UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Preventive measures for dental erosion

Estratégias preventivas para a erosão dentária

BAURU
2018

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Tese constituída por artigos apresentada à Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutora em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração de Odontopediatria.

Orientadora: Prof[a]. Dr[a]. Daniela Rios Honório

Versão corrigida

BAURU
2018

Ionta, Franciny Querobim
Io6p Preventive measures for dental erosion / Franciny Querobim Ionta. – Bauru, 2018.

142 p. : il. ; 31cm.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientadora: Profa. Dra. Daniela Rios Honório

Comitê de Ética da FOB-USP

Protocolo nº: CAAE 48753115.0.0000.5417

Data: 24/11/2015

Protocolo nº: CAAE 48729115.0.0000.5417

Data: 24/11/2015

Protocolo nº: CAAE 45792415.8.0000.5417

Data:29/07/2015

(FOLHA DE APROVAÇÃO)

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DADOS CURRICULARES

Nascimento 27 de setembro de 1989 – Bauru, SP

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SBPqO - Sociedade Brasileira de Pesquisa Odontológica

IADR - International Association for Dental Research

DEDICATÓRIA

Dedico essa tese aos que participaram intensamente no dia-a-dia de minha luta, me dando força e razões para seguir em frente. Sou uma pessoa muito rica, por possuir uma ótima família, um companheiro de vida incrível, amigos que são como irmãos e os melhores filhos de quatro patas que eu poderia ter.

A Deus,

Obrigada por guiar meus passos durante todo esse caminho, por me fortalecer e me amparar durante os momentos difíceis. Ele me fez enxergar mais longe quando tudo parecia embaçado. Obrigada por nem sempre fazer minhas vontades, mas sim atender as minhas necessidades! Sou infinitamente grata pela vida que tenho e pela pessoa que sou.

Á minha amada família,

Meus pais Jose Antonio Ionta e Maria Ivone Querobim Ionta,

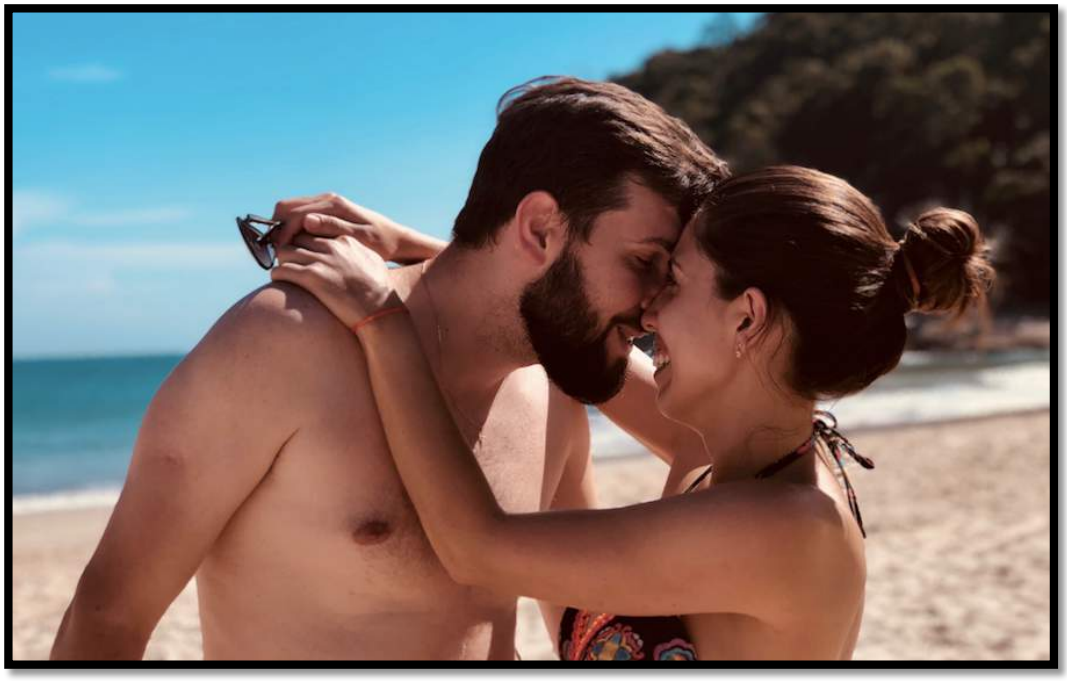
Vocês sonharam junto comigo e me proporcionaram TUDO que era preciso para conquistar esse título. Obrigada pelo amor incondicional e pelo apoio em todos os momentos. Amo muito vocês! Não tenham dúvidas de que foi por vocês que consegui.

Meus irmãos Fabio Querobim Ionta e Emily Querobim Ionta Dacacch e minha sobrinha Sofia da Silva Ionta,

Ter vocês como irmãos é saber que nunca estou e nunca estarei sozinha na caminhada da vida. No final, sempre seremos nós, crescendo com nossas diferenças e acolhendo-nos em nossas semelhanças. Obrigada por me incentivarem durante todo o caminho. E a princesa Sofia, que trouxe muitas alegrias e momentos incríveis para nossa família. Amo vocês!

Ao meu companheiro de vida Bruno Moreno Nascimento,

Essa conquista também é sua, por ter vivido comigo cada momento desde os tempos de graduação e por hoje partilharmos nossas vidas e nossos mundos. Todas as vezes em que surgiam dificuldades, você estava lá para me apoiar e me mostrar que eu seria capaz de superá-la. Sempre muito amoroso e atencioso, você tornou esse caminho mais fácil e certamente mais prazeroso! Obrigada por todo apoio e incentivo. Te amo Vi!



Aos meus filhos de quatro patas, **minha cachorrinha Lady e meu gatão Snow**, agradeço por existirem e me completarem. Todos os dias me recebiam carinhosamente e alegremente em casa me fazendo esquecer dos problemas.

AGRADECIMENTOS ESPECIAIS

O meu profundo agradecimento as pessoas que, desde o início do doutoramento, contribuíram para a concretização dessa tese. Sem aqueles contributos, este estudo não teria sido possível.

À minha professora e orientadora, **Profa. Dra. Daniela Rios Honório,** sou muito grata por todo o aprendizado desde 2010 durante minha graduação e especialmente a partir de 2012, durante todo o meu período de pós-graduação. Dani, poder observar de perto o quanto você é excelente profissional e o quanto amor coloca em tudo que faz foi a minha maior inspiração durantes todos esses anos na Odontopediatria. Obrigada por insistir e lutar por minhas conquistas mesmo quando, algumas vezes, eu queria desistir. Você enxergou minha capacidade de ir além do que eu imaginava e sempre me impulsionou a lutar por isso. Agradeço pelas oportunidades que me forneceu, onde pude me doar ao máximo e crescer muito! Se hoje sou a profissional completa que você diz e eu custo a acreditar, é porque você me estimulou e me serviu de exemplo em todas as esferas: na clínica, na pesquisa e na docência. Saiba que algumas vezes, ao fim de uma aula ministrada por mim, alguns comentários de colegas como “sua aula me lembrou muito das aulas que tive com a Prof.ª Dani” ou mesmo, quando eu terminava algum artigo e você, que é uma pessoa extremamente exigente com a qualidade de tudo que faz, me dizia que estava ótimo, foram os maiores elogios que eu poderia receber. Meu profundo agradecimento!

À minha parceira de doutorado e amiga **Natália Mello dos Santos**, não tenho palavras para te agradecer por tudo que fez por mim e por essa pesquisa. Sua ajuda foi fundamental em três das quatro pesquisas que compuseram essa tese. Você doou seu tempo, energia e disposição para a realização desse trabalho. Nos momentos de desesperos, você me dava forças e me impulsionava a continuar mesmo sendo 2h da manhã morrendo de fome e de sono, com tudo dando errado no laboratório e sabendo dos afazeres das 8h da manhã. Sem sua ajuda, eu não teria conseguido! Nossa parceria de laboratório, aos poucos passou para clínica como duplinha e agora não tenho dúvidas de que é para a vida. Obrigada por entrar na minha vida e tomar parte de um lugar tão especial, onde coloco pouquíssimas pessoas, que são meus amigos irmãos.

Às queridas alunas de Iniciação Científica que tive o prazer de co-orientar, **Bianca Tozi Portaluppe, Isabela Maníglia Mesquita, Marcela de Azevedo Garcia Bassoto** e **Poliana Pacifico Val**, pois sem vocês a realização de todos esses projetos simultaneamente nunca seria possível. Tivemos dificuldades no caminho e superamos cada uma e depois rimos juntas de cada fase difícil. Ao lado de vocês, tudo foi mais leve, laboratório, corte, dureza, moldagens, fluxo, correria com os voluntários, aparelhos, análises, apresentação de trabalho, relatórios... E vocês fizeram isso tudo mesmo com pouco tempo disponível pois tinham aulas, clínicas, seminários, provas. Vocês deram conta do recado e me orgulharam muito do resultado final. Vocês, juntamente com a Nat, formaram o #teamfran e fizeram essa pesquisa acontecer! Obrigada pela parceria meninas e pela experiência única em ensinar e aprender com vocês! Vocês estão para sempre marcadas em meu coração.

Ao técnico de laboratório da Odontopediatria e amigo, **Evandro José Dionísio**, agradeço muito por toda ajuda durante a realização em meus experimentos. Você sempre teve boa vontade em ajudar, tornando mais fácil e mais rápido o desenvolvimento desta pesquisa. Tenho um grande carinho por você, pela pessoa extremamente educada, correta e calma. Obrigada por ser também um amigo durante esse processo.

À ex-colega de pós-graduação, hoje Prof.ª Dr.ª **Catarina Ribeiro Barros de Alencar**, por ser a mentora principal do projeto dos óleos vegetais, ao qual apenas teve a ideia lapidada por mim. Cat, obrigada por confiar e ceder a realização desse projeto ao qual tinha tanto apreço, juntamente com os espécimes que havia preparado para a realização do mesmo. Certamente, o resultado final foi maravilhoso porque pensamos juntas nesse processo. Você sempre foi um exemplo de dedicação e excelência profissional.

AGRADECIMENTOS

Agradeço também as pessoas que contribuíram direta ou indiretamente para a concretização dessa tese.

Aos meus cunhados, **Henrique Daccach Manoel** e **Livea da Silva Ionta**, agradeço pela amizade, momentos de descontração e ajuda em diversos momentos. Obrigada pelo carinho e por preencherem a família de maneira especial.

À família de Sorocaba, meus sogros **Daniel Nascimento** e **Claudete Moreno Nascimento**, minhas cunhadas e seus respectivos, **Karen Moreno Nascimento** e **Henri Korkes**, **Priscilla Moreno Nascimento** e **Augusto Fazoli** e meus sobrinhos, **Maria Laura Moreno Canineu**, **Miguel Moreno Fazoli** e **Julia Korkes**. Vocês sempre me receberem sempre tão bem e me acolheram como parte da família. Obrigada pelo apoio, pelos momentos de descontração e por me deixar considerá-los como minha segunda família.

Às minhas amigas-irmãs desde os tempos da adolescência, **Luiza do Amaral Virmond, Georgia Terra Lustre de Flora, Ana Carolina Zago e Thatiana Porto Martelli**, que estiveram disponíveis para meus desabafos e também torciam e comemoravam junto comigo minhas conquistas, mesmo que muitas vezes à distância. Obrigada pelos conselhos, pelos momentos de descontração e por ser, cada uma com seu jeitinho, exatamente o que a palavra AMIGA significa. Não posso imaginar minha vida sem vocês! Amo vocês!

Aos meus amigos sorocabanos, **Júlia Cacciatore, Guilherme Bissoli e Felipe Bissoli**, obrigada pelo carinho com o qual me acolheram nessa cidade e pelos momentos de descontração.

À **Faculdade de Odontologia de Bauru** da Universidade de São Paulo, na pessoa de seu Diretor, Prof. Dr. Carlos Ferreira dos Santos. A infraestrutura da FOB é algo incrível e o ensino fornecido é de alta qualidade. Agradeço a essa instituição, por viabilizar minha capacitação profissional durante 10 anos de minha vida, desde a época de minha graduação.

Aos professores do departamento de Odontopediatria da FOB-USP:

Prof.ª Dr.ª Maria Aparecida de Andrade Moreira Machado, um dos nomes mais consagrados da Odontopediatria brasileira em que tive o orgulho em aprender direto com a Mestre. Professora Cidinha, obrigada por todo conhecimento transmitido, principalmente quanto a prática clínica durante o meu período de especialização, quando pudemos conviver mais frequentemente. Durante sua vigência como Diretora da FOB, lutou e trouxe muitas para melhorias para a estrutura e qualidade do ensino, como em especial pude acompanhar mais de perto na Odontopediatria.

Prof.ª Dr.ª Thais Marchini de Oliveira Valleri, a senhora foi meu maior exemplo durante minha graduação, o modo como tratava os alunos e como atendia os pacientes, sempre com seu jeito especial e seu bom humor, me trouxeram para a Odontopediatria. Durante toda minha pós-graduação esteve presente transmitindo conhecimentos e conselhos, sempre com muito carinho e com sua marca especial, o seu sorriso contagiante. Querida professora Thais, se hoje sou Odontopediatra, sinta-se responsável por ter me estimulado como aluna.

Prof. Dr. Thiago Cruvinel da Silva, em que tive o prazer de tê-lo como professor durante o meu Doutorado. Além de ser um excelente professor, suas características de bondade e de acolhimento o fazem destacar. Além disso, coloca muito amor e carinho em tudo que faz. Agradeço por ter sido banca de meu exame de qualificação de doutorado, e de forma crítica, porém atenciosa e gentil, conseguiu me estimular e contribuir para a melhora na qualidade técnica meu projeto.

Prof.ª Dr.ª Cleide Felício de Carvalho Carrara, mesmo durante nossa breve convivência, se fez marcante como professora. Observar o seu atendimento clínico e sua orientação com os alunos de graduação foram enriquecedores no meu processo de aprendizado. Além disso, seu bom humor e suas conversas, mostram o quanto é uma pessoa simples e que se coloca disponível e acessível ao aluno. Te admiro muito como professora da FOB e também, devido ao belíssimo trabalho no Centrinho (HRAC-USP).

Prof. Dr. Natalino Lourenço Neto, ao qual tive oportunidade de conviver como colega de pós-graduação e hoje, tê-lo como professor. Agradeço pelos ensinamentos transmitidos, por ensinar e motivar o aprendizado por meio das novidades no mundo odontológico com muito entusiasmo e também pela relação de carinho.

Aos ex-professores do departamento de Odontopediatria da FOB-USP, **Prof.ª Dr.ª Salete Moura Bonifácio da Silva, Prof. Dr. José Eduardo de Oliveira Lima, Prof. Dr. Ruy César Camargo Abdo**, agradeço por toda paciência, conhecimento e experiência transmitidos durante minha graduação, especialização e mestrado.

Ao **Prof. Dr. Heitor Marques Honório**, agradeço por sempre se mostrar disponível para ajudar, quer seja na realização de análises estatísticas e fluxogramas em horários imprevistos, ou mesmo no compartilhamento de ideias. Agradeço também por me inspirar para sempre querer montar aulas didáticas e visualmente atraentes. O senhor é um excelente professor, admiro muito seu trabalho e sua dedicação.

À **Prof.ª Dr.ª Marília Afonso Rabelo Buzalaf**, agradeço por todos os ensinamentos desde a graduação. Para mim, a senhora é um exemplo profissional e pessoal, pois é muito boa no que faz, sendo reconhecida mundialmente pelo seu trabalho, e também é uma pessoa extremamente humilde e gentil. Tive o prazer de tê-la como banca em minha defesa de mestrado e em minha qualificação de doutorado, nas quais com sua vasta experiência nessa área de pesquisa, ressaltou os melhores aspectos dos trabalhos e contribuiu de forma significativa. Muito obrigada por disponibilizar seu tempo para leitura e participação de minha qualificação. Suas dicas e sugestões elevaram muito a qualidade dessa tese.

Ao **Prof. Dr. Paulo Sérgio da Silva Santos**, por me aceitar como aluna na prática profissionalizante de Odontologia em Oncologia e permitir meu crescimento profissional e pessoal. Querido professor, essas clínicas e seus ensinamentos significaram muito para mim. Poder conviver com uma equipe tão capacitada e prestativa, foi um imenso prazer. O carinho e atenção demandado aos pacientes oncológicos são características que sempre busquei em todos os meus atendimentos e pude encontrar e aprender com maestria. Admiro muito seu trabalho e o ser humano que é. Agradeço também a toda equipe **Prof.ª.Dr.ª Cassia Fischer Rubira, Anderson Prestes, Luciana Viti Betti, Marcelo Zanda, Poliane de Araújo, Sueli Ribeiro,** por todos os ensinamentos que vocês me forneceram e também pela incrível relação de trabalho que vocês criaram, tornando muito prazeroso participar do Centro de Pesquisas Clínicas.

Á **Prof[a]. Dr[a]. Juliane Avansini Marsicano**, que guiou meus primeiros passos em minha vida acadêmica e me orientou durante minha iniciação científica de forma muito compreensiva e estimulante, sempre demonstrando empenho, dedicação e amor no que fazia. Obrigada por ser meu primeiro exemplo nessa caminhada.

