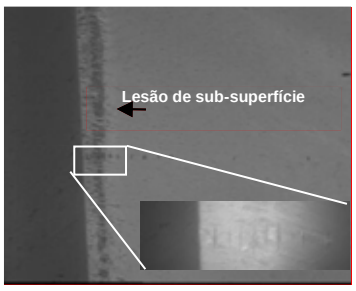
[a] Takeshita *et al.* In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate. Caries Res 2009;43(1): 50-56.

Anexo G: Análise da dureza em secção longitudinal (capítulo 2).

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

Corpo de prova – plano longitudinal voltado para a superfície da resina acrílica.

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



Fotomicrografia das impressões. (Aumento: 1000x)

Sequência do polimento de esmalte:

- ✓ Lixas de granulação 320 (1 minuto), 600, 800 e 1200 (2minutos) e refrigeração a água. Limpeza em lavadora ultrassônica e água destilada/ deionizada por 2 minutos, entre cada lixa;
- ✓ Acabamento final com disco de papel feltro Microcloth Supreme PSA (Buehler) durante 2 minutos com suspensão de diamante 1/4 micron base-água (Buehler);

- ✓ Lavagem durante 30 segundos com jato de água deionizada;
- ✓ Limpeza em lavadora ultrassônica utilizando água destilada/deionizada (2 minutos).

A análise da dureza em secção longitudinal foi determinada segundo Takeshita et al. (2009)[a]. Uma secção foi feita no centro de cada bloco e uma das metades incluída em resina acrílica, posteriormente polida. Utilizou-se o mesmo microdurômetro utilizado para a análise de dureza de superfície com penetrador Knoop operando com carga de 5 gramas por 10 segundos, em aumento de 1000 vezes. Uma seqüência de 14 impressões nas distâncias de 5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 e 330 µm da superfície externa do esmalte foi realizada na área central dos blocos, e outras duas a 100 µm acima e abaixo. Os valores médios dos três pontos medidos foram calculados em cada distância. A área integrada da dureza (KHN x µm) da lesão até o esmalte hígido foi calculada utilizando a regra trapezoidal (GraphPad Prism, versão 3.02) e subtraída da área integrada da dureza do esmalte hígido obtendo a perda integrada da dureza (ΔKHN) (Spiguel et al., 2009)[b].