Aos colegas atuais, **Natália Mello dos Santos, Bianca Tozi Portalupe Bergantin, Camila Di Leone**, e ex-colegas, **Fernanda Lyrio Mendonça, Maísa Camillo Jordão, Gabriela Cristina de Oliveira, Catarina Ribeiro Barros de Alencar e Priscilla Santana Pinto Gonçalves**, de pós-graduação orientados pela Prof.[a] Dr.[a] Daniela Rios, agradeço pela amizade, troca de conhecimento e pela grande oportunidade de aprender e conviver com vocês. Nat e Bi, muito obrigada pela grande ajuda durante a fase experimental do estudo. Ca, Maísa, Cat, Gabi e Pri, nossa convivência foi muito prazerosa e proveitosa, agradeço pelo companheirismo de vocês e por toda contribuição durante o doutorado. Ferzinha, nossa amizade se iniciou durante nossa especialização e se fortaleceu durante o mestrado, e hoje mesmo com a distância geográfica imensa entre a gente, sei que posso contar com minha baianinha arretada sempre; tenho muito orgulho da pessoa e profissional que você é, meu enorme carinho e consideração por você!

À minha primeira co-orientada de iniciação científica e hoje, colega de pós-graduação em Dentística, **Ana Paula Boteon**. Obrigada pela disponibilidade e prontidão ao ajudar-me em um dos projetos de meu doutorado, mesmo aos finais de semana, e ainda pelas ideias sugeridas que colaboraram para esse estudo. Agradeço também, a colega de pós-graduação em prótese, e Prof.[a] **Brunna Mota Ferrairo** pelos conselhos e pela amizade. Meu carinho e admiração por vocês duas.

Ás colegas de doutorado em Odontopediatra, **Daniela Cusicanqui Mendez, Estefania Ayala Aguirre, Luciana Lourenço Vitor, Paula Karine Jorge, Bianca Zeponi Mello, Mariel Prado Bergamo, Tássia Carina Stafuzza, e Eloá Ambrosio**, pelo aprendizado e pelo carinho durante o período de convivência. Em especial, agradeço a Dani, Esteff, Lu e Paulinha, com as quais tive oportunidade de conviver mais intensamente por meio de conversas, conselhos e momentos de descontração, e que possuo muito carinho por tudo que vivemos.

Às alunas e ao aluno de mestrado em Odontopediatra, **Maiara Falzoni, Anna Paola Strieder, Giuliana Campos Chaves, Camila de Leone, Bianca Bergantin, Bianca Zalaf** e **Kim Tanabe**. Vocês são pessoas muito especiais e fizeram essa turma ser única! Agradeço pelo ótimo convívio e pelos momentos de descontração. Não tenho palavras para agradecer tanto carinho que tiveram por mim em tão pouco tempo, por meio da organização, juntamente com os alunos de doutorado, de uma festa surpresa de despedida tão linda e emocionante. Todos esses momentos estarão para sempre guardados em meu coração.

Aos funcionários do departamento de Odontopediatria FOB-USP:
Estela Ferrari e Lilian Rosana Cândida, pela organização da clínica, agendamento de pacientes, disponibilidade e prontidão em ajudar. Obrigada também pela amizade desenvolvida ao longo desses anos. Agradeço também a **Cleonice Selmo**, que mesmo sendo funcionária da Ortodontia, sempre nos ajudou com muita boa vontade. **Alexandre Montilha,** obrigada pela disponibilidade e pela paciência ao me ajudar a resolver burocracias e problemas sempre rapidamente e com boa vontade.
Lourisvalda Celestino, obrigada pela convivência agradável durante esse processo.

Ao departamento de Ciências Biológicas, disciplina de Bioquímica da FOB- USP, em nome da **Prof.ª Dr.ª Marília Afonso Rabelo Buzalaf**, por permitir a utilização dos equipamentos necessários do laboratório para a realização desse estudo. Aos funcionários do laboratório de Bioquímica da FOB-USP, em especial à **Larissa Grizzo**, pela disponibilidade e prontidão em ajudar.

Aos funcionários da biblioteca, especialmente ao **José Roberto Plácido Amadei**, pelo auxílio e atenção principalmente referente à normativa desse trabalho.

Aos funcionários da pós-graduação da FOB-USP, em especial à **Fatima Cassador Carvalho e Leila Regina da Silva Yerga Sanchez**, pela atenção e auxílio durante a resolução de questões burocráticas. Agradeço também a auxiliar de consultório da clínica de pós-graduação, **Hebe de Freitas Pereira,** que apesar de não ter convivido tão frequentemente, estava sempre disposta a ajudar esbanjando bom humor e amor no que faz.

Aos **pacientes Odontopediátricos** e seus responsáveis, obrigada pela confiança, disposição na realização de fotografias intra-bucais e pelo carinho, contribuindo para o meu crescimento pessoal e profissional.

Aos **voluntários** de meus 4 estudos, sem vocês a realização do protocolo experimental não seria possível. Obrigada por confiarem em meu trabalho e doarem tempo e disposição para realizarem essas pesquisas com tanto zelo.

À **Central de Esterilização ACECIL**, agradeço pela esterilização dos blocos de dentes utilizados neste estudo.

À agência financiadoras de pesquisa **CAPES**, agradeço pela concessão de bolsa de doutorado durante o início de meu doutorado.

À agência financiadoras de pesquisa **FAPESP**, agradeço pela concessão de bolsa de doutorado, por meio do processo **2015/21552-7**.

À **banca avaliadora**, agradeço aos professores por aceitarem o convite para participar da banca avaliadora dessa tese, e por contribuírem para o enriquecimento da mesma.

Agradeço a todos que de alguma forma contribuíram para a realização deste trabalho, ou que me impulsionaram de alguma forma para conquistar esse título.

“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

Marthin Luther King

ABSTRACT

Preventive measures for dental erosion and erosive tooth wear

In recent years, due to the high prevalence of dental erosion, therapies to prevent the occurrence or inhibit the progression of this condition have been searched. The purpose of this thesis was to present four articles that evaluated possible preventive measures for enamel erosion. Specifically, it was evaluated: article I - the effect of five types of vegetable oils against initial enamel erosion; article II - protective effect of palm oil alone or associated with a fluoride solution against erosive enamel wear (chemical-mechanical/toothbrushing); article III - the protective potential against erosive tooth wear of an aspartame solution, used as mouthwash prior to acid exposure; article IV - the effectiveness of a dentifrice with calcium silicate, phosphate and fluoride on the prevention of erosive wear (chemical-mechanical/toothbrushing). In all articles, deionized water (DW) was used as negative control and a solution (SS) or dentifrice (SD) containing fluoride and stannous as positive control. The response variable adopted was loss of surface hardness for article I and enamel loss in height for articles II, III and IV. In article I, two volunteers used the intraoral appliance for 2 hours to form the acquired pellicle and then the enamel blocks of each study group were treated *in vitro* by 5 different vegetable oils at 2 different concentrations (5 or 100%). Then, the blocks were immersed in artificial saliva for 2 minutes and subjected to 0.5% citric acid for 2 minutes. Data were analyzed by one-way ANOVA and Tukey's test ($p < 0.05$). Among the evaluated vegetable oils, palm oil was the only one that presented protective potential against initial enamel erosion, resulting in less hardness loss than DW and SS. In Article II, volunteers used intraoral appliances *in situ* for 5 days, in which 4 ex vivo erosive cycling with 0.5% citric acid for 2 minutes was carried out. Prior to the first and third erosive challenge, DW, SS and palm oil associated or not to SS were applied on enamel blocks by administration of one drop of the respective solution, followed by acid immersion. Then, the abrasive challenge was performed (brushing for 15 seconds). Data were submitted to 2-way ANOVA and Tukey's test ($p < 0.05$). For both, erosion and erosion + abrasion, palm oil alone or associated to SS resulted in less enamel loss than DW, but did not differ from SS. In article III, 4x/day volunteers performed in situ mouthwashes with DW, SS or 0.024% aspartame solution. Then half enamel blocks were immersed ex vivo in intrinsic (0.01M hydrochloric acid pH 2.3) and the other half in extrinsic (0.03 citric acid pH 2.4 for 2 minutes) acid challenge for 5 days. After statistical analysis (2-way ANOVA and Tukey's test, $p < 0.05$), it was observed that aspartame was similar to DW and resulted in greater loss of enamel than SS. Hydrochloric acid promoted higher enamel loss than citric acid. In article IV, volunteers used intraoral appliances *in situ* for 5 days and 4 erosive cycling with 0.5% citric acid for 2 minutes was carried out. Right after the first and third cycling of the day the dentifrices, including the one with addition of calcium silicate, phosphate and fluoride, were applied for 1 minute and then, half of the enamel specimens were brushed for 15 seconds (abrasion). Statistical analysis was performed by 2-way ANOVA and Fischer's exact test ($p < 0.05$). The dentifrice containing calcium silicate, sodium phosphate and fluoride protected the

enamel against erosion similar to SD; but when subjected to abrasion by brushing, it showed similar enamel loss than DW, demonstrating no protective effect. Among the tested preventive measures, palm oil presented promising results in the prevention of erosive tooth wear, similarly to a stannous-solution. Aspartame, however, did not present a preventive effect against erosive tooth wear caused by intrinsic or extrinsic acid. The dentifrice containing calcium silicate, sodium phosphate and fluoride only presented a preventive effect against erosion but it did not show a protective effect against erosive tooth wear.

Keywords: Dental erosion. Erosive enamel wear. Prevention. In situ.

RESUMO

Estratégias preventivas para erosão dentária e desgaste dentário erosivo

Devido à alta prevalência de erosão dentária encontrada nos últimos anos, tem se buscado terapias para prevenir a ocorrência ou inibir o avanço desta condição. O propósito deste estudo foi apresentar quatro artigos que avaliaram possíveis medidas preventivas para erosão dentária do esmalte. Especificamente, foram avaliados: artigo I - o efeito de cinco tipos de óleos vegetais contra a erosão inicial; artigo II - capacidade protetora do óleo de dendê sozinho ou associado uma solução fluoretada contra desgaste dentário erosivo (químido-mecânico/escovação); artigo III - o potencial protetor contra o desgaste dentário erosivo de uma solução com aspartame, utilizada como bochecho previamente a exposição ácida; artigo IV - a eficácia da aplicação do dentifrício com adição de silicato de cálcio, fosfato e flúor na prevenção do desgaste erosivo (químido-mecânico/escovação). Em todos os artigos adotou-se água deionizada (AD) como controle negativo e solução (SE) ou dentifrício (DE) contendo fluoreto e estanho como controle positivo. A variável de resposta adotada foi perda de dureza de superfície para o artigo I e perda de tecido dental duro em altura para os artigos II, III e IV. No artigo I, dois voluntários utilizaram o dispositivo intrabucal durante 2 horas para formação da película adquirida e em seguida, os blocos de esmalte de cada grupo em estudo foram tratados in vitro por 5 diferentes óleos vegetais em 2 concentrações distintas (5 ou 100%). A seguir, os blocos foram imersos em saliva artificial por 2 minutos e então, em ácido cítrico 0,5% por 2 minutos. Os dados foram analisados por ANOVA 1 critério e teste de Tukey ($p<0,05$). Dentre os óleos vegetais avaliados, o óleo de dendê foi o único que apresentou potencial protetor contra erosão inicial do esmalte pois resultou em menor perda de dureza quando comparado a AD e SE. No artigo II, voluntários utilizaram aparelhos palatinos in situ por 5 dias, sendo realizadas 4 ciclagens erosivas ex vivo em ácido cítrico 0,5% por 2 minutos, anteriormente a primeira e a terceira ciclagem a AD, o SE e o óleo de dendê associado ou não à SE foram aplicados nos blocos de esmalte por meio da administração de uma gota da respectiva solução, seguida da imersão ácida e então, o desafio abrasivo foi realizado (escovação por 15 segundos). Os dados foram submetidos a ANOVA 2 critérios e teste de Tukey ($p<0,05$). Tanto para erosão como para erosão+abrasão, o óleo de dendê sozinho ou associado à SE resultou em menor perda de esmalte do que AD, porém não diferiu da SE. No artigo III, os voluntários realizaram bochechos in situ 4x/dia com 0,024% de aspartame e então, metade dos blocos de esmalte foram submetidos ex vivo ao desafio com ácido intrínseco (ácido clorídrico a 0.01M pH 2.3) e a outra metade ao extrínseco (ácido cítrico 0.03M pH 2.4) durante 5 dias. Após análise estatística (ANOVA 2 critérios e teste de Tukey, $p<0,05$), constatou-se que o aspartame foi similar à AD e resultou em maior perda de esmalte do que SE; sendo que o ácido clorídrico promoveu maior perda de esmalte do que o ácido cítrico. No artigo IV, voluntários utilizaram aparelhos palatinos in situ por 5 dias e foram realizadas 4 ciclagens erosivas em ácido cítrico 0,5% por 2 minutos, sendo que logo após a primeira e terceira ciclagem do dia, os dentifrícios, incluindo o com adição de silicato de cálcio, fosfato e flúor foram aplicados por 1 minuto e metade dos espécimes foram escovados por 15 segundos (abrasão). A análise estatística foi

realizada por ANOVA 2 critérios e teste exato de Fischer ($p<0.05$). O dentifrício contendo silicato de cálcio, fosfato de sódio e flúor protegeu o esmalte contra a erosão semelhantemente ao DE. Porém quando a abrasão por escovação foi associada, o mesmo resultou em perda de esmalte semelhante a AD e portanto, não houve efeito protetor. Dentre as medidas preventivas testadas o óleo de dendê apresentou resultados promissores na prevenção do desgaste dentário erosivo, se assemelhando ao estanho. Já o aspartame, não apresentou efeito preventivo contra o desgaste dentário erosivo causado por ácido intrínseco ou extrínseco. O dentifrício contendo silicato de cálcio, fosfato de sódio e flúor, só apresentou efeito preventivo contra a erosão, mas não foi capaz de proteger o esmalte contra o desgaste dentário erosivo.

Palavras-chave: Erosão dentária. Desgaste erosivo do esmalte. Prevenção. In situ.

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1 Introduction

1 Introduction

The high prevalence and severity of dental erosion (SALAS et al., 2015; TSCHAMMLER et al., 2016; BRUSIUS et al., 2018; SCHLUETER; LUKA, 2018) is a cause of concern to dental clinicians and researchers since this condition can interfere on patient's quality of life impairing aesthetics and chewing (PAPAGIANNI et al., 2013). At routine examination, erosive wear becomes clinically visible in later stages, when the appearance and shape of the teeth are compromised. Studies have been conducted to better understand the mechanisms involved in this condition and to seek for early preventive therapies (BUZALAF; MAGALHÃES; WIEGAND, 2014; HUYSMANS; YOUNG; GANSS, 2014; WEGEHAUPT; KUMMER; ATTIN, 2017; BUZALAF; MAGALHÃES; RIOS, 2018). Dental erosion is characterized by the loss of structure, with progressive surface softening and dissolution of the minerals, due to the exposure to intrinsic or extrinsic acids of non-bacterial origin (TENCATE; IMFELD, 1996; LUSSI et al., 2011). Currently, dental erosion has also been called as erosive tooth wear (ETW) since in clinical practice, the chemical action of the acids occurs in association of mechanical forces, such as attrition and abrasion (CARVALHO; LUSSI, 2015; SHELLIS; ADDY, 2014). The prevention of ETW can be complicated, since patient-related and nutritional factors are involved in the etiology (LUSSI; CARVALHO, 2014; BUZALAF; MAGALHÃES; RIOS, 2018).

Saliva is considered the most important patient-related protective factor on the etiology of ETW (HARA; LUSSI; ZERO, 2006; BAUMANN et al., 2016; BUZALAF; MAGALHÃES; RIOS, 2018). One key factor in the salivary mechanism for erosion protection is the formation of the acquired enamel pellicle (AEP) (HANNIG; JOINER, 2006; SIQUEIRA; CUSTODIO; MCDONALD, 2012; BUZALAF; HANNAS; KATO, 2012; HANNIG; HANNIG, 2014; VUKOSAVLJEVIC et al., 2014), which diminishes the direct contact between acids and enamel, reducing and retarding enamel softness and loss (AMAECHI et al., 1999; HANNIG; JOINER, 2006; HANNIG; HANNIG, 2014). The AEP is a non-bacterial organic film formed by the adsorption of proteins, peptides, lipids and other macromolecules available in the saliva (HANNIG; JOINER, 2006;

HANNIG; HANNIG, 2009; SIQUEIRA; CUSTODIO; MCDONALD, 2012; BUZALAF; HANNAS; KATO, 2012; HANNIG; HANNIG, 2014). The protective capacity of the AEP against ETW depends on physical properties, including the thickness and time of maturation (VUKOSAVLJEVIC et al., 2014; HANNIG; HANNIG, 2014; MENDONÇA et al., 2017; MENDONÇA et al., 2017). In terms of protein adsorption, the formation of AEP begins within a few seconds after the exposure of dental surface to saliva (HANNIG et al., 2004) producing a dense basal layer (ERICSON et al., 1982). Studies have shown that acquired pellicle formed for 1 hour to 2 hours offers the maximum protection against demineralization without significantly improving with longer maturation periods (AMAECHI et al., 1999; HANNIG et al., 2003; WETTON et al., 2006; MENDONÇA et al., 2017). After the consumption of erosive drinks, the outer layers of AEP are removed, however, the basal layer does not seem to be affected (HANNIG et al., 2009). In order to delay its removal during the erosive challenge, a possible strategy could be modifying AEP composition. About 25% of the acquired pellicle dry weight is composed by lipids (HANNIG et al., 2003); lipophilic components can modulate the composition and ultrastructure of the pellicle (KENSCHE et al., 2013). It has been speculated that lipid-enriched pellicles are more resistant to acids hampering dental erosion process (HANNIG et al., 2012; KENSCHE et al., 2013).