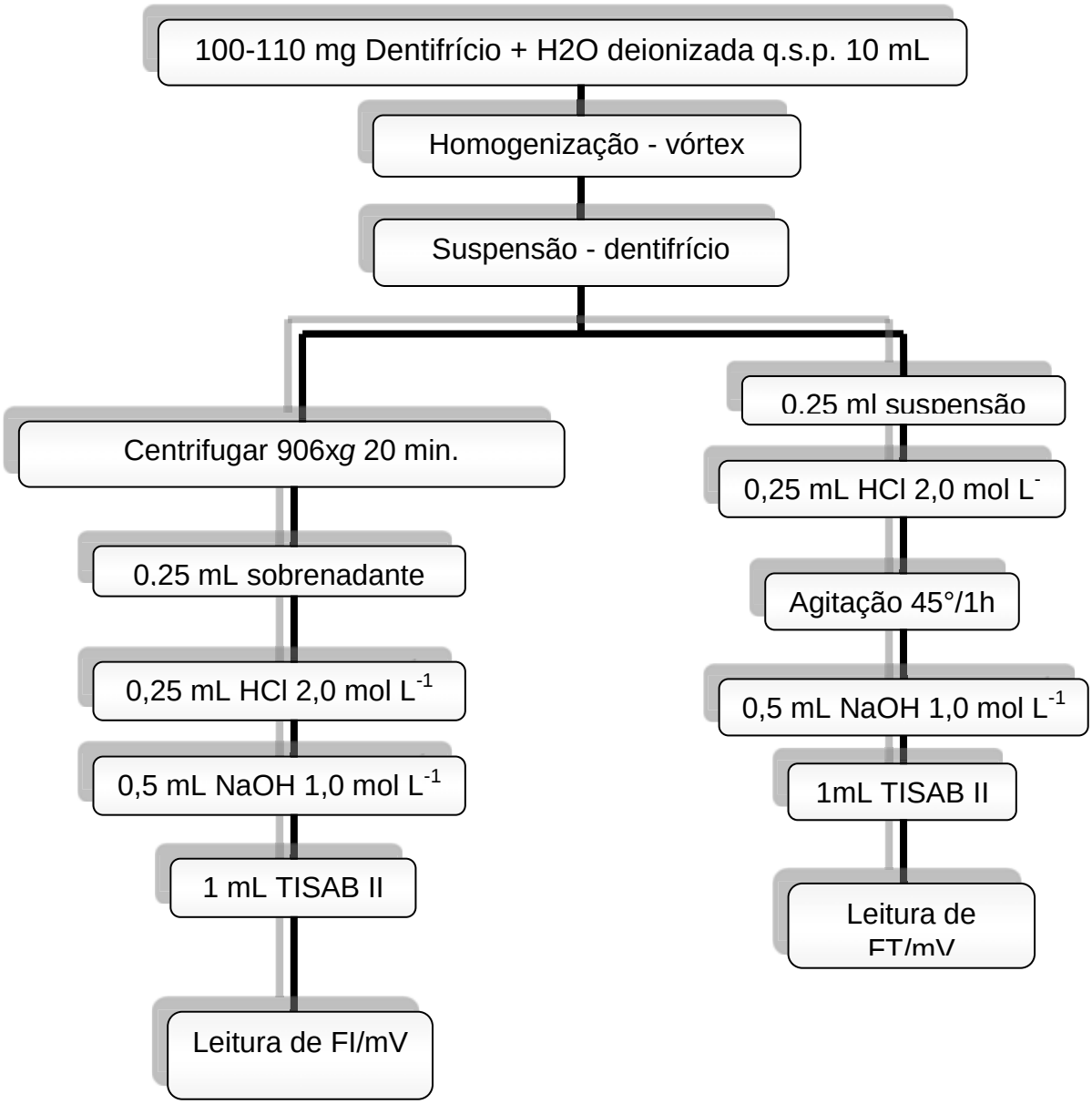
[a] Takeshita *et al.* In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate. Caries Res 2009;43(1): 50-56.

[b] Spiguel *et al.* Evaluation of laser fluorescence in the monitoring of the initial stage of the de-/remineralization process: an in vitro and in situ study. Caries Res 2009;43:302-307.

Anexo H: Esquema representativo da dosagem de fluoreto dos dentifrícios (capítulos 1 e 2).

A dosagem de fluoreto dos dentifrcícios foi realizada segundo método descrito em:

Delbem *et al.* Anticaries effect of toothpastes with calcium citrate and sodium trimetaphosphate. J Appl Oral Sci 2012;20:94-98.



Anexo I: Dosagem de fluoreto nos dentifrícios experimentais (capítulos 1 e 2).

Capítulo 1:

Tabela 1. Valores de fluoreto iônico (FI) e fluoreto total (FT) (média ± dp) nos dentifrícios experimentais