Vegetable oils have been widely studied in preventive dentistry since they are a natural, edible, low-cost, and worldwide accessible source (BUCHALLA et al., 2003; HANNIG et al., 2012; KENSCHE et al., 2013). Previous study showed that 2% olive oil and 2% olive oil associated to fluoride mouthwash had some preventive effect against dental erosion, but to a lower extent when compared with the positive control (acidulated fluoride solution, 250 ppm, pH 3.88) (WIEGAND; GUTSCHE; ATTIN, 2007). Considering the preventive potential of vegetable oils on dental erosion inhibition, it would be important to evaluate the use of different vegetable oils and its addition to a stannous(Sn)-containing solution, as a strategy to prevent ETW.

Nutritional related factors are determinant on the development of ETW. High consumption of soft drinks and specific diets with high consumption of acid fruits (e.g. vegan, vegetarian and raw food diets), can influence on the risk of development of dental erosion (SCHLUETER; TVEIT, 2014). In last decades, dietary habits have been changed with an increasing on the consumption of soft drinks (LUSSI; JAEGGI; ZERO, 2004; LUSSI; CARVALHO, 2014). In this way, researches have investigated the

erosive effects of different versions of soft drinks (GROBLER; SENEKAL; LAUBSCHER, 1990; LUSSI; JAEGGI; ZERO, 2004). Light colas have demonstrated less erosive potential compared to their traditional versions (RIOS et al., 2009; RIOS et al., 2011). The main chemical differences between the two versions is the pH, 2.6 on the regular version and 3.0 on the light version (HANNIG et al., 2005; RIOS et al., 2009; RIOS et al., 2011), and the type of sweetener in the formulation, sugar in regular version and aspartame in light version (RIOS et al., 2009; RIOS et al., 2011). Aspartame in contact with saliva can undergo hydrolysis producing phenylalanine - an aromatic amino acid with a carboxylic grouping - in which the acid protons could bind to, diminishing the dental enamel demineralization (RANGAN; BARCELOUX, 2009; ASHOK; SHEELADEVI, 2014). The anti-erosive potential of aspartame, regardless of its association with colas, has not been evaluated. Thus, it would be important to evaluate whether an aspartame mouthwash prior to immersion in acid would be able to prevent intrinsic and extrinsic erosive enamel wear in situ.

Patient-related ETW etiological factor also involves the patients' behavior regarding to intraoral habits and products adopted to daily oral hygiene (WIEGAND; SCHLUETER, 2014; HELLWIG; LUSSI, 2014; CARVALHO et al., 2015). Dentifrices could work as suitable delivery for preventive agents for dental erosion since they are routinely used once or twice a day by most individuals (FALLER; EVERSOLE; TZEGHAI, 2011; HORNBY et al., 2014; JOINER et al., 2014; JONES et al., 2014; FALLER; EVERSOLE; SAUNDERS-BURKHARDT, 2014). However, eroded enamel is more susceptible to toothbrush abrasion than sound enamel (JAEGGI; LUSSI, 1999); thus, specific anti-erosive dentifrices must have a mechanism to compensate the impact of brushing forces. In vitro studies have demonstrated that specific types of calcium silicate – such as Ca_3SiO_5 – can induce the formation of hydroxyapatite in saliva, which could be deposited on acid-eroded enamel, suggesting a protective effect against erosive enamel wear (DONG et al., 2010; PARKER et al., 2014). However, the efficacy of calcium silicate seems to be similar to that of 1000 ppm sodium fluoride solution in a pH cycling erosive model and better results were obtained when Ca_3SiO_5 was associated with fluoride (WANG et al., 2012).

Greater rehardening capacity of eroded enamel have been demonstrated by dentifrices containing calcium silicate, sodium phosphate, and fluoride compared to conventional fluoride dentifrices (HORNBY et al., 2014; JOINER et al., 2014; JONES

et al., 2014). However, the evidence about the mechanism and the protective capacity of calcium silicate is limited since only a few studies were reported and most of those studies have not assessed the impact of toothbrushing. In addition, only the enamel surface hardness or whether or not there was formation of hydroxyapatite on the tooth surface was measured. It would be also important to consider the amount of dental hard tissue lost when this product is applied under episodes of erosion associated to abrasion.

Based on these findings, the overall aim of this study was to investigate the effectiveness of possible preventive measures for dental erosion and erosive tooth wear. Each article has specifically evaluated:

• Article I: The effect of five different vegetable oils, in pure form or as emulsions, applied on AEP formed *in situ*, on the protection of enamel against initial erosive demineralization *in vitro*.

• Article II: The effect of palm oil alone or associated with a Sn-containing solution on ETW prevention (chemical and mechanical/abrasive challenge) using an in situ protocol.

• Article III: The protective potential against ETW of regular aspartame solution mouthwash prior to immersion in citric and hydrochloric acids using an in situ protocol.

• Article IV: The effect of a dentifrice that contains calcium silicate, sodium phosphate, and fluoride on the prevention of ETW (chemical and mechanical/abrasive challenge) using an in situ protocol.

2 Articles

2 Articles

The articles presented in this thesis were written according to the instructions and guidelines for article submission of the corresponding journals.

- ARTICLE 1 – Effect of vegetable oils applied over acquired enamel pellicle on initial erosion. Journal of Applied Oral Science.
- ARTICLE 2 – Effect of palm oil alone or associated to stannous solution on enamel erosive-abrasive wear: a randomized in situ/ex vivo study. Archives of Oral Biology. (Submitted)
- ARTICLE 3 - Protective effect of aspartame against erosive enamel wear by intrinsic and extrinsic acids: an in situ/ex vivo study. International Dental Journal. (Submitted)
- ARTICLE 4 - Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear? an in situ/ ex vivo study. Clinical Oral Investigations. (Submitted)

2.1. Article 1[1] - Effect of vegetable oils applied over acquired enamel pellicle on initial erosion.

Original Article
http://dx.doi.org/10.1590/1678-7757-2016-0436

Effect of vegetable oils applied over acquired enamel pellicle on initial erosion

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Abstract

Objective: The prevalence of dental erosion has been recently increasing, requiring new preventive and therapeutic approaches. Vegetable oils have been studied in preventive dentistry because they come from a natural, edible, low-cost, and worldwide accessible source. This study aimed to evaluate the protective effect of different vegetable oils, applied in two concentrations, on initial enamel erosion. Material and Methods: Initially, the acquired pellicle was formed *in situ* for 2 hours. Subsequently, the enamel blocks were treated *in vitro* according to the study group (n=12/*per* group): GP5 and GP100 – 5% and pure palm oil, respectively; GC5 and GC100 – 5% and pure coconut oil; GSa5 and GSa100 – 5% and pure safflower oil; GSu5 and GSu100 – 5% and pure sunflower oil; GO5 and GO100 – 5% and pure olive oil; CON– – Deionized Water (negative control) and CON+ – Commercial Mouthwash (Elmex® Erosion Protection Dental Rinse, GABA/ positive control). Then, the enamel blocks were immersed in artificial saliva for 2 minutes and subjected to short-term acid exposure in 0.5% citric acid, pH 2.4, for 30 seconds, to promote enamel surface softening. The response variable was the percentage of surface hardness loss [((SHi - SHf) / SHf)×100]. Data were analyzed by one-way ANOVA and Tukey's test ($p<0.05$). Results: Enamel blocks of GP100 presented similar hardness loss to GSu100 ($p>0.05$) and less than the other groups ($p<0.05$). There was no difference between GP5, GC5, GC100, GSa5, GSu100, GSa100, GSu5, GO5, GO100, CON– and CON+. Conclusion: Palm oil seems to be a promising alternative for preventing enamel erosion. However, further studies are necessary to evaluate a long-term erosive cycling.

Keywords: Tooth erosion. Plant oils. Primary prevention. Dental enamel.

Submitted: September 5, 2016
Modified: November 16, 2016
Accepted: November 27, 2016

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Introduction

The prevalence of dental erosion has been increasing in recent years[1-7]. Dental erosion is defined as a chemical process that involves gradual loss of dental hard tissue by intrinsic or extrinsic acids of non-bacterial origin[2,7]. Advanced stages of this condition may impair esthetics and function, affecting the patient's quality of life[15]. Therefore, establishing effective preventive and therapeutic approaches focused on the etiopathogenesis of the lesion is required. Preventive measures should start as early as possible and involve causal measures, such as dietary advice, to reduce the erosive challenges. In addition, the development of strategies to enhance biological protective factors may help preventing dental erosion. Saliva has been considered the most important biological factor on the pathogenesis of dental erosion[3,14]. The protective mechanism of saliva includes the formation of the acquired enamel pellicle (AEP)[9,25], a non-bacterial organic film formed over the enamel surface by the adsorption of proteins, peptides, lipids, and other macromolecules available in saliva[8,9]. The AEP plays an important role on the prevention of dental erosion, working as a mediator that diminishes the direct contact of acids with the enamel surface[9]. The protective potential of the AEP depends on its physical properties, including thickness and maturation time[9]. Studies have shown that pellicles formed during two hours or less offer maximum protection against erosive demineralization, without any increase in enamel erosion prevention with longer periods of maturation[10,26]. One possible strategy to increase AEP protection may be the modification of its composition, to improve the protective effect during an erosive challenge by the maintenance of the AEP on the enamel. Lipids consist of about 25% of the dry weight of acquired pellicle[10], and it is known that lipophilic components are able to modulate the composition and ultrastructure of the AEP[12]. Therefore, it is believed that lipid-rich AEPs are more resistant to acid challenges, protecting against enamel erosion[12].

The preventive potential of vegetable oils has been widely studied, since they are a natural, edible, low-cost, and worldwide accessible source[2,8,12]. A previous study showed that 2% olive oil and 2% olive oil associated to fluoride mouthwash were able to prevent erosion, but to a lower extent when compared with the positive control (acidulated fluoride solution, 250 ppm, pH 3.88)[27]. Various types of vegetable oils are available and their anti-erosive potential might be different according to their composition, including the types of fatty acids and other components. This study aimed to evaluate the *in vitro* effect of different types of vegetable oils, in pure form or as emulsions, applied on AEP formed *in situ*, on the protection of enamel against initial erosive demineralization.

Material and methods

Experimental design

This study was conducted according to the Declaration of Helsinki. The protocol was approved by the local Research Ethics Committee (Protocol 1.173.522/2015). All individuals signed an informed consent form before the confirmation of their eligibility for the study.

This study evaluated the *in vitro* potential of distinct vegetable oils, in different concentrations, to inhibit enamel erosive demineralization. The experimental design is shown in Figure 1. Before applying the oils, the AEP was formed *in situ* on 24 enamel blocks worn by two volunteers (1 block for each group per volunteer) for 2 hours. Subsequently, the enamel blocks were treated *in vitro* according to the groups (n=12/per group): GPS – 5% palm oil; GP100 – pure palm oil; GCS – 5% coconut oil; GC100 – pure coconut oil; GSaS – 5% safflower oil; GSa100 – pure safflower oil; GSuS – 5% sunflower oil; GSu100 – pure sunflower oil; GOS – 5% olive oil; GO100 – pure olive oil; Control– – negative control, deionized water; Control + – positive control, mouthwash commercial solution containing 125 ppm F^- as AmF, 375 ppm F^- as NaF, 800 ppm Sn^{2+} as $SnCl_2$; pH 4.5 (Elmex® Erosion Protection Dental Rinse/EP – CP GABA GmbH; Hamburg, Germany). After application of the oils (5 drops, 30 seconds), the blocks were immersed in 0.5% citric acid (nascent pH 2.4) during 30 seconds to promote the softening of enamel surface. The percentage of surface hardness change was assessed (response variable). The mentioned procedures were repeated for 6 days, in which one sample *per* group was fixed in each volunteer intraoral appliance *per* day.

Sample size

A pilot study was conducted with six enamel blocks of 100% palm oil and negative control (deionized

water) *per* group. Thus, a standard deviation of 8.5% was obtained. Twelve samples per group were set, considering 12 groups with a minimally detectable difference of 15% in hardness loss and 8.5% of standard deviation, with alpha and beta errors of 5% and 20%, respectively.

Enamel blocks preparation

Enamel blocks (4×4×3 mm³, n=160) were prepared from the labial surfaces of bovine incisor crowns. The blocks were cut using a IsoMet® low speed saw cutting machine (Buehler Ltd.; Lake Bluff, Illinois, United States) with two diamond disks (Extec Corp.; Enfield, Connecticut, United States), which were separated by a 4-mm thickness spacer. The blocks' surfaces were smoothed with water-cooled silicon carbide discs (320, 600, and 1200 grade papers; Buehler Ltd.; Lake Bluff, Illinois, United States), and wet polished with felt paper and diamond spray (1 µm; Buehler Ltd.; Lake Bluff, Illinois, United States). The blocks were cleaned using an ultrasonic device for 2 min and verified regarding the presence of white spots and cracks using a microscope (40×). Knoop surface hardness (SHi) was determined by the mean values of five indentations performed 100 µm away from each other, with 25 g for 10 seconds (Micromet® 5114 hardness tester; Buehler Ltd., Lake Bluff, Illinois, United States). One hundred and forty four enamel blocks were selected by excluding values 10% higher or lower than the mean microhardness of all specimens (interblock variability), to avoid bias regarding initial enamel condition. The blocks were allocated using Microsoft Excel to distribute blocks with lower and higher initial hardness values equally into all groups. The randomization was done to divide the enamel blocks between the groups and the two volunteers (position of the block in the intraoral device and 6 days of experiment).

Before the *in situ* phase for AEP, the blocks were sterilized with ethylene oxide[23].

In situ phase – acquired enamel pellicle formation

Two healthy adult volunteers with the same age (22 years old), residing in the same fluoridated area (0.70 mg F/l), participated in the study, after satisfying the following inclusion criteria: physiological salivary parameters (stimulated >1 ml/min; unstimulated >0.1 ml/min; neutral pH 7.0-7.5); absence of erosive tooth wear, untreated carious lesions, or periodontitis. The exclusion criteria were: presence of systemic diseases; use of medicines that affect the salivary characteristics (antidepressants, narcotics, diuretics, or antihistamines); undergoing

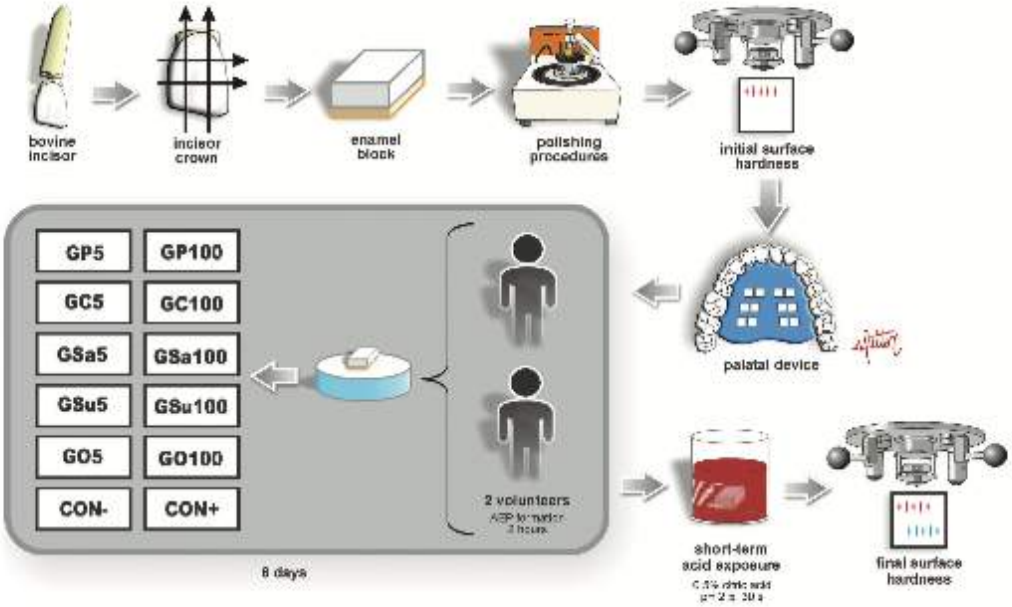

Figure 1- Illustration of the experimental design adopted in this study

radiation or chemotherapy; gastro-esophageal reflux; frequent regurgitation and/or vomiting; pregnancy or breastfeeding; smoking; practicing pool activities (exposure to low-pH treated water); working in low-pH environment (e.g., batteries industry); or fluoride topical application in the past two months. The intraoral palatal appliances were made with acrylic resin containing six sites (9×6×3 mm) for two blocks fixation in each (n=12).

The position of the blocks in the intraoral appliance was randomly determined for each volunteer. Seven days prior to and during the experiment, the volunteers brushed their teeth with commercial fluoride toothpaste containing 1,450 ppm F (Tripla Ação® – Colgate-Palmolive Comercial Ltda; São Paulo, São Paulo, Brazil). The volunteers were also warned not to use any other fluoride product. Toothbrushing with fluoride toothpaste was performed by the volunteers one hour prior to the insertion of the intraoral appliance. During 6 days, at the same time (8-10 AM), two volunteers used the intraoral appliances containing one block for each studied group during 2 hours to allow the formation of the AEP. The volunteers did not eat or drink in this period.