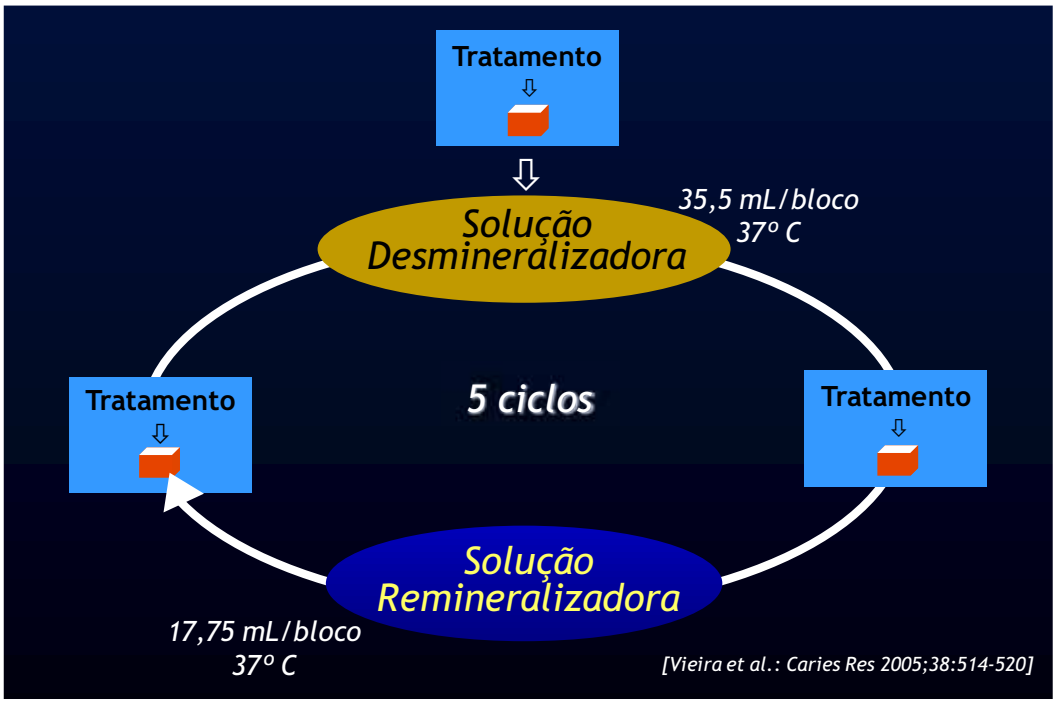
Dentifrício	Análise	
	FT (ppm F)	FI (ppm F)
Placebo	8,8 ± 0,5 (n=2)	10,7 ± 2,1 (n=2)
250	267,1	269,5
250 + 0,05% TMP	260,2	268,9
250 + 0,05% TMPnano24	274,1	270,8
250 + 0,05% TMPn 48h	261,7	263,9
250 + 0,1% TMP	275,35	273,45
250 + 0,1% TMPnano24	283,1	271,4
250 + 0,1% TMPnano48	275,7	276,7
250 + 0,25% TMP	268,7	269,4
250 + 0,25% TMPnano24	269,3	275,1
250 + 0,25% TMPnano48	262,5	268,2
1100	1184,2	1178,6

Capítulo 2:

Tabela 1. Valores de fluoreto iônico (FI) e fluoreto total (FT) (média ± dp) nos dentifrícios experimentais

Dentifrício	Análise	
	FT (ppm F)	FI (ppm F)
Placebo	8,4 ± 0,6	9,3 ± 0,3
250	274,7 ± 5,2	274,5 ± 5,5
250 TMPnano	262,1 ± 7,0	261,0 ± 5,9
1100	1184,2 ± 41,4	1137,3 ± 10,3

Anexo J: Esquema representativo da ciclagem de pH (capítulo 1).



Anexo K: Instruções aos voluntários (capítulo 2).

Projeto: Ação do TMP nanoparticulado associado a dentifrício de baixa concentração de fluoreto sobre a desmineralização do esmalte bovino. Estudo in situ.

Prezado voluntário,

Muito obrigado por participar do nosso estudo. Para que tudo ocorra da melhor forma possível, leia com atenção as instruções que se seguem, qualquer dúvida entrar em contato nos telefones indicados.

Para cada fase você receberá 1 maleta com o Kit contendo:

- ✓ 1 dispositivo palatino com 4 blocos de esmalte bovino cobertos por tela plástica
- ✓ 1 estojo porta-aparelho
- ✓ 1 creme dental para a higiene bucal
- ✓ frasco conta-gotas contendo o *tratamento* (creme dental misturado a água que será gotejado sobre os blocos)
- ✓ frasco conta-gotas contendo solução de sacarose 30%
- ✓ 1 escova dental
- ✓ 1 rolo de fio dental sem flúor
- ✓ 1 pacote de gaze
- ✓ 1 frasco com água deionizada para enxaguar o aparelho

Serão 4 fases experimentais, com duração de sete (7) dias cada, com 7 dias de descanso entre elas.

NSTRUÇÕES GERAIS:

1. O voluntário não deverá utilizar qualquer tipo de produto fluoretado (exceto beber água de abastecimento) ou anti-séptico bucal uma semana antes e durante todo o experimento. Não utilize produtos para bochecho ou outros agentes tópicos de qualquer natureza, nem vitaminas ou suplementos sistêmicos que contenham flúor durante a fase experimental.
2. Não será feita restrição alimentar, apenas pede-se não consumir chá, principalmente o mate, pois contém grande quantidade de flúor.
3. Não beber e nem comer nada usando o aparelho.
4. Uma semana antes do experimento e durante todo o período de descanso o voluntário deverá fazer a higiene bucal usando somente o creme dental não fluoretado, escova e o fio dental, fornecidos pela pesquisadora.

PROCEDIMENTOS

Na noite que antecede cada fase, após a última escovação, o voluntário deverá colocar o dispositivo palatino na boca e dormir com ele. A partir do dia seguinte (1º dia da fase experimental) o voluntário deverá iniciar o tratamento dos blocos de esmalte e continuar durante mais 6 dias, como descrito a seguir:

a) Com o aparelho fora da boca, colocado sobre o porta-aparelho, gotejar solução de sacarose 30%, sendo 2 gotas em cada bloco, 6 x ao dia nos horários pré-determinados

(8:00, 11:00, 14:00, 17:00, 19:00 e 21:00h). Tenha o cuidado de não tocar a ponta do conta-gotas no dispositivo, evitando assim a contaminação da solução. Após o gotejamento o dispositivo palatino será deixado em descanso por 5 minutos, e, após esse tempo deverá ser reinstalado na cavidade bucal. A cada 2 dias o voluntário receberá uma solução de sacarose 30% recém-preparada;

b) Duas vezes ao dia nos horários pré-determinados (7:30 e 21:30h) gotejar o *tratamento* (solução de creme dental) sobre todos os blocos, sendo 2 gotas por bloco, e, após, o dispositivo deverá ser reinstalado na cavidade bucal. Homogeneizar (agitando o frasco fechado) a solução por 2 minutos antes da aplicar.
No 8º dia o voluntário deverá remover o aparelho pela manhã para tomar o café e após a higiene bucal reposicioná-lo na boca, no entanto não deverá mais realizar o tratamento dos blocos. O dispositivo deverá ser mantido na boca e apenas será removido no momento em que será entregue à pesquisadora a qual fará a coleta do biofilme e dos blocos de esmalte, que serão submetidos à avaliação.

Em seguida, por 7 dias o voluntário não usará dispositivo palatino – período de descanso – utilizando somente o creme dental, a escova e o fio dental fornecidos pela pesquisadora. Dar-se-á, então, o início da 2ª fase e as outras fases na sequência seguirão os mesmos procedimentos. Ao início da cada nova fase o voluntário receberá um novo kit de materiais e um novo dispositivo palatino.

ATENÇÃO:
O dispositivo deverá ser utilizado durante todo o dia e durante a noite, sendo removido da boca **apenas** durante as refeições ou quando o voluntário for ingerir algum alimento ou água. Quando estiver fora da boca, em nenhum momento o dispositivo deve ser deixado à seco. Guarde-o no porta-aparelho, com gaze umedecida em água deionizada sobre a área dos blocos de esmalte. Troque a gaze diariamente ou sempre que julgar necessário. Se necessário, peça mais gaze ao pesquisador.

Os blocos NÃO deverão ser escovados. Os voluntários poderão realizar a escovação do dispositivo, 1 VEZ POR DIA, somente NA FACE VOLTADA PARA O PALATO e com o creme dental fornecido pela pesquisadora e depois enxágue com a água deionizada fornecida.