In vitro phase – treatment and acid exposure

Immediately after the formation of the AEP, the enamel blocks were removed from the intraoral appliance and fixed in an acrylic disk to receive the treatment. The commercial brands of the vegetable oils used in this study were: GP5 and GP100: palm oil (Kidendê - Dendê Light Indústria de Produtos Alimentícios Ltda; Valença, Bahia, Brazil); GC5 and GC100: extra virgin coconut oil (COPRA - COPRA Indústria Alimentícia Ltda; Maceió, Alagoas, Brazil); GSa5 and GSa100: extra virgin safflower oil (Giroil - Giroil Agroindústria Ltda, Entre-Ijuís, Rio Grande do Sul, Brazil); GSu5 and GSu100: extra virgin sunflower oil (Pazze - Pazze Indústria de Alimentos Ltda; Panambi, Rio Grande do Sul, Brazil); GO5 and GO100: extra virgin olive oil (Cirio - Sandeleh Alimentos; Paranaguá, Paraná, Brazil).

The 5% emulsions of the vegetable oils in deionized water were daily prepared prior to the application by using a high-speed household mixer, resulting in a finely dispersed emulsion[27].

The treatment consisted in applying five drops on each enamel block of the respective group during 30 seconds. Then, the enamel block was separately immersed in 17.6 ml of artificial saliva (0.33 g KH_2PO_4, 0.34 g Na_2HPO_4, 1.27 g KCl, 0.16 g NaSCN, 0.58 g NaCl, 0.17 g $CaCl_2$, 0.16 g NH_4Cl, 0.2 g urea, 0.03 g glucose, 0.002 g ascorbic acid, pH 7[13] – without mucin) for 2 minutes, under constant agitation, to simulate a natural rinsing process occurring in the oral cavity. After that, the enamel blocks were subjected to short-term erosive demineralization by immersing each block in 17.6 ml of 0.5% citric acid pH 2.4, under constant agitation, for 30 seconds. Then, the blocks were washed with deionized water for 30 seconds.

Surface hardness assessment

After the short-term acid exposure, the surface hardness determination was performed again (SH*f*) with five indentations performed at 100 µm distance in relation to initial indentations (Micromet® 5114 hardness tester; Buehler Ltd., Lake Bluff, Illinois, United States). The percentage of hardness loss was calculated [((SH*i* - SH*f*) / (SH*i*)) × 100] for each block and averaged to represent the studied groups.

Statistical analysis

Statistical analysis was performed with SigmaPlot™ version 12.3 (Systat Software GmbH; Erkrath, Germany). Assumptions of equality of variances and normal distribution of errors were verified by Bartlett's and Shapiro–Wilk tests, respectively. Once the assumptions were satisfied, two-way ANOVA (for the factors "volunteers" on two levels and "treatments" on 12 levels) and Tukey's *post hoc* test were applied. The significance level was set at 5%.

Results

We found no statistically significant difference for the factor "volunteers" ($p=0.911$), and no interaction between "volunteers" and "treatments" ($p=0.634$), but we found significant difference between "treatments" ($p=0.002$). The percentage of hardness loss of the evaluated groups is shown in Table 1. Only GP100 (pure palm oil) was statistically different from the control group, showing the lowest surface hardness loss ($p<0.05$). All the other studied oils presented surface hardness loss similar to the control groups ($p>0.05$). We found no significant difference between GP100 and GSu100 (pure sunflower oil) ($p>0.05$).

Table 1- Mean and standard deviation (±SD) of the percentage of hardness loss of enamel treated with the studied vegetable oils

Groups	SHi	SHf	% Hardness Loss
GP5 – 5% palm oil	329.92 (±35.81)	253.90 (±45.15)	23.24 (±8.436)[a]
GP100 – pure palm oil	337.58 (±28.41)	310.80 (±34.58)	7.89 (±7.5)[b]
GC5 – 5% coconut oil	334.16 (±26.88)	250.92 (±37.49)	24.65 (±11.50)[a]
GC100 – pure coconut oil	336.24 (±30.50)	240.26 (±48.45)	28.47 (±13.37)[a]
GSa5 – 5% safflower oil	341.19 (±31.87)	241.90 (±37.23)	28.74 (±11.53)[a]
GSa100 – pure safflower oil	337.85 (±29.91)	248.89 (±43.01)	26.56 (±9.51)[a]
GSu5 – 5% sunflower oil	335.76 (±26.05)	259.23 (±50.07)	22.92 (±12.94)[a]
GSu100 – pure sunflower oil	338.51 (±27.63)	265.02 (±55.67)	21.78 (±14.83)[ab]
GO5 – 5% olive oil	337.27(±32.29)	252.81 (±57.71)	25.35 (±12.76)[a]
GO100 – pure olive oil	337.36 (±29.49)	249.86 (±46.95)	25.91(±12.51)[a]
CON− – deionized water (negative control)	335.45 (±29.71)	240.90 (±38.02)	28.09 (±9.95)[a]
CON+ – fluoride mouthrinse (positive control)	337.19 (±28.92)	256.44 (±22.04)	23.74 (±6.15)[a]

In the fourth column, different letters show significant differences between the groups (two-way ANOVA and Tukey's test, $p<0.05$)

Discussion

Lipids consist of about 25% of the pellicle's dry weight[19], and lipophilic components are able to modulate the pellicle composition and ultrastructure[12]. Therefore, authors have suggested that lipid-rich pellicles might be more resistant to acids[23], thus reducing erosion[12].

This study aimed to elucidate the protective effect of different vegetable oils, applied after *in situ* formation of AEP, against initial erosion demineralization. Two of the vegetable oils assessed here (coconut oil and palm oil) have not been previously studied regarding their anti-erosive potential, requiring an initial *in vitro* evaluation of their effect. However, *in vitro* studies are not able to accurately replicate the biological characteristics of the oral cavity, such as the presence of human saliva and the formation of AEP, which could interfere with the development of erosion. Some limitations occur in protocols using human saliva *in vitro*, such as fast extraoral protein decomposition[18]. Natural and *in vitro* formed AEPs also present differences in their characteristics, e.g., the natural pellicle is more hydrophobic than the one formed *in vitro*[24]. Therefore, a combined *in situ*/*in vitro* protocol was chosen in this study to allow the physiological formation of the AEP *in situ* prior to the *in vitro* application of the vegetable oils and short-term exposure to citric acid. A single short-term erosive challenge was performed to more precisely evaluate the protective ability of the AEP modified by the studied oils against initial enamel erosion. Studies have shown that the hardness test is an adequate method to evaluate the initial softening of enamel surface[11,20].

Vegetable oils are extracted from oil plants and seeds and are commonly used in foods, cosmetics, and medical products[12]. Studies have shown that, when hard tooth tissue is exposed to vegetable oils, the superficial layer of the AEP gets rich in lipids micelles[4,7]. However, the protective effect of these oils against caries and erosion demineralization processes remains unclear, because only a few evidence-based researches are available in the literature[2,8,27].

Only one study evaluated the direct effect of 5 and 50% olive oil emulsions compared to distilled water and fluoride solution on dentin caries demineralization[2]. Dentin samples were subjected to three cycles *per* day of 5 min treatment application followed by 8 hours of immersion in demineralization solution (pH 5.0) during 9 days. The olive oil emulsions showed a decrease in mineral loss in comparison with deionized water, and the fluoride solution presented better results[2].

Our results showed that pure palm oil was capable to protect enamel against initial erosion demineralization, but the same was not found for the 5% palm oil emulsion. No protective effect was observed to 5% emulsion and pure form of coconut, safflower, sunflower, and olive oils. The effect of olive oil-based emulsions (100%, 2%, and 2% associated with mouthwash) on enamel subjected to 10 erosive cycles was previously assessed using profilometry analysis[27]. Each cycle consisted of samples treatment with oil-based emulsions during 5 min, immersion in artificial saliva for 30 min, demineralization in 1% citric

acid for 3 min, and remineralization in artificial saliva for 60 min. The researchers found that 2% emulsion or 2% olive-oil containing mouthrinse offered protection against tooth erosion, but in a lesser extent than the positive control (250 ppm acidic fluoride solution), and that pure olive oil did not offer protection[27]. The authors hypothesized that the adhesion properties of olive oil might increase when applied as emulsion[27]. In contrast, our study did not find any protective potential for 5% and pure olive oil. The different results between the studies might be explained by the different methodologies used. We adopted a short-term erosive demineralization model and the abovementioned study used an erosive cycling model.

The effect of safflower oil on the protective properties of the AEP formed *in situ* against the exposure to hydrochloric acid for 2 min was previously described[8]. Enamel mineral loss was determined by measurement of calcium and phosphate release, and the ultrastructure of the AEP was evaluated by transmission electron microscopy. The results showed that the surface of AEP was rich in lipids, but no substantial lipids integration was found in the pellicle's basal layer. The *in situ* AEP modified by safflower oil rinsing was more susceptible to acid degradation than the *in situ* physiological AEP[8]. In contrast, our study showed that safflower oil (GSa5 and GSa100) did not present a negative impact on enamel demineralization when compared to the control groups.

To our knowledge, palm oil has never been investigated for the prevention of erosion. Palm oil is the second largest produced and consumed vegetable oil in the world, due to its high productivity, low production cost, and rich nutritional content[21]. It is rich in tocotrienols that have presented health benefits[1]. Tocotrienols allow an efficient penetration into tissues that have saturated fatty layers and exhibit antioxidant protection of cellular membranes against oxidative damage[1]. The antioxidant property has been attributed to its ability of distribution in lipid layers of the cell membrane[1].

In previous studies, the outer layer of the AEP was modified by the increase of lipids micelles[4,7]. However, the outer layers of the AEP are supposed to be easily removed after an erosive challenge, in contrast to the basal layer that might not be affected[8]. In this study, despite the ultrastructure of the AEP not being analyzed, it is hypothesized that palm oil might have modified the basal layer of the acquired pellicle, increasing its protective potential. The tocotrienols contained in the palm oil might have allowed its penetration and distribution into the basal layers of the acquired pellicle, increasing its protective role[1]. We also highlight that we found no differences between the protective effect of pure palm oil and pure sunflower oil. This result can be explained by the tocotrienols content of the sunflower oil, but in a lesser extent when compared to palm oil[1], which enables a borderline behavior between palm oil and the other tested oils (coconut, safflower, and olive oil).

In this study, the commercial mouthwash solution – Elmex® Erosion Protection Dental Rinse/EP, 125 ppm F^- as AmF, 375 ppm F^- as NaF, 800 ppm Sn^{2+} as $SnCl_2$; pH 4.5 (CP GABA GmbH; Hamburg, Germany) – did not present any effect on the inhibition of initial enamel erosion, being similar to deionized water (negative control). The role of fluoride on tooth erosion is not fully evidenced[14]. Differing from our result, some studies have shown a preventive capacity of fluoride solution containing AmF/NaF (500 ppm F) and $SnCl_2$ (800 ppm Sn) against enamel erosion[5,13].

Although palm oil has shown superior protective capacity against tooth erosion, its effect to prevent the enamel erosive wear needs to be further evaluated under long-term erosive challenges. Moreover, the effect of palm oil on the physical properties, quality, and composition of the acquired pellicle should also be assessed.

Conclusion

Considering our study design, palm oil seems to be a promising alternative for the prevention of initial enamel erosion.

Acknowledgements

The authors would like to gratefully acknowledge the volunteers who participated in this study and FAPESP – São Paulo Research Foundation for the financial support (process numbers 2014/00102-0, 2015/26520-6, and 2015/21552-7).

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2.2 Article 2[2] – Effect of palm oil alone or associated to stannous solution on enamel erosive-abrasive wear: a randomized in situ/ex vivo study.

ABSTRACT

Objective: Palm oil has demonstrated preventive potential against initial erosive demineralization *in vitro*. This *in situ* study evaluated the effect of palm oil alone or associated with stannous-containing (Sn) solution on preventing enamel loss from an erosive/abrasive challenge. **Design:** This single-blind, randomized, crossover *in situ/ex vivo* study was developed in four phases (one per group of five days) with sixteen volunteers. Enamel blocks (n=256) were allocated to groups according to the treatment: Palm oil; Palm oil plus Sn solution; Sn solution – positive control; and Deionized water - negative control. Half of the enamel blocks of each group was subjected to erosion and the other half to erosion+abrasion. The daily *ex vivo* protocol consisted of four citric acid immersions (2 minutes). Before the first and third acid exposure, the blocks were treated with the test solutions (1 drop/block) for 1 minute followed by acid immersion and abrasive challenge (toothpaste was applied on all blocks and half were brushed for 15 seconds/block). Enamel loss was quantified profilometrically and data were analyzed by two-way ANOVA and Tukey's test ($p<0.05$). **Results:** A significant difference was found for type of treatment ($p<0.001$); wear condition ($p=0.38$) and the treatment x condition interaction ($p=0.33$) were non-significant. Palm oil associated or not to Sn solution significantly reduced enamel wear in comparison with the negative control but did not differ from the positive control ($p>0.05$). **Conclusions:** Palm oil was able to prevent enamel loss under erosive and erosive+abrasive challenges in a similar extent to stannous-containing commercial solution.

Keywords: palm oil; tooth erosion; dental enamel; prevention

INTRODUCTION

Erosive tooth wear is a result of a chemical-mechanical process involving intrinsic or extrinsic acids of non-bacterial origin and mechanical forces, such as attrition and abrasion (Carvalho, & Lussi, 2015; Shellis, & Addy, 2014). The preventive

[2] Effect of palm oil alone or associated to stannous solution on enamel erosive-abrasive wear: a randomized in situ/ex vivo study. Archieves of Oral Biology. (Submitted)

management of this condition should start as early as possible and focus on intercepting causal factors such as dietary habits, gastroesophageal reflux, general medical conditions, oral hygiene habits, and functional problems (Carvalho, & Lussi, 2015; Buzalaf, Magalhães, & Rios, 2018). As the identification and reversal of the causal factors is difficult, strategies to enhance biological protective factors can help prevent erosive tooth wear.

Among the biological factors, saliva has a significant role in the physiological control of dental erosion (Hara, Lussi, & Zero, 2006; Magalhães, Wiegand, Rios, Honório, Buzalaf, 2009; Buzalaf, Hannas, & Kato, 2012). One key factor in the salivary mechanism for erosion protection is the formation of the acquired enamel pellicle (AEP) (Hannig, & Joiner, 2006; Siqueira, Custodio, & McDonald 2012), which protects the enamel surface from direct contact with acids, reducing and retarding enamel demineralization (Amaechi, Higham, Edgar, & Milosevic, 1999; Hannig, & Joiner, 2006; Hannig, & Hannig, 2014; Vukosavljevic, Custodio, Buzalaf, Hara, & Siqueira, 2014). However, the AEP cannot protect against severe erosive challenges (Hara et al., 2006). As about 25% of the acquired pellicle dry weight are lipids (Hannig, Hess, Hoth-Hannig, & De Vrese 2003), lipophilic components can modulate the composition and ultrastructure of the pellicle (Kensche, Reich, Kümmerer, Hannig, & Hannig, 2013). Thus, it has been speculated that lipid-enriched pellicles are more resistant to acids and might hinder dental erosion (Hannig et al., 2012; Kensche, Reich, Kümmerer, Hannig, & Hannig, 2013).

Vegetable oils are a natural, edible, low-cost, and worldwide accessible product and thus, have been widely studied in dental research (Buchalla, Attin, Roth, & Hellwig 2003; Hannig et al., 2012; Kensche, Reich, Kümmerer, Hannig, & Hannig, 2013). However, only a few studies have evaluated the effect of vegetable oils on erosion prevention (Buchalla, Attin, Roth, & Hellwig 2003; Wiegand, Gutsche, & Attin, 2007; Hannig et al., 2012; Ionta et al., 2017). Olive oil emulsions associated or not to a fluoride mouthwash have demonstrated a preventive potential against erosion, however, this effect occurred in lower extent compared with fluoride compounds (Buchalla, Attin, Roth, & Hellwig 2003; Wiegand, Gutsche, & Attin, 2007). Rinsing with safflower oil resulted in a lipid-rich AEP, but the pellicle was more susceptible to acid degradation than the *in situ* physiological AEP (Hannig et al., 2012). A previous study demonstrated great preventive potential for palm oil applied over the AEP against initial

erosive demineralization when compared to four other vegetable oils, deionized water, and stannous-containing solution (Ionta et al., 2017). However, that study was conducted *in vitro* and a single short-term erosive challenge was performed (Ionta et al., 2017). An *in situ* erosive cycling that exposes the enamel samples to biological factors and to mechanical forces from tongue and abrasion could better represent the process that occurs on erosive enamel wear *in vivo*.

Thus, the aim of this *in situ-ex vivo* study was to evaluate the effect of palm oil alone or associated with a Sn-containing commercial solution on enamel loss prevention after 5 days of erosive and abrasive cycling regimen.