O acúmulo de biofilme (placa bacteriana) sob a tela plástica nesta fase é desejável; não tente removê-la. A placa bacteriana será objeto de análise.

Utilize 1 escova dental para cada fase experimental e uma outra nova escova para o período de descanso. Ao concluir cada fase experimental, devolva a escova que foi utilizada para que seja descartada.

Pede-se atenção aos voluntários quanto aos procedimentos a serem realizados, seguindo as instruções fornecidas. Os mesmos devem estar cientes de que o não cumprimento das instruções poderá prejudicar os resultados da pesquisa.

Qualquer dúvida entre em contato. Obrigada!

Contato: Maria Daniela Basso de Souza
Telefones: (45) 91417129 (VIVO) / (45) 99563406 (TIM)
e-mail mdanibs@gmail.com

Anexo L: Preparo e montagem dos blocos para a microabrasão do esmalte. Análise de F, Ca e P no esmalte (capítulos 1 e 2).

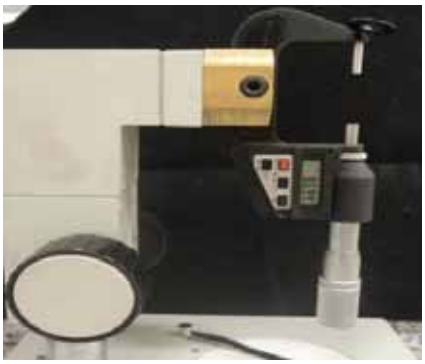
O preparo e montagem dos blocos para a microabrasão do esmalte seguiu método descrito por Alves et al. (2007)[a] e Takeshita et al. [2009][b], o qual teve por base o estudo de Weatherell et al. (1985) [c.]

Após a análise da SH*f* os blocos foram seccinados longitudinalmente. Uma das metades foi seccionada em 2 blocos (2x2 mm); um deles foi fixado com cianocrilato (Super Bonder, Loctite, Brazil) em mandril para peça reta, e o conjunto bloco/mandril conectado a um micrômetro (Micrometer 733 MEXFLZ-50, Starret Company, São Paulo) acoplado a uma base de microscópio. Após, a superfície de esmalte foi gentilmente posta em contato com a base, e uma camada de esmalte (~50 µm) foi removida com o auxílio de disco de lixa auto-adesiva (13 mm de diâmetro) de carbureto de sílica, granulação 400 (Buelher) fixado em frasco de poliestireno cristal (J-10, Injeplast, Brasil).

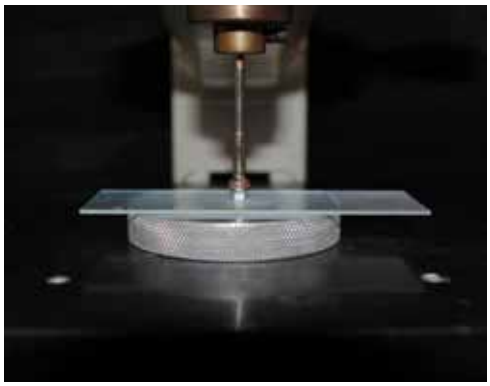
[a]Alves *et al.* In vitro evaluation of the effectiveness of acidic fluoride dentifrices. Caries Res 2007;41:263-267.

[b]Takeshita *et al.* In vitro evaluation of toothpaste with low fluoride content supplemented with trimetaphosphate. Caries Res 2009;43(1): 50-56.

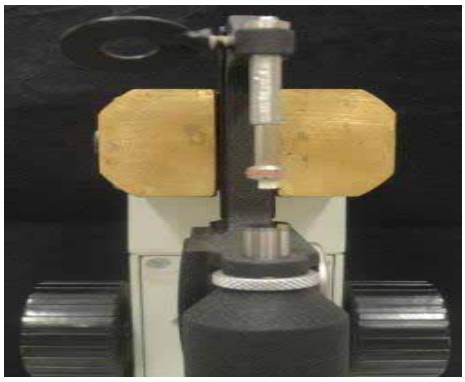
[c]Weatherell *et al.* Micro-sampling by abrasion. Caries Res 1985;19:97-102.



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

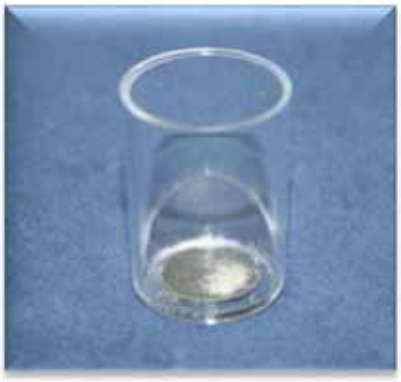


Fixação do bloco (superfície de esmalte voltada para baixo) em mandril para peça reta estabelecendo paralelismo com superfície plana.



Conjunto bloco/mandril fixado ao micrômetro.

Bloco de esmalte sendo submetido à microabrasão, com desgaste de 50 µm, para posterior análise do conteúdo de F, Ca e P no esmalte.



Pó de esmalte presente na lixa adaptada em frascos de poliestireno cristal (J - 10, Injeplast, Brasil), após o desgaste.

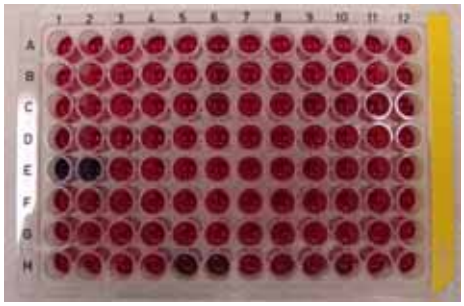
Para análise do conteúdo de F no esmalte utilizou-se:

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

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

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

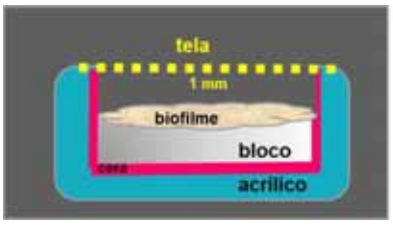
Espectrofotômetro (Microplate Spectrophotometer EONC Biotek, Winooski, USA) utilizado para as dosagens de cálcio e fósforo.



Dosagem de cálcio. Método colorimétrico Arsenazzo III. Placa de 96 poços com água deionizada + amostra + Arsenazo III.

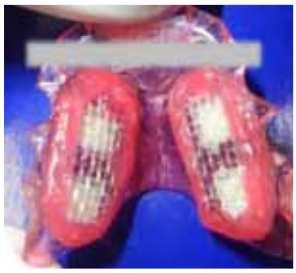
Dosagem de fósforo. Método colorimétrico. Placa de 96 poços com água deionizada + amostra + molibdato + reativo redutor.

Anexo M: Confecção, instalação do dispositivo palatino e aspecto do biofilme (capítulo 2).

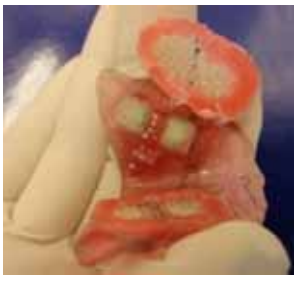


Esquema representativo da confecção do dispositivo palatino.

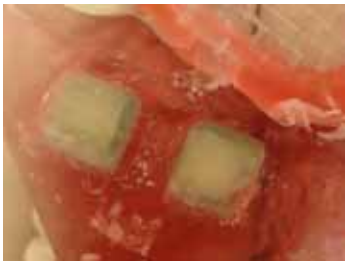
Dispositivo palatino instalado no palato do voluntário.



Dispositivo palatino no dia da coleta de biofilme.