MATERIALS AND METHODS

Experimental Design

This *in situ-ex vivo* experiment followed a single-center, single-blind (researcher), randomized crossover design with four phases (according to treatments) of five days, with a wash-out period of 1 week, and 16 volunteers. A total of 256 bovine enamel blocks were randomized by stratified allocation considering their initial surface hardness were allocated among the volunteers and the treatments: Palm oil (Kidendê, Dendê Light Indústria de Produtos Alimentícios Ltda, Valença, Bahia, Brazil); positive control - Sn-containing commercial solution [Elmex® Erosion Protection Dental Rinse / EP, 500 ppm F^- (125 ppm F^- from amine fluoride, 375 ppm F^- from sodium fluoride) and 800 ppm Sn^{2+} (from stannous chloride); pH 4.5, CP GABA GmbH, Hamburg, Germany]; Palm oil after the application of Sn-solution*;* and negative control – deionized water*.* Half of the enamel blocks of each group was subjected to erosion and the other half to erosion+abrasion. In each phase, the volunteers wore a palatal removable appliance containing 4 blocks (2 for erosion and 2 for erosion+abrasion) eight hours per day for five days (Santos et al., 2018). The daily *ex vivo* protocol consisted of four immersions of the appliance in citric acid (0.5%, pH 2.4) for 2 minutes. Before the first and third daily acid challenges, the blocks were treated with the test solution (1 drop per block) for 1 minute. After acid immersion, a toothpaste slurry was applied on the four blocks (1 drop per block); two of the blocks were immediately abraded with power toothbrushing for 15 seconds per block, totaling approximately 35 seconds. Enamel loss was quantified profilometrically. Fig. 1 shows a flowchart of the

study according to Consolidated Standards of Reporting Trials (CONSORT) (Moher, et al., 2010; Schulz, Altman, & Moher, 2010).

Enamel Blocks Preparation

Enamel blocks (4 x 4 x 3 mm) were prepared from the labial surfaces of the bovine incisors crowns. The teeth were cut using a cutting device (ISOMET Low Speed Saw, Buehler Ltd., Lake Bluff, IL, USA), with two double-sided diamond discs (XL 12205, "High concentration", 102 x 0,3 x 12,7 mm^3 Extec Corp., Enfield, CT, USA/ Ref: 12205), which were separated by a 4-mm thickness spacer.

The blocks' surfaces were ground flat with water-cooled silicon carbide discs (320, 600, and 1200 grade papers; Buehler, Lake Bluff, IL, USA), and polished with felt paper wet by 1-μm diamond spray (Buehler, Ltd., Lake Bluff, IL, USA). The blocks were cleaned using an ultrasonic device (T7 Thornton, Unique Ltda., São Paulo, SP, Brazil) with deionized water for 2 min between each silicon carbide discs change and for 10 min at the end of the polishing procedures. Two hundred and fifty-six blocks were selected and randomized among 8 study groups and 16 volunteers according to their surface hardness by stratified allocation (Hardness tester from Buehler, Lake Bluff, IL, USA; five indentations in each block using Knoop diamond with 25 g for 10 seconds; enamel mean surface hardness of 362.34 ± 33 KHN). Before the *in situ* phase, the blocks were sterilized by ethylene oxide gas exposure.

Baseline Profilometric Analysis

The surface of the enamel blocks were marked with a scalpel blade (Embramac, Itapira, SP, Brazil) for definition of the reference areas of 1.0 mm (at the border) and test area of 2.0 mm (at the center). Subsequently, five baseline surface profiles were obtained from the blocks using a profilometer (MarSurf GD 25, Göttingen, Germany) and a contour software (MarSurf XCR20). To standardize the position, blocks were fixed to a special holder and the location was recorded allowing their exact repositioning after the *in situ* phase. The surface profiles were obtained at the following distances of relative position of the block on the y-axis: 0.5, 0.75, 1.0, 1.25 and 1.5 μm. After the initial profilometry, the reference areas (at the border of enamel surface) were covered with nail varnish (Maybelline Colorama, Cosbra Cosmetics Ltda, São Paulo, SP, Brazil), to be used as a reference for intact enamel during measurements of enamel loss.

Volunteers

This study was conducted according to the guidelines of good clinical practice and conformed to the Declaration of Helsinki for experiments involving humans. Ethical approval was granted by the local institutional ethics committee (CAAE nº 45792415.8.0000.5417). Informed written consent form was obtained from each volunteer at the beginning of the study, prior to confirmation of their eligibility. The participants had the right to withdraw from the study at any time and for any reason without prejudice.

For this study, a sample size of fourteen volunteers (dentistry graduation and post-graduation students at local institution) was required considering a minimally detectable difference of 2.5 µm of enamel wear and 1.65 µm of standard deviation obtained in a pilot study with 2 volunteers, 5% α error and 20% β error. An extra two volunteers were added to account for possible drop out. Sixteen healthy adult volunteers (dental students and graduate students of the local institution, aged 18–35 years) participated in the study. The inclusion criteria were residing in the same fluoridated area (0.70 mg F/L), stimulated salivary flow rate >1 mL/min, non-stimulated salivary flow rate >0.25 mL/min, and adequate oral health with no caries, erosion lesions, or gingivitis/periodontitis. The exclusion criteria were systemic illness, gastroesophageal reflux, pregnant or breastfeeding women, current orthodontic intervention, professional application of highly concentrated fluoride compounds in the last 2 months, smokers, and users of acidic medications.

In situ Phase

Before starting the *in situ* phase, volunteers were properly trained on how to perform the protocol. They received the study material and written instructions. Seven days prior to and during the experimental phase, the volunteers brushed their teeth after meals with a standard toothbrush (Curaprox 5460 ultra-soft, Curaden Swiss, Switzerland) and fluoride toothpaste (Triple Action, 1.450 ppm F, Colgate, Brazil) without the intraoral appliance. In addition, volunteers were informed not to use any other fluoride product.

Four acrylic resin removal palatal appliances were made for each volunteer to be used in each phase of the study. The appliances had four 6 × 4 × 3 mm cavities, two on each side, for block fixation. The blocks were fixed with wax (Asfer, Asfer Indústria Química, São Caetano do Sul, SP, Brazil) and adjusted to the level of the appliance surface. In one side of the appliance the enamel blocks were fixed with green

wax (erosion group) and on the other side with blue wax (erosion+abrasion group) to identify the blocks that would to be brushed after the application of toothpaste slurry. Volunteers were allocated to study phases (according to the treatment) following simple randomization procedures (computerized random numbers). The data analysts were blinded to the allocation of the volunteers and enamel blocks.

The four test treatments were conducted in different phases; erosion and erosion+abrasion challenges were conducted in the same phase. Treatments were done twice daily by the volunteer before the erosive and abrasive challenges. For the Palm oil group one drop of the oil was applied on each enamel block for 1 minute; for the positive control, one drop of Sn solution was applied over each enamel block for 1 minute; for the Palm oil + Sn solution, a drop of Sn solution, and right after, a drop of oil were applied and maintained for 1 minute; and in the negative control, one drop of deionized water was applied over each block for 1 minute.

The night before the beginning of each phase, the volunteers wore the intraoral appliances overnight (11.00 until 7.00) to allow AEP formation. During the experimental phases, the appliances were worn during 5 working days from 7.45 until 18.00 (times could vary +/- 15 min), with a washout period of 1 week. During the experiment, the oral hygiene was performed normally after meals, without the intraoral devices. The participants were instructed not to eat while wearing the appliances but they were allowed to drink water. The appliance was stored in a plastic box wrapped in wet gauze (tap water: 0.70 mg F/L) during meals (12.00 until 13.45) and from 18.00 until 7.45 of the next day to prevent dehydration of the enamel blocks.

The experimental protocol consisted of: 7:45 AM - appliance worn for pellicle rehydration; 8.00 AM - treatment + erosive/abrasive challenges; 10.00 AM - erosive challenge; 12.00 AM - lunch time (stored in wet gauze); 1.45 PM - appliance worn for pellicle formation; 2.00 PM - treatment + erosive/abrasive challenge; 4.00 PM - erosive challenge; 6.00 PM - appliance removal (Fig. 2).

The erosive and abrasive challenges were conducted *ex vivo* to protect the volunteers from potential teeth damage. The erosive challenges (at 10.00 AM and 4.00 PM) consisted of immersing the intraoral appliance in 80 mL of citric acid (0.5%; pH 2.4) for 2 minutes (Ganss et al., 2012) and washing in tap water. This protocol was chosen to simulate a patient at high risk of developing erosive wear. For treatment + erosive/abrasive challenge (at 8.00 AM and 2.00 PM), the enamel blocks were treated

according to the group under study followed by the erosive challenge. Then, the appliance was washed and a toothpaste slurry (ratio=1:3, dentifrice Triple Action, 1.450 ppm F, Colgate, Brazil) / deionized water) was applied (1 drop/block) over the four blocks. The two blocks fixed with blue wax were immediately brushed with an electric brush (Oral-B Precision Clean, Brazil) during 15 seconds each, and then the appliance was washed in tap water. The time of slurry action was approximately 35 to 40 seconds. The blocks were exposed to the oral environment (*in situ* exposure) for 2 h, between the challenges. During this period, the volunteers were asked not to remove the appliance.

Final Profilometric Analysis

After the *in situ* phase, the nail varnish was removed and the profilometric analysis was performed at the same sites of the baseline measurements. Since the enamel samples could be precisely repositioned on the profilometer table, the respective baseline and final profiles could be matched. The graphs were superimposed and analyzed using a specific software program (MarSurf XCR 20, Göttingen, Germany). The vertical difference (average depth of the surface) between the baseline and final surface profiles were analyzed to quantify the enamel loss, reported as the mean of five graphs. The accuracy of the method was around 0.5 µm and the standard deviation of repeated analyses of a given sample was 0.4 µm.

Statistical Analysis

Statistical analysis was performed with SigmaPlot version 12.3 (2011 Systat Software, Germany), following the recommendations for dental research (Hannigan, & Lynch, 2013). The assumptions of equality of variances and normal distribution of errors were checked using Shapiro–Wilk test. Since the assumptions were met, two-way ANOVA and Tukey's *post hoc* test were applied. The significance level was set at 5%.

RESULTS

Of the sixteen volunteers, two were excluded for not following the *in situ* protocol properly. Table 1 contains the average and the standard deviation for enamel loss of each experimental group.

A significant difference was observed among treatments ($p<0.001$). The erosion and erosion+abrasion challenges did not yield different enamel loss ($p=0.38$). There

was no interaction between type of challenge and treatment (p=0.33). Palm oil, palm oil associated to Sn solution, and Sn solution significantly reduced enamel wear in comparison to the negative control (deionized water) with no difference among them.

DISCUSSION

The AEP protects the hard dental tissue against acids by modulating the demineralization/remineralization process; however, it does not prevent the acid diffusion completely (Hannig, & Balz, 1999; Hannig, & Hannig, 2014). The basal layer of the AEP is formed by the initial adsorption of single peptides and proteins from saliva and other oral fluids, characterizing a densely packed zone (Carvalho, Baumann, & Lussi, 2016; Hannig, & Hannig, 2014; Siqueira, Custodio, & McDonald 2012). Then, a loosely arranged structure consisting of protein-protein interactions and the adsorption of proteins and macromolecules called external layer covers the basal layer (Hannig, & Balz, 1999). When an erosive challenge occurs, the AEP is gradually dissolved from the external to the basal layer (Amaechi, Higham, & Edgar, 1999; Hannig, Hess, Hoth-Hannig, & De Vrese 2003; Hannig et al., 2007). Due to its densely arranged formation, the basal layer is more difficult to dissolve than the external layer; however, it is permeable to ions (Hannig et al., 2007). Thus, one possible strategy to improve the protective effect of AEP consists on modifying the composition of the external layer in an attempt to obtain a less permeable membrane during acid dissolution, or of the basal layer, hampering the ion exchange during an erosive process.

Previous studies have shown that the outer layer of the AEP absorbs lipids micelles when teeth are exposed to vegetable oils (Das, Adhikary, & Bhattacharyya, 1976; Hannig et al., 2012). Nonetheless, the protective effect of these oils against acid challenge remains unclear because only a few evidence-based studies are available in the literature (Buchalla, Attin, Roth, & Hellwig, 2003; Hannig et al., 2012; Ionta et al., 2017, Wiegand, Gutsche, & Attin, 2007). In one of these studies, the application of palm oil prior to a single short-term acid exposure resulted in 7.8% of enamel surface hardness loss compared to 23.7% of the fluoride solution (positive control) and 28.1% of the deionized water (negative control) (Ionta et al., 2017). Because of the promising effect of the palm oil against initial erosion, the present study aimed to elucidate its protective effect when applied alone or in association with a Sn-containing solution on enamel loss after an erosive and erosive/abrasive cycling challenge. The profilometry

was adopted as the response variable because it is the gold standard for *in vitro* analysis of erosive wear thickness (height) (Paepegaey et al., 2013). The results showed that both treatments protected against erosive and abrasive dental wear, resulting in less enamel wear than deionized water. However, the effect was not significantly different compared to the Sn-containing mouthwash solution.

Palm oil is naturally rich in carotenes, giving the oil its dark red color (Sundram, & Sambanthamurthi, 2003), and in tocotrienols, which have demonstrated many health benefits such as antioxidant properties (Sundram, & Sambanthamurthi, 2003). The antioxidant effect has been attributed to the ability of tocotrienols to diffuse in the lipid layer of the cell membrane (Ahsan, Ahad, & Siddiqui, 2015; Kamat, Sarma, Devasagayam, Nesaretnam, & Basiron, 1997). Thus, it is hypothesized that palm oil might have been distributed into the lipids of the AEP basal layer retarding its disintegration (Ionta et al., 2017). One study has analyzed *in situ* the effect of a vegetable oil (safflower oil) on the ultrastructure of the AEP against the exposure to hydrochloric acid (Hannig et al., 2012). By transmission electron microscopy, the authors showed a lipid-rich AEP surface, but with no substantial integration of the lipids to the basal layer. In addition, the AEP modified by safflower oil rinsing was more susceptible to acid degradation than the *in situ* physiological AEP. Considering the preventive effect demonstrated in the present study, the alteration of the AEP ultrastructure by palm oil should be assessed in the future, to better understand its mechanism of action.

The stannous-containing mouthwash adopted in the present study as positive control contains stannous chloride, amine fluoride, and sodium fluoride, and has demonstrated preventive effects on dental erosion studies (Algarni et al., 2015, Esteves-Oliveira et al., 2015; Oliveira, Scaramucci, Nogueira, Simões, & Sobral, 2015). The mechanism of action of stannous ions, such as SnF_2 or $SnCl_2$, is based on the formation of an acid-resistant layer on the surface of teeth (Ellingsen 1986; Ganss, Schlueter, Hardt, Schattenberg & Klimek, 2008). This might result in the incorporation of ions into the enamel due its low pH (Schlueter, Klimek & Ganss, 2009) and high reactivity to hydroxyapatite (Yu et al., 2010), increasing the substrate resistance. In addition, it was shown that the association of $SnCl_2$ and fluoride ions can modify quantitatively and qualitatively the AEP (Algarni et al., 2015) leading to the formation of more stable and acid-resistant precipitates on the tooth surface (Schlueter et al.,

2009). In the present study, to test the combined treatment, the stannous-containing solution was applied before the palm oil, considering the stannous ion incorporation into the enamel and the hydrophobic properties of the oil, which could negatively interfere with the preventive effect of the stannous solution. However, this association was not able to improve significantly the protective potential in comparison with palm oil or stannous-containing mouthwash alone. Perhaps the stannous-containing mouthwash led to the formation of a barrier on the surface of the enamel, impairing the penetration of the palm oil in the basal layer of the AEP.

Sound enamel is more resistant against mechanical forces than eroded enamel, which is highly unstable and potentially more susceptible to abrasion (Eisenburger, Shellis, & Addy, 2003). The outer zone of softened erosive lesion consists of thinned fragile crystals separated by large spaces (Eisenburger, Shellis, & Addy, 2004), which can be easily removed by relatively slight abrasive or frictional forces, causing further enamel substance loss (Eisenburger, Shellis, & Addy, 2003; Shellis, Featherstone, & Lussi, 2014). However, the inner part of the lesion is less demineralized, mainly limited to the prism boundaries (Eisenburger, Shellis, & Addy, 2004) and requires a greater mechanical impact (e.g. excessive brushing) to be removed (Wiegand, Wegehaupt, Werner, & Attin, 2007; Wiegand, Köwing, Attin, 2007; Wiegand, & Schlueter, 2014). In the present study, a slight increase in enamel wear was observed in groups subjected to erosion and abrasion by toothbrushing; around 4% for palm oil, 4% for palm oil+Sn solution, 21% for Sn solution and 19% for deionized water compared to the groups subjected to erosion only. Although there was no statistical difference between erosion and erosion+abrasion challenges, it seems that the lubricating properties of the oil may play a role in protecting against abrasive toothbrushing, which can indicate a new possibility of research for palm oil-containing dentifrices. A possible explanation for the statistical similarity between erosion and erosion+abrasion is the enamel exposure to tongue friction, since it was previously demonstrated that the tongue produces an abrasive effect on enamel softened by erosion (Gregg, Mace, West, & Add, 2004). Therefore, in the present protocol, the specimens were exposed to friction by the tongue, interfering with the results. To avoid the mechanical impacts of the tongue, it is recommended that the enamel blocks are protected by an orthodontic wire (Ionta et al., 2017; Jordão et al., 2017; Mendonça et al., 2017); however, in the pilot study, the

placement of a wire caused a higher oil retention and greater difficulty to remove the oil.

Before the brushing procedures, a toothpaste slurry was applied on all enamel blocks to simulate the daily oral hygiene. In a clinical situation, dentifrices are always used for toothbrushing. A question that could be raised is that the fluoride dentifrice could mask the effect of the tested treatments, because in a previous study, fluoride dentifrice showed a protective effect on eroded enamel subjected to brushing abrasion (Magalhães, Rios, Delbem, Buzalaf, & Machado, 2007). However, the negative control group (deionized water) of our study, which received the dentifrice slurry, presented worse results than the other groups, showing that the effect of the fluoride dentifrice was neglectable.