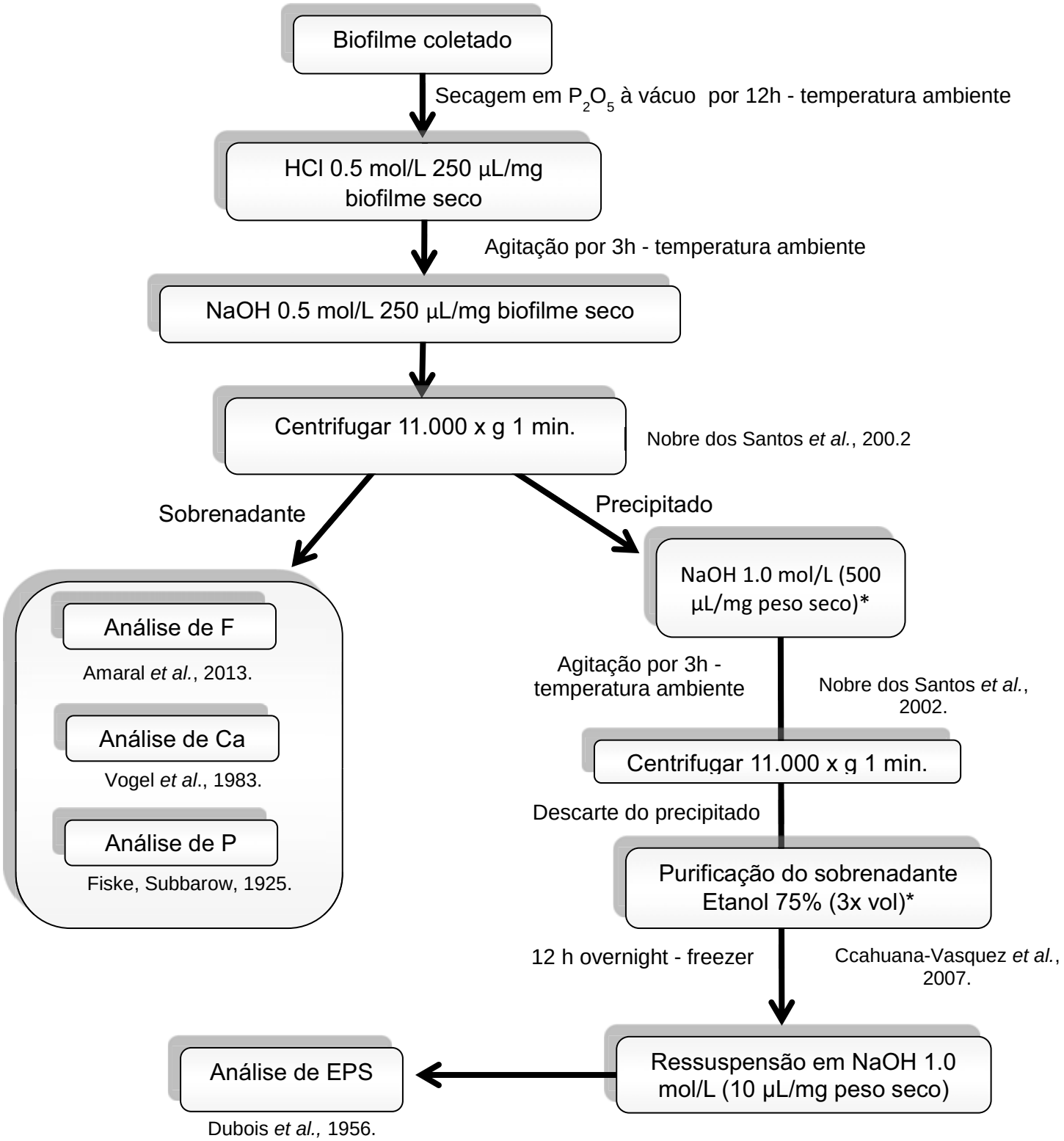


Remoção da tela plástica.



Aspecto do biofilme acumulado sobre os blocos de esmalte no dia da coleta.

Anexo N: Protocolo para análise bioquímica do biofilme seco (capítulo 2).



Anexo O: Secagem do biofilme dentário e procedimentos para posterior análise (capítulo 2).

Pentóxido de fósforo em placa de petri no fundo da jarra.

Amostras de biofilme acondicionados em microtubos abertos, colocados sobre uma base acima da placa de petri contendo pentóxido de fósforo. Secagem do biofilme em jarra de anaerobiose (sob vácuo), por 12 horas.

Pesagem do biofilme seco.

Agitação em incubadora TE-420 (Tecnal, Piracicaba-SP) por 3 horas a temperatura ambiente, após adição de HCl 0,5 mol. L^{-1} ao biofilme seco.

Centrifugação das amostras em centrífuga de bancada Combi 514R (Hanil BioMed Inc., Korea) por 3 horas. Tubos posicionados.

Anexo P: Dosagem de polissacarídeos extracelulares (capítulo 2).

A dosagem dos polissacarídeos extracelulares (carboidratos álcali-solúveis) foi realizada pelo método fenol-sulfúrico conforme descrito por Dubois et al. (1956), empregando os seguintes reagentes:

1 – Fenol 5%:

2 – Ácido Sulfúrico concentrado

A reação foi feita de acordo com a tabela abaixo:

	Blank	**Padrão**	**Padrão**	**Padrão**	**Padrão**	**Padrão**	**Amostra**
Água deionizada	0,5 mL	0,4 mL	0,3 mL	0,2 mL	0,1 mL	_	0,3 mL
Solução padrão (Glicose)	_	0,1 mL	0,2 mL	0,3 mL	0,4 mL	0,5 mL	_
Amostra	_	_	_	_	_	_	0,2 mL
Fenol 5%	0,5 mL em todos						
Ác. sulfúrico	2,5 mL em todos, agitar imediatamente, esperar 20 minutos e ler a 490 nm						

Inicialmente foi colocada água deionizada nos tubos de ensaio de acordo com os volumes descritos na tabela acima. A seguir foram colocados os padrões de glicose ou as amostras, em duplicata. Então, foram adicionados 0.5 mL de fenol 5% em todos os tubos e em seguida colocou-se 2.5 mL de ácido sulfúrico e agitou-se imediatamente. A coloração da reação foi desenvolvida pela adição do ácido sulfúrico concentrado. Após 20 min da colocação do ácido sulfúrico realizou-se a leitura em espectofotômetro (Espectrophotometer UV-1800 Shimadzu) a 490 nm de absorbância. Esses valores foram transferidos para uma planilha (programa Excel-Microsoft) e convertidos para μg de glicose.

Precipitado obtido após processamento, para posterior dosagem de polissacarídeos extracelulares.

Amostras processadas para dosagem de polissacarídeos extracelulares, após adição de fenol 5% e ácido sulfúrico concentrado.

Espectrofotômetro (Espectrophotometer UV-1800 Shimadzu)

Transferência de amostra para cubeta para leitura em Espectrofotômetro (Espectrophotometer UV-1800 Shimadzu)

Anexo Q: Análise de concentração de fluoreto, cálcio e fósforo no biofilme (capítulo 2).

DOSAGEM DE FLÚORETO

Analisador de íons Orion 4 Star (Orion Research, Inc.). Eletrodo de referência invertido Orion 900100 (Orion Research, Inc.).

Pipetagem da amostra para leitura em Eletrodo de referência invertido Orion 900100 (Orion Research, Inc.).

DOSAGEM DE CÁLCIO E FÓSFORO

Espectrofotômetro (Microplate Spectrophotometer EONC Biotek, Winooski, USA) utilizado para as dosagens de cálcio e fósforo, empregando-se placa de 96 poços.

Dosagem de cálcio. Método colorimétrico Arsenazzo III.

Dosagem de fósforo. Método colorimétrico (molibdato + reativo redutor).

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Araçatuba

Programa de Pós Graduação em Ciência Odontológica
Área de concentração Saúde Bucal da Criança

Rua José Bonifácio, 1193 - Fone: (18) 3636-3235 - Araçatuba-SP