Ideally, the abrasive procedure should be performed with the appliance in the mouth of the volunteers. However, in a pilot study, the volunteers were unable to distinguish the blocks that should be brushed (erosion+abrasion groups) from those that should not (erosion groups). Thus, the abrasive procedure was conducted *ex vivo*. In addition, the *ex vivo* erosive challenge prevented injuries to the participants teeth's. Due to the absence of saliva, the extraoral procedures can be considered a limitation of the study. It is also important to bear in mind that in *in situ* protocols, there are variations among individuals biological factors, resulting in different values of wear among volunteers, this could be the reason for the high standard deviations found in the study.

Based on these findings, palm oil and commercial fluoridated mouthwash - alone or combined - presented a preventive potential against erosion and erosion/abrasion when compared to deionized water. Thus, further studies should be carried out to eventually create a palm oil-containing emulsion that could be used as a mouthwash, by improving gustatory properties - as flavor and smell - and preventive effect. Moreover, the effect of the palm oil on the physical properties, quality, and composition of the acquired pellicle should be assessed.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge Dionísio EJ for the technical and laboratory assistance, the volunteers who participated in this study, and the financial support from Fundação de Amparo a Pesquisa de São Paulo [FAPESP grant numbers

2014/00102-0, 2015/26520-6, 2015/21552-7, 2016/05347-7] and Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq grant numbers 431264/2016-3, 310679/2015-0].

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: There is no conflict of interest by any of the authors.

Funding: The work was supported by Fundação de Amparo a Pesquisa de São Paulo [FAPESP grant numbers 2014/00102-0, 2015/26520-6, 2015/21552-7, 2016/05347-7] and Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq grant numbers 431264/2016-3, 310679/2015-0].

Ethical approval: All procedures performed in human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (protocol number 45792415.8.0000.5417).

Informed consent: Informed consent was obtained from all individual participants included in the study.

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Table 1. Mean (µm) and the standard deviation (sd) of enamel loss of the studied groups.

Treatment	**Palm Oil**		**Palm oil + Sn solution**		**Sn solution**		**Deionized Water**	
Wear Condition	**erosion**	**erosion plus abrasion**	**erosion**	**erosion plus abrasion**	**erosion**	**erosion plus abrasion**	**erosion**	**erosion plus abrasion**
Enamel Loss	4.78 (±1.68) a	4.99 (±1.73) a	4.26 (±2.12) a	4.45 (±1.92) a	3.93 (±1.67) a	4.75 (±2.30) a	5.97 (±2.05) b	7.12 (±2.64) b

*Distinct letters indicate significant differences among groups (two-way ANOVA and Tukey's test, $p < 0.05$).

Fig. 1. Flowchart of the experiment according to Consolidated Standards of Reporting Trials (CONSORT).

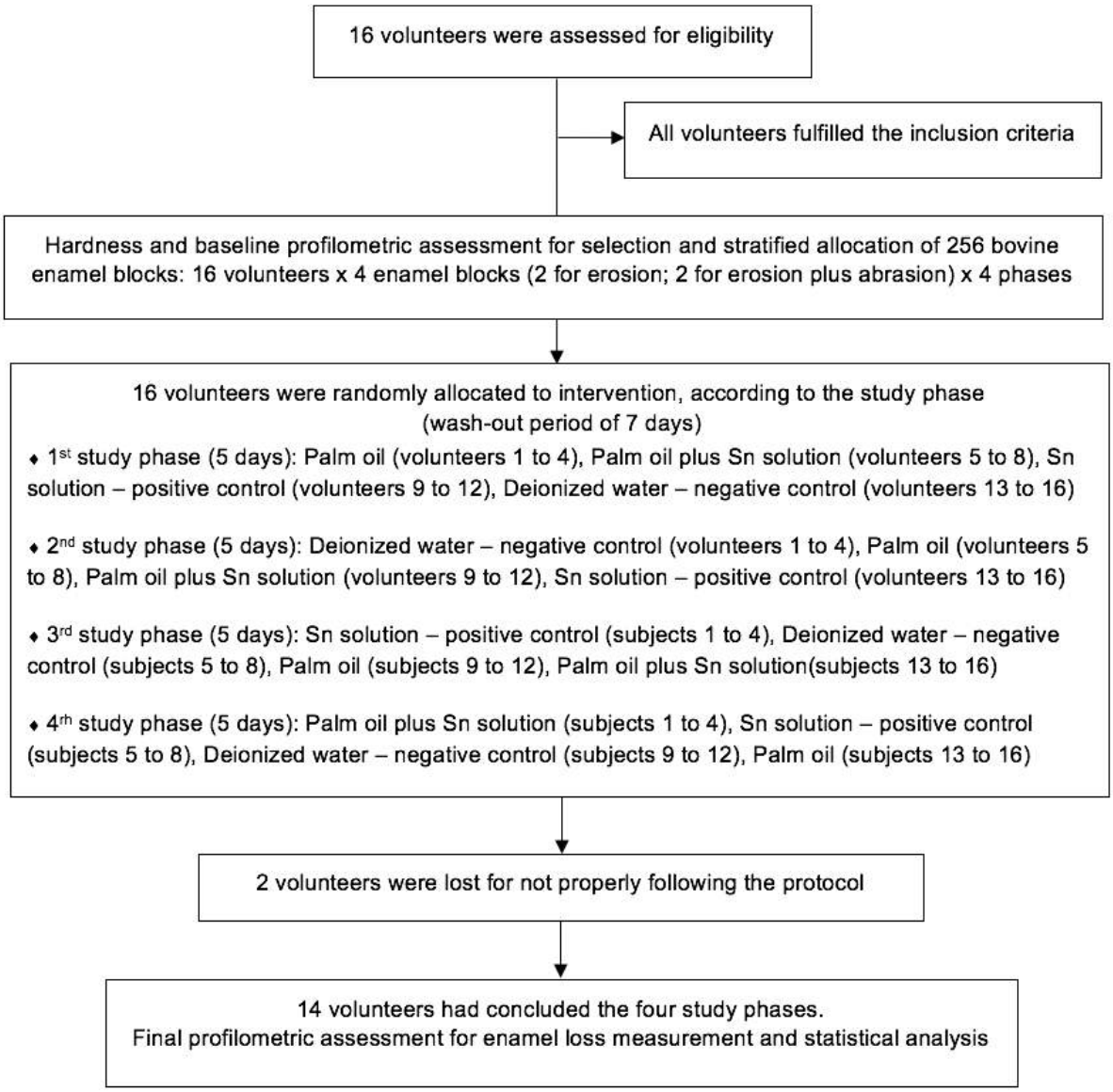
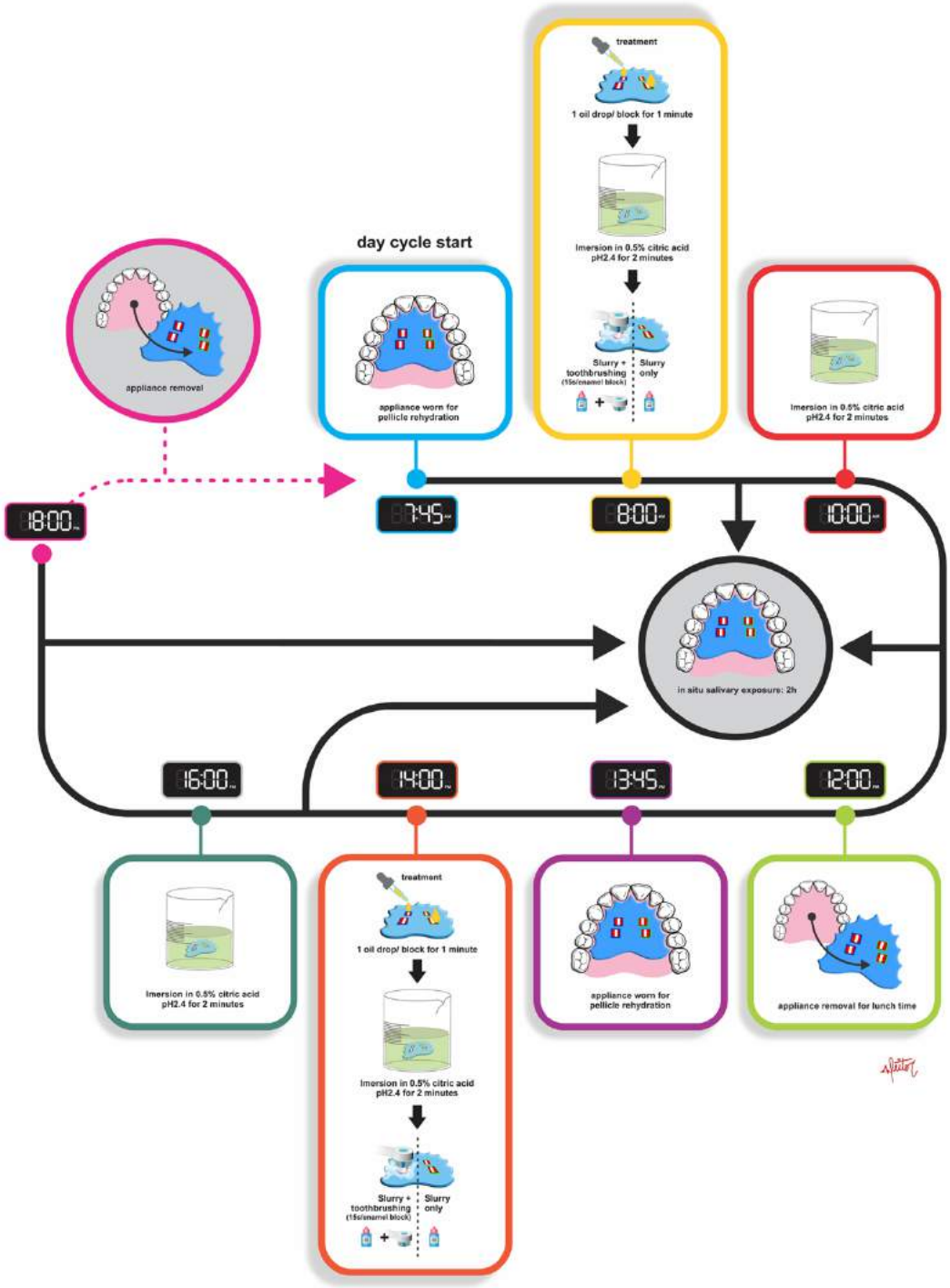


Fig. 2. In situ / Ex vivo daily protocol repeated for 5 days in each phase. Treatment for Palm oil, Deionized water and Stannous solution were done by application of 1 drop for 1 minute; for the Palm oil + Stannous solution, a drop of Sn solution, and right after, a drop of oil was applied and maintained for 1 minute.



2.3. Article 3[3] - Effect of aspartame against erosive enamel wear by intrinsic and extrinsic acids: an in situ/ex vivo study

ABSTRACT

Previous study suggests that the presence of aspartame as well as a slight increase in pH are responsible for the lower erosive potential of light cola drink. The anti-erosive potential of aspartame, regardless of its association with colas, has not been evaluated. The aim of this study was to evaluate whether aspartame regular mouthwash prior to erosive challenges with citric or hydrochloric acids would be able to prevent erosive enamel wear. This randomized, single blind study was conducted with 3 crossover phases of 5 days. Polished bovine enamel blocks (n=252) were randomly divided among 6 groups/ 3 phases/ 21 volunteers. The groups under study were: aspartame solution (0.024% of aspartame in deionized water - experimental groups), deionized water (negative-control) and stannous-containing solution (Elmex® Erosion Protection Dental Rinse; positive-control); half of the enamel blocks was subjected to erosion on citric acid and the other half in hydrochloric acid. Four times per day the volunteers rinsed the intraoral appliance with the respective solutions *in situ* prior to extraoral immersion of half of the appliance in 0.05M citric acid and the other half in 0.01M hydrochloric acid for 120 seconds. The response variable was enamel loss by profilometry. Data were analyzed by ANOVA and Tukey's test ($p<0.05$). No difference on enamel loss was found between aspartame solution and deionized water. Stannous-solution resulted in less enamel loss compared to deionized water. Hydrochloric acid resulted in higher enamel loss than citric acid. In this model, aspartame was not able to prevent erosive enamel wear against citric or hydrochloric acids.

INTRODUCTION

Dental erosion can be defined as the softening of dental surface due to the exposure of dental hard tissue to extrinsic or intrinsic acids of non-bacterial origin[1]. Whether the acid exposure is prolonged or under the incidence of mechanical forces,

[3] Protective effect of aspartame against erosive enamel wear by intrinsic and extrinsic acids: an in situ/ex vivo study. International Dental Journal. (Submitted)

this vulnerable softened layer can be removed causing tooth hard substance loss[2]. At routine examination, erosive wear becomes clinically visible in later stages, when the appearance and shape of the teeth are compromised. In patients with gastroesophageal reflux or with eating disorders such as bulimia, stomach acids can be constantly present in the oral cavity leading to severe erosion with extensive loss of enamel and dentine[3]. The risk of development of dental erosion is also related to nutritional habits, such as high consumption of soft drinks and specific diets with increased consumption of acid fruits (e.g. vegan, vegetarian and raw food diets)[4]. The high prevalence of this condition[5-8]and the impact on patient's quality of life[9] is a cause of concern to dental clinicians and researchers.

Due to the high consumption of soft drinks, researches have been conducted regarding the erosive effects of different versions of soft drinks[10,11]. Previous studies had found that light cola, which contains aspartame and a slight increase in the pH, presents less erosive potential compared to its traditional version[12,13]. It was speculated that the aspartame could be responsible for the lower erosive potential of light cola[12,13]. However, the anti-erosive potential of aspartame, regardless of its association with cola drinks, has not been previously evaluated. Thus, the aim of this *in situ*/*ex vivo* study was to evaluate whether aspartame regular mouthwash prior to immersion in citric or hydrochloric acids would be able to prevent erosive enamel wear when compared to deionized water and stannous-containing solution. The null hypothesis tested was that there is no difference among the tested solutions against both kinds of acid challenges.

METHODS

Study Design

This experiment followed a single-blind (for research), placebo-controlled, randomized, 3 *in situ/ex vivo* crossover phases in which the independent variables were type of treatment (in three experimental levels) and type of challenge (in two experimental levels). A washout period of 7 days was established between each phase. Polished bovine enamel blocks (n=252) were random divided among groups (n=6) and volunteers (n=21). The groups under study were: 0.024% of aspartame in deionized water (experimental groups), deionized water (negative-control) and stannous (Sn)- containing solution (Elmex® Erosion Protection Dental Rinse; positive-

control); half of the enamel blocks was subjected to erosion in citric acid and the other half in hydrochloric acid (both Sigma Alderich - Merck, Darmstadt, Germany). The dependent variable was enamel loss (in µm) quantified profilometrically.

Enamel Blocks Preparation

Enamel blocks (4 x 4 x 3 mm) were prepared from the labial surfaces of bovine incisors crowns. The teeth were cut using a cut device (ISOMET Low Speed Saw, Buehler Ltd., Lake Bluff, IL, USA), with two double-sided diamond discs (XL 12205, “High concentration”, 102 x 0,3 x 12,7 mm3 Extec Corp., Enfield, CT, USA/ Ref: 12205), which were separated by a 4- mm thickness spacer.

The blocks’ surfaces were ground flat with water-cooled silicon carbide discs (320, 600, and 1200 grade papers; Buehler, Lake Bluff, IL, USA), and polished with felt paper wet by 1 µm diamond spray (Buehler, Ltd., Lake Bluff, IL, USA). The blocks were cleaned using an ultrasonic device (T7 Thornton, Unique Ltda., São Paulo, SP, Brazil) with deionized water for 2 min between each silicon carbide discs change and for 10 min at the end of the polishing procedures. Two hundred and fifty-two blocks were selected and randomized among 8 studied groups and the 16 volunteers according to their surface hardness (Hardness tester from Buehler, Lake Bluff, IL, USA; five indentations in each block using Knoop diamond with 25 g for 10 seconds; enamel mean surface hardness of 341.20 ± 33 KPa/mm^2). Before the *in situ* phase, the blocks were sterilized by ethylene oxide gas exposure.

Baseline Profilometric Analysis

The surface of the enamel blocks were marked with a scalpel blade (Embramac, Itapira, SP, Brazil) for definition of the reference areas of 1.0 mm (at the border) and test area of 2.0 mm (at the center). Subsequently, five baseline surface profiles were obtained from the blocks using a profilometer (MarSurf GD 25, Göttingen, Germany) and a contour software (MarSurf XCR20). Blocks were fixed to a special holder to standardize the position and the location was recorded allowing their exact repositioning after the *in situ* phase. The surface profiles (3.0 mm in length) were obtained at the following distances of relative position of the block on the y-axis: 0.5, 0.75, 1.0, 1.25 and 1.5 µm. After the initial profilometry, the reference areas (at the border of enamel surface) were covered with nail varnish (Maybelline Colorama,

Brazil), to be used as a reference for intact enamel during measurements of enamel loss.

Volunteers and In situ/Ex vivo phase

This study was conducted according to the guidelines of good clinical practice and conformed to the Declaration of Helsinki. The experiments were undertaken with the understanding and written consent of each subject and according to the mentioned principles. The participants had the right to withdraw from the study at any time and for any reason without prejudice. The study has been independently reviewed and approved the local institutional ethics committee (CAAE nº 48729115.0.0000.5417).

For this study, a sample of nineteen healthy volunteers was required considering a minimally detectable difference of 0.35 µm of enamel loss and 0.34 µm of standard deviation obtained in a pilot study with 3 volunteers, an 5% α error, and 20% β error was adopted. Two extra volunteers were added to account for possible drop out. Twenty-one healthy adult volunteers (dental students and graduate students of the local institution, aged 18–35 years) participated in the study. The inclusion criteria were residing in the same fluoridated area (0.70 mg F/L), stimulated salivary flow rate >1 mL/min, non-stimulated salivary flow rate >0.25 mL/min, and adequate oral health with no caries, erosion lesions and gingivitis/periodontitis. The exclusion criteria were systemic illness, gastroesophageal reflux, pregnant or breastfeeding women, current orthodontic intervention, professional application of highly concentrated fluoride compounds in the last 2 months, smokers, and users of acidic medications.

Before starting the *in situ* phase, volunteers were properly trained on how to perform the protocol. They received the study material and written instructions. Seven days prior to and during the experimental phase, the volunteers brushed their teeth after meals with a standard toothbrush (Curaprox 5460 ultra-soft, Curaden Swiss, Switzerland) and fluoride toothpaste (Triple Action, 1.450 ppm F, Colgate, Brazil) without the intraoral appliance. In addition, volunteers were informed not to use any other fluoride product.

Three acrylic resin removal palatal appliances were made for each volunteer to be used in each phase of the study. The appliances had four 6 × 4 × 3

mm cavities, two on each side, for block fixation. The blocks were fixed with wax (Asfer, Asfer Indústria Química, São Caetano do Sul, SP, Brazil) and adjusted to the level of the appliance surface. In one side of the appliance the enamel blocks were fixed with green wax (citric acid) and on the other side with blue wax (hydrochloric acid) to identify which blocks should be immersed in citric or hydrochloric acids. Volunteers were allocated to study phases (according to the treatment: aspartame, stannous-containing solution, or deionized water) following simple randomization procedures (computerized random numbers). The data analyst was kept blinded to the allocation of the volunteers and enamel blocks.

Each type of treatment was conducted in different study phases; erosion with citric and hydrochloric acids were performed in the same phase. A washout period of 7 days was established between the phases. The night before the beginning of each phase, the volunteers wore the intraoral appliances overnight (11.00 until 7.00) to allow formation of acquired enamel pellicle. Thereafter, volunteers wore the appliance for 5 working days from 7.45 to 18.00 (times could vary +/- 30 min). The experimental procedure was as follows (all times +/- 15 min): at 7.45 the appliance was worn for pellicle formation; at 8.00 and 10.00 the treatment + erosive challenge was performed; at 12.00 the appliance was removed and stored in wet gauze during lunch; at 13.45 the appliance was worn for pellicle formation; at 14.00 and 16.00 the treatment + erosive challenge; and at 18.00 the appliance was removed and stored in wet gauze during night sleep. The participants were instructed not to eat while wearing the appliances; drinking water was allowed.

Four times per day the volunteers rinsed the respective solutions (10 mL for 60 seconds) with the intraoral appliance *in situ.* Immediately after the mouthwash, the volunteers placed the appliance into an ethylene vinyl acetate dipositive that allows the immersion of half of the appliance in acid, while the other half floated without contacting the acid (Figure 1)[14.] Therefore, half of the appliance were immersed in 80 mL of 0.03M citric acid (natural pH 2.4)[15,16] and the other half in 80 mL of 0.01M hydrochloric acid (adjusted with NaOH pH 2.3, to approximate that found in stomach acid[17,18]), both for 120 seconds without agitation and then washed in tap-water.

A 2-hour period was planned for *in situ* exposure between challenges, during which the volunteers were asked not to remove the appliance[19-21].

Final Profilometric Analysis

After the *in situ* phase, the nail varnish was removed and the profilometric analysis was performed at the same sites of the baseline measurements. Since the enamel samples could be precisely repositioned on the profilometer table, the respective baseline and final profiles could be matched. The graphs were superimposed and analyzed using a specific software program (MarSurf XCR 20, Göttingen, Germany). The vertical difference (average depth of the surface) between the baseline and final surface profiles were analyzed to quantify the enamel loss, reported as the mean of five graphs.

Statistical Analysis

Statistical analysis was performed with SigmaPlot version 12.3 (2011 Systat Software, Germany), following the recommendations for dental research[22]. The assumptions of equality of variances and normal distribution of errors were checked using Shapiro–Wilk test. Since the assumptions were satisfied, two-way ANOVA and Tukey's *post hoc* test were applied. The significance level was set at 5%.

RESULTS

Of the twenty-one volunteers, one was excluded for not following the *in situ* protocol properly. Table 1 contains the average and the standard deviation for enamel loss of each experimental group.

A significant difference was observed among treatments (p=0.000) and between type of acid (p=0.001). There was no interaction between type of acid and treatment (p=0.14). Hydrochloric acid promoted higher enamel loss than citric acid. Aspartame did not differ from negative control (deionized water) on enamel loss. Sn-containing solution significantly reduced enamel wear in comparison to the negative control and aspartame.

DISCUSSION

The prevention of erosive tooth wear can be challenging since it is a multifactorial condition in which patient-related and nutritional factors are involved[5]. Specific anti-erosive products could be useful when is not possible to intercept and

reverse these causal factors. The present study evaluated the effect of an aspartame mouthwash against enamel erosive challenges by citric and hydrochloric acids, however it was not able to prevent enamel loss. Therefore, the null hypothesis that there was no difference in the preventive effect for the tested solutions was partly rejected, due to a reduction in enamel loss by the stannous-solution.

Since 1981 to the present days, the Food and Drug Administration Agency (FDA) approves the use of aspartame and considers it safe as a general-purpose sweetener in food[23], being used as a non-nutritive sweetener in low-calorie products. Aspartame is a synthetic dipeptide formed by the reaction of L-aspartic acid with L-phenylalanine methyl ester, which can be released in contact with saliva[24]. Amino acids, such as phenylalanine, may present potent antioxidant effect and may protect against inflammatory diseases [25]. Phenylalanine has showed to attenuate ulcerogenic parameters and to improve the gastric hemorrhagic erosion in acid-irrigated stomachs of rats [26]. Light cola – which contains aspartame – had demonstrated less erosive potential than regular cola[12,13]. In the present study, the experimental aspartame mouthwash prior to acid challenge showed a reduction of only 16% of enamel loss in comparison with deionized water, without statistical differences. The stability of aspartame depends on pH and temperature; in acquous solution, when the pH ranges between 4 and 5, the degradation is minimal[27]. However, under extremely acidic conditions with pH below 4, aspartame can be degraded in phenylalanine, aspartic acid and methanol. Phenylalanine is considered to be an aromatic amino acid, having a carboxylic group and the amino group[28]. Light cola soft drink has pH around 3 and thus, the protons of the acid might be possibly captured by the carboxylic (COO^-) or aminic (NH_2) groups of phenylalanine, diminishing deleterious action on the tooth enamel[12,13]. In the present study, the pH of aspartame solution was 5.6, which may have impaired its anti-erosive effect. Different results might be obtained in solutions with lower pH (<4.0) which should be tested in future studies. Previous studies have found that at high concentrations, aspartic acid can bind to the N-methyl-D-aspartate receptor, causing an influx of calcium ions into brain cells[29]. Also, the hydrogen peroxide and peroxyl radicals formed during the ingestion of aspartame seem to be involved in enhanced calcium mobilization in brain cells[28,30]. Perhaps, in higher concentration, it could have some effect on tooth remineralization by enhancing

calcium mobilization to acquired enamel pellicle, this hypothesis needs to be further evaluated.

The Sn-containing solution was the only treatment able to prevent enamel loss from citric and hydrochloric acids. This mouthwash solution contains 800 ppm of Sn^{2+} from stannous chloride, 125 ppm of F^- from amine fluoride, and 375 ppm of F^- from sodium fluoride and was adopted as positive control since it has previously showed preventive effect against tooth erosion[31-33]. The stannous ions can form an acid-resistant layer on the surface of teeth increasing the substrate resistance[34,35]. When it is associated with fluoride ions, the acquired enamel pellicle can be quantitatively and qualitatively modified[31] forming a more stable and acid-resistant precipitates[36]. Considering the pH of the Sn-solution (4.5), it could be interest to investigate in future studies its association with aspartame in order to improve the anti-erosive effect.

Considering the endogenous sources of acids, the mainly compound of gastric contend is hydrochloric acid[37], being frequently adopted in experimental studies simulating this condition[3,38-43]. Citric acid is one of the main acidic compound of exogenous sources, such as foodstuffs and drinks[11]; thus, it is frequently adopted in laboratorial studies to represent extrinsic causes of dental erosion[38,44-46]. In the present study, citric and hydrochloric acid were adopted with the pH of 2.4 and 2.3 respectively, since many acidic drinks range at this level[11] and gastric juice has been simulated in this range of pH[38-43]. It should be considered that in this experimental setup, acidic solutions of similar pH but different concentrations (0.01M for hydrochloric acid and 0.03M for citric acid) were used for characterization of erosive effect of intrinsic and extrinsic acids. Different acidic concentrations can impair the comparison with data, however previous study has found that concentration of the acids and the amount of titratable acid are of minor importance to determine the acidic erosive capacity when compared to pH and type of acid[44]. Each erosive challenge was set in 120 seconds as in previous studies[14,40-42]; it was demonstrated that the pH on tooth surfaces stays low during this time after exposition to an acid until the salivary clearance[47,48]. Hydrochloric acid is considered as a strong monovalent acid (pKa = −6.3 – HCl) and can dissolves and removes the mineral surface more quickly than weaker polyvalent acids, such as citric acid (pKa1 = 3.15; pKa2 = 4.77; pKa3 = 6.40)[45]. Thus, tooth erosion from intrinsic acids seems to be more severe than from extrinsic acids[3,49]. In the present study,

hydrochloric acid promoted higher enamel loss than citric acid for all tested solution, which confirm this hypothesis.

Researches about preventive measures for dental erosion should be ideally conducted *in vivo*[50,51]. However due to the difficult to yield very precise intra-oral measurement of the progression of erosive wear in vivo[50,51]; the present study was conducted *in situ*/ *ex viv*o. The intraoral appliance was exposed to the oral environment and the mouthwash was performed intra-orally, but the acid challenge was conducted extra-orally to avoid damage to the volunteer's teeth. The enamel loss was measured by profilometry since it is considered a suitable method to measure the erosive wear thickness *in vitro*[51-54] The present study adopted bovine teeth, which is widely used in enamel erosion studies and it is easier to obtain in large quantities with a more uniform structure[54-57] compared to human teeth. However, the presence of some chemical and structural differences should be taken into account when extrapolling the results to the clinical practice[54-57].

Based on the results of this study, the aspartame solution was not able to prevent the enamel loss when used before an erosive challenge independently of type of acid. Sn-solution promoted less enamel loss than deionized water and aspartame solution. Hydrochloric acid promoted higher enamel loss than citric acid. Further studies should be conducted evaluating the anti-erosive effect of an aspartame solution with lower pH.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the volunteers who participated in this study and the financial support from Fundação de Amparo a Pesquisa de São Paulo [FAPESP grant numbers 2015/26520-6, 2015/21552-7, 2013/26684-3] and Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq grant numbers 431264/2016-3, 310679/2015-0]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. There is no conflict of interest by any of the authors.

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TABLE

Table 1. Means and standard deviations values of enamel loss (μm) of the studied groups.

TREATMENT	CITRIC ACID [A]	HYDROCLORIC ACID [B]
Aspartame [a]	2.60 (± 1.28)	3.59 (± 1.84)
Deionized Water [a]	3.09 (± 0.99)	4.27 (± 1.49)
Sn-containing Solution [b]	0.87 (± 0.76)	0.99 (± 0.96)

*Different lower-case and uppercase letters show significant differences among the treatment and between the types of acid, respectively (two-way ANOVA and Tukey's test, $p < 0.05$).

FIGURE LEGEND

Figure 1. Intraoral appliance placed into an ethylene vinyl acetate dipositive allowing the immersion of half of the appliance in acid, while the other half floated without contacting the acid.

FIGURE 1

2.4. Article 4[4] - Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear? an in situ/ ex vivo study.

ABSTRACT

Objectives: The aim of this study was to investigate the effect of a dentifrice that contains calcium silicate, sodium phosphate, and fluoride on erosive-abrasive enamel wear. Material and Methods: This randomized, single-blind *in situ/ex vivo* study was conducted with 4 crossover phases of 5 days (1 group tested per phase). Bovine enamel blocks (n=256) were allocated to 16 volunteers and 8 groups. The groups under study were: test dentifrice, with calcium silicate, sodium phosphate, and 1450 ppm sodium monofluorophosphate; tin dentifrice, with 3500 ppm stannous chloride, 700 ppm amine fluoride, and 700 ppm sodium fluoride; conventional dentifrice, with 1450 ppm sodium monofluorophosphate; and control (deionized water). Half of the enamel blocks was subjected to erosion and the other half to erosion plus abrasion. The daily extraoral protocol consisted in 4 citric acid exposures (2 minutes) and two applications of dentifrice slurry on all blocks for 30 seconds; after, half of the blocks were brushed for 15 seconds. The response variable was enamel loss. Data were analyzed by two-way ANOVA and Fisher's test ($p<0.05$). Results: For erosion, the test dentifrice promoted less enamel loss than water (4.7±3.1 and 5.8±2.5μm, respectively, $p<0.05$), and did not differ from tin (4.8±2.5μm) and conventional (4.8±1.4μm) dentifrices ($p>0.05$). However, the test dentifrice (7.7±3.8μm) promoted higher wear after erosive plus abrasive procedures than tin (5.4±1.5 μm) and conventional (6.2±1.7μm, $p<0.05$) dentifrices, and did not differ from water (6.9 ±2.0μm). Conclusions: The test dentifrice reduced enamel loss against acid challenge but had no effect against acid and brushing challenge.

Clinical Relevance: Little is known regarding the preventive effect of dentifrices indicated for dental erosion. The tested anti-erosive dentifrice was unable to protect enamel when erosion was associated to toothbrushing abrasion.

Keywords: Tooth Erosion, Tooth Abrasion, Calcium Silicate, Stannous Fluoride

[4] Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear? an in situ/ ex vivo study. Clinical Oral Investigations. (Submitted)

INTRODUCTION

Erosive tooth wear (ETW) is defined as a chemical-mechanical process mainly induced by intrinsic or extrinsic acids of non-bacterial origin [1, 2]. The prevalence of ETW has been increasing in the last decade, affecting about half of the population under 18 years of age [3, 4]. Specific measures are required to prevent the initiation and progression of erosive lesions [5]; however, since patient-related and nutritional factors are involved in the development of erosive tooth wear, prevention can be complicated [6]. Therefore, when causal factors are not identified and reversed, the use of specific protective products could be beneficial.

Routinely used once or twice a day by most individuals, dentifrices could work as suitable delivery systems for dental erosion preventive therapies [6-10]. However, data about the role of fluoride dentifrices on ETW process are controversial [11], because on one hand, fluoride or any other anti-erosive agent can be delivered through it, but on the other hand, the mechanical impact of toothbrushing commonly enhances the loss of demineralized tissue [2]. In addition, toothbrushing abrasive forces can disrupt or partially remove the mineral precipitation promoted by fluoride action [12, 13]. Since eroded enamel is more susceptible to toothbrush abrasion than sound enamel [14], dentifrices for tooth wear prevention must have a mechanism to compensate the impact of brushing forces.

The use of oral hygiene products containing calcium is one of the strategies to increase the availability and retention of fluoride in the oral cavity [15]. *In vitro* studies have demonstrated that specific types of calcium silicate – such as Ca_3SiO_5 – can induce the formation of hydroxyapatite in saliva, which is deposited on the acid-eroded enamel forming a remineralizing layer, suggesting a protective effect against erosive demineralization [16,17]. When considering the pH cycling model of erosive demineralization, the efficacy of calcium silicate seems to be similar to that of 1000 ppm sodium fluoride solution; better results were obtained when Ca_3SiO_5 was associated with fluoride [18].

Dentifrices containing calcium silicate, sodium phosphate, and fluoride have shown greater rehardening capacity of eroded enamel than conventional fluoride dentifrices [6-8]. However, the evidence about the mechanism and the protective capacity of calcium silicate is limited since only a few studies were reported, which were conducted under highly artificial conditions. In addition, those studies assessed

the effect of calcium silicate applied as slurry, without assessing the impact of toothbrushing. Only one study evaluated erosion and toothbrushing *in situ*, but the efficacy of the dentifrice was measured by hardness recovery, not by the decrease of enamel loss. Thus, although the evidence is limited, these dentifrices are available in the market for patients with or at risk of erosive tooth wear. Therefore, the objective of this study was to evaluate the effect of a dentifrice that contains calcium silicate, sodium phosphate, and fluoride on the prevention of ETW, simulating a clinical condition by associating erosion to abrasion using an *in situ* protocol.

SUBJECTS, MATERIAL AND METHODS

Experimental Design

This experiment followed a single-blind, placebo-controlled, randomized, crossover *in situ/ex vivo* design in which the independent variables were type of treatment (in four experimental levels) and type of challenge (in two experimental levels). The 256 bovine enamel blocks were randomly allocated into groups (n = 8) and volunteers (n = 16). The groups under study were: test dentifrice with calcium silicate, sodium phosphate, and 1450 ppm sodium monofluorophosphate (REGENERATE™ ENAMEL SCIENCE, France); tin dentifrice with 3500 ppm stannous chloride, 700 ppm amine fluoride, and 700 ppm sodium fluoride (elmex® EROSION PROTECTION, Austria); conventional dentifrice with 1450 ppm sodium monofluorophosphate (Colgate Tripla Ação®, Brazil); and control (deionized water). Half of the enamel blocks was subjected to erosion and the other half to erosion and abrasion. The protocol comprised four phases and the groups were randomly divided according to the type of dentifrice, since erosion and erosion+abrasion conditions were evaluated in the same phase. A wash-out period of 7 days was established between each phase. The dependent variable was enamel loss (in μm) quantified profilometrically.

Enamel Blocks Preparation

About 300 enamel blocks (4×4×3 mm) were prepared from the labial surfaces of bovine incisor crowns. The blocks were cut using an ISOMET low speed cutting machine (Buehler Ltd., Lake Bluff, IL, USA) with two diamond disks (XL 12205, “High concentration”, 102×0.3×12.7 mm^3 Extec Corp., Enfield, CT, USA/ Ref: 12205)

separated by a 4 mm spacer. The blocks' surfaces were ground flat with water-cooled silicon carbide discs (320, 600, and 1200 grade papers; Buehler, Lake Bluff, IL, USA), and polished with felt paper wet by diamond spray (1 μm; Buehler, Ltd., Lake Bluff, IL, USA). The blocks were cleaned using an ultrasonic device (T7 Thornton, Unique Ltda., São Paulo, SP, Brazil) with deionized water for 2 min between each silicon carbide disc change and for 10 min at the end of the polishing procedures. Two hundred and fifty-six blocks were allocated by stratified randomization according to their surface hardness (Hardness tester from Buehler, Lake Bluff, IL, USA; five indentations in each block using Knoop diamond with 25 g for 10 seconds; enamel mean surface hardness of 342 ± 26 KPa/mm^2) among the 8 groups and the 16 volunteers. Before the *in situ* phase, the blocks were sterilized with ethylene oxide gas.

Initial Profilometric Analysis

Enamel blocks were marked with a scalpel blade (Embramac, Itapira, SP, Brazil) to indicate two reference areas with 1.0 mm (at the border) and a test area with 2.0 mm (at the center). Subsequently, five baseline surface profiles were obtained from the blocks using a profilometer (MarSurf GD 25, Göttingen, Germany) and a contour software (MarSurf XCR20). To standardize their position, samples were fixed to a special holder and their locations were recorded allowing their exact replacement after the *in situ* phase. The surface profiles were obtained at the following distances of relative position of the block on the y-axis: 0.5, 0.75, 1.0, 1.25, and 1.5 μm. After the initial profilometry, the reference areas (at the border of enamel surface) were covered with nail varnish (Maybelline Colorama, Cosbra Cosmetics Ltda, São Paulo, SP, Brazil), serving as a guide for measurement of enamel loss.

Volunteers and In Situ /Ex Vivo Phase

This study was conducted according to the guidelines of good clinical practice and conformed to the Declaration of Helsinki. Ethical approval was granted by the local institutional ethics committee (CAAE nº 48753115.0.0000.5417). Informed written consent form was signed by volunteers at the beginning of the study, prior to eligibility confirmation. The participants had the right to withdraw from the study at any time and for any reason without prejudice.

For this study, sixteen healthy volunteers (aged 20–30 years) were recruited, considering a minimally detectable difference of 2 µm of enamel loss and 1.55 µm of standard deviation obtained in a pilot study with 2 volunteers, 5% α error, and 20% β error. The inclusion criteria were: reside in the same fluoridated area (0.70 mg F/L), stimulated physiological salivary flow rate >1 mL/min and non-stimulated physiological salivary flow rate >0.25 mL/min, and adequate oral health with no caries, erosion lesions, or gingivitis/periodontitis. The exclusion criteria included systemic illness, presence of reflux conditions, pregnant or breastfeeding women, current orthodontic intervention, use of fluoride compounds in the last 2 months, smokers, and users of acidic medications.

The volunteers were properly trained prior to the experimental *in situ/ex vivo* phase and received all the necessary material for the study and written instructions. Seven days prior to and during the experimental period, the volunteers brushed their teeth with a standardized toothbrush (Curaprox 5460 ultra-soft, Curaden Swiss, Switzerland) and a non-fluoride commercial dentifrice (Cocoricó baby care, Bifufo, Itupeva, SP, Brazil) and they were asked not to use any fluoride product.

Four palatal appliances for each volunteer were made of acrylic resin on plaster models of the upper arches. Each appliance had four cavities (6×4×3 mm) for block fixation, two on the right side and two on the left. The enamel blocks were fixed with green wax (Asfer, Asfer Indústria Química, São Caetano do Sul, SP, Brazil) in one side of the appliance (erosion groups) and with blue wax (erosion+abrasion groups) on the other side, leveled to the resin surface of the appliance.

The volunteers wore the appliances for 4 crossover phases, each one with a different dentifrice or water. A randomization schedule was followed to indicate the dentifrice order for the study phases. Each phase consisted in wearing the appliance for 5 working days from 7.45 to 18.00 (times could vary +/- 30 min), with a washout period of 7 days. The participants were instructed not to eat while wearing the appliances; drinking water was allowed. They were asked to wrap the appliance in wet gauze and store it in a plastic box during meals (12.00 to 13.45) and from 18.00 to 7.45 to prevent dehydration of the enamel. One night before the beginning of each phase, the appliances were worn during sleep (11.00 to 7.00) to allow the formation of acquired enamel pellicle. On the following 5 days, the experimental procedure was as follows (all times +/- 15 min): at 7.45 the appliance was worn for pellicle formation; at

8.00 the erosive+abrasive challenge was performed; at 10.00, erosive challenge; at 12.00 the appliance was stored in wet gauze during lunch; at 13.45 the appliance was worn for pellicle formation; at 14.00, erosive+abrasive challenge; at 16.00, erosive challenge; at 18.00, appliance was stored in wet gauze during night sleep. The erosive and abrasive challenges were conducted extraorally.

For the erosive challenge, the appliances were immersed in 80 mL of citric acid (0.5%; pH 2.4) for 2 minutes without agitation and then washed in tap-water. For the erosive+abrasive challenge, the appliance underwent the acidic exposure and then a dentifrice slurry (1:3 ratio of dentifrice and deionized water) was applied (2 drops/block) on the 4 enamel blocks for 30 seconds. Two of the blocks were then immediately brushed with a power toothbrush (Oral-B Precision Clean, Queimados, RJ, Brazil) during 15 seconds per block and the appliances were washed with tap water. Thus, the time of action of the dentifrice was 1 minute per application. A 2-hour period was planned for *in situ* exposure between challenges, during which the volunteers were asked not to remove the appliance.

The dentifrice slurry of the calcium silicate dentifrice was prepared twice a day (at the time of the erosion-abrasion challenge) by the researcher to avoid reactions (hydroxyapatite formation) before the contact with enamel. The dentifrice composition and the pH of slurries are described in Table 1.

Profilometric Analysis

After the *in situ* phase, the nail varnish was removed from the reference areas and the profilometric analysis was performed at the same sites of the initial measurements. Baseline and final profiles were perfectly matched, since the enamel samples could be precisely repositioned in the profilometer wells. The enamel wear was quantitatively determined using a specific software (MarSurf XCR 20) by calculating the vertical difference (average depth of the surface) between baseline and final surface profiles.

Statistical Analysis

Statistical analysis was performed with SigmaPlot, version 12.3 (2011 Systat Software, Germany), following the recommendations for dental research [19]. The assumptions of equality of variances and normal distribution of errors were satisfied;

therefore, two-way ANOVA and Fisher's exact test were applied. The significance level was set at 5%.

RESULTS

All volunteers completed the study and followed the protocol. Table 2 shows the results for enamel loss of each experimental group. A significant difference was found for type of treatment ($p=0.002$), and type of challenge ($p=0.000$); their interaction was also significant ($p=0.006$). The dentifrice with calcium silicate, sodium phosphate salts, and fluoride (test dentifrice) presented significantly less enamel loss compared to the negative control (deionized water) after the erosive challenge. The other two dentifrices (tin and conventional) did not differ from the test dentifrice and water.

The use of stannous fluoride (tin) dentifrice resulted in less enamel loss after the erosive+abrasive challenge compared to the control and calcium silicate/phosphate fluoride dentifrice ($p<0.05$), which resulted in the highest wear among the tested toothpastes and did not differ from the control.

In respect to wear condition, the calcium silicate/phosphate fluoride and monofluorophosphate dentifrices resulted in higher enamel loss after the erosion-abrasion procedure compared to erosion alone ($p<0.05$). For stannous dentifrice and deionized water, enamel loss did not differ between erosion-abrasion and erosion alone ($p>0.05$).

DISCUSSION

Patients with erosive tooth wear are advised to use any fluoride toothpaste; however, dentifrices specifically indicated for the prevention and treatment of dental erosion are available in the market. The effect of toothpastes containing calcium and phosphate on dental erosion have been studied due to their potential to provide a topical source of calcium and form of hydroxyapatite [7]. A new dentifrice containing calcium silicate, sodium phosphate, and fluoride has now been available with the proposal to regenerate the enamel lost due to erosive acids. In the present study, the effectiveness of this product was evaluated and compared to two other commercial dentifrices, one specifically indicated for dental erosion and the other for general use. The purpose was to compare commercial brands and not the effect of specific compounds of each dentifrice because we used commercial products with different

overall compositions. We aimed to determine the best preventive product and better orient patients.

The results showed that the calcium silicate/phosphate and fluoride dentifrice presented limited protective action against erosion, reducing enamel loss by approximately 18% after erosive challenges, which was better than deionized water but did not differ from the other two dentifrices analyzed. In previous studies, the use of calcium silicate dentifrice caused a higher gain in surface hardness and greater formation of hydroxyapatite on the tooth surface [6-8, 18]. Although these variables were not analyzed in our study, we believe that clinically the amount of hydroxyapatite formed on enamel might be similar or higher than the amount that might be lost by acid exposure, as a mechanism to guarantee enamel soundness. Therefore, it is hypothesized that the erosive challenge applied was able to demineralize the hydroxyapatite both from the calcium silicate dentifrice and the enamel, leading to tissue loss.

The partially demineralized eroded enamel is extremely fragile and susceptible to physical forces of the oral environment, such as soft tissue and tongue friction and toothbrushing abrasion [2, 20]. *In situ* studies demonstrated that the thickness of enamel removed by erosion associated to toothbrushing abrasion is greater than the losses resulting from erosion or abrasion alone [20, 21]. Most dentifrices provide a degree of protection against erosive demineralization when applied as slurries, but this cannot predict their effect against combined erosive and brushing challenges [22]. In the present study, when erosion and abrasion episodes were associated, the calcium silicate dentifrice resulted in higher enamel loss than the other evaluated dentifrices, showing similar effect to deionized water. This result was not expected, but the hydroxyapatite layer that supposedly is formed by the dentifrice could be easily removed by abrasive forces, hindering a preventive effect. In addition, this limited effect could have been a result of the short 60-second contact between enamel blocks and the dentifrice slurry. Previous studies showed that 1 to 3 minutes application of calcium silicate dentifrice slurry resulted in better erosion protection [6, 8]. Therefore, longer exposures of the enamel to the dentifrice might lead to a more resistant layer of hydroxyapatite.

The stannous-containing dentifrice [1400 ppm F (700 amine fluoride and 700 sodium fluoride) and 3500 ppm Sn^{2+} as stannous chloride], was used as the positive

control. The addition of tin to solutions as SnF_2 or combinations of different fluorides with $SnCl_2$ has been extensively studied [12, 23, 24]. The mechanism of action of tin is based on its deposition on enamel surface, resulting in the formation of a stannous-containing layer, which may incorporate insoluble stannous compounds into the softened enamel during erosive challenges [25, 26]. In previous studies, a solution containing Sn, amine fluoride, and NaF was able to reduce enamel loss when subjected to an *in vitro* erosive cycling protocol, even under severe erosive challenges [27, 28]. Nonetheless, the preventive effect of Sn-containing dentifrices against tooth erosion has not been well studied [22-24, 29].

It is known that Sn can be adsorbed by silica reducing the availability of Sn-ions on the mouth, differing from Sn solutions, which do not contain silica and could act as a great reservoir of Sn-ions. [22, 24]. When the enamel blocks were subjected to erosion alone, the Sn-containing dentifrice promoted limited protection, similar to deionized water. On the other hand, when erosion was followed by brushing abrasion, only the Sn dentifrice resulted in less enamel wear than the negative control. Probably, the Sn-containing layer formed by this dentifrice presented higher mechanical stability against toothbrushing abrasion when compared to the other dentifrices tested. A previous *in situ* study that tested the effect of two dentifrices containing Sn against erosive and abrasive challenges (3×/day 0.05 M citric acid, pH 2.3 for 5 min and 2×/day application of slurry for 2 min and brushing 10 strokes with 150 g) showed 24 and 35% increased protection compared to water, which was similar to the 21% observed in the present study [29]. However, other studies found higher protective potential (67-69%) [23, 24]. The differences in enamel loss reduction might be due to methodological variations between *in vitro* and *in situ* studies, since *in vitro* studies seem to provide greater protection values than *in situ* ones [12, 30-34]. Additionally, the erosive and abrasive cycling protocols are extremely variable among studies; methodology standardization is required for effective comparisons of results.

Conventional dentifrices used in daily oral care usually have 1100-1450 ppm monovalent fluoride [35]. There is some evidence about the preventive potential of these dentifrices on dental erosion [15], although this effect seems limited. Some studies, especially *in situ* ones, demonstrated limited or no protection to enamel and dentin erosion of conventional fluoride formulations with 1000-1500 ppm NaF [30, 32, 36, 37]. In *in situ* models, enamel blocks are exposed to tongue friction in addition to

the acid challenge, removing the softened enamel layer [38], which can explain the greater protection obtained in *in vitro* studies. It was previously shown that the *in vitro* application of fluoride dentifrice slurries was able to protect 18 to 39% more that the control against erosive tooth wear [12, 33, 34, 39]. When erosion was associated with abrasion, the effect varied between none to 17-26% protection [12, 31, 33, 34]. *In situ* studies have demonstrated a lower protection; two studies did not show any protective effect [30, 32], one study showed 7% protection [29], and two studies showed around 21% protection [40, 41]. In the present study, when compared with the negative control, conventional fluoride dentifrices demonstrated a 10% reduction on erosive and abrasive wear.

Besides active ingredients such as fluoride and Ca_3SiO_5, other dentifrice components are important in the erosion/abrasion process, like the abrasives [42]. Abrasives vary in size and shape according to specific properties expected for each product [22]. The role of abrasives on enamel wear seems to be controversial. On one hand, a recent study showed that the amount of particles interferes on dentifrice efficacy under erosion and abrasion conditions; very low or very high radioactive enamel abrasion (REA) values influenced enamel loss whereas a broad range of REA was not very relevant [22]. On the other hand, another study have demonstrated no interference on enamel loss by dentifrices with low or high abrasivity [43]. The present study did not evaluate amount of particles and the radioactive dentin/enamel abrasion (RDA and REA) values of the dentifrices, but tin-containing and conventional fluoride dentifrices are both classified as having medium abrasivity according to RDA values by ADA classification. *In vitro* methods are commonly used for dental erosion studies due to the difficulties found in *in vivo* studies, such as lengthy time scale and inability to promote accurate measurements of tooth wear [44]. Nevertheless, *in vitro* studies cannot replicate the biological characteristics of the oral cavity [44]. The *in situ* methodology used in this study partially overcomes these limitations having the advantage of exposing specimens to the oral environment while allowing the sensitivity and accuracy of laboratory analysis [21, 44]. The present study was conducted *in situ* but the erosive and abrasive procedures were performed *ex vivo* to avoid any injuries to participants and to guarantee that toothbrushing was performed in the designated blocks. The extraoral procedures can be considered a limitation due to the absence of saliva. The phosphatases present in saliva play an important role on enzymatic

hydrolysis of sodium monofluorophosphate [45]. This fluoride compound is covalently bound to Na_2FPO_3 and its hydrolysis is essential for the release of fluoride ions [45]. This aspect may have influenced the results for the dentifrices with sodium monofluorophosphate (test and conventional dentifrices), which showed limited protective effect in the present study. In addition, artificial saliva could have been used for the dilution of the dentifrice. However, the different formulations of the available options can influence the level of enamel softening [46, 47]; thus, to eliminate the bias of saliva type used, the slurries were prepared with water in the present study. Nevertheless, most studies testing dentifrices with calcium silicate and sodium monofluorophosphate were conducted *in vitro* and with deionized water for slurry preparation, and found positive results in the demineralization and remineralization process [6, 8]. Another limitation of the present study is the use of bovine enamel, as it is known that bovine teeth present some chemical and structural differences to human teeth [48-52] that should be considered when projecting the results to human enamel. However, bovine enamel has been largely adopted as a substrate for *in vitro* and *in situ* dental erosion experiments [48-52], since it is easier to obtain in large quantities and their structure is more uniform than human teeth.

Based on our findings, we conclude that the application of dentifrice containing calcium silicate, sodium phosphate, and fluoride was able to diminish enamel loss after erosive acid exposure. Nevertheless, when toothbrushing abrasion was combined, this dentifrice promoted higher enamel wear than the other dentifrices and did not differ from water. The tin-containing toothpaste showed the best preventive effect against erosive and abrasive challenges.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest: The authors declare there is no conflict of interest.

Funding: The work was supported by Fundação de Amparo a Pesquisa de São Paulo [FAPESP grant numbers 2015/26520-6, 2015/21552-7, 2015/23911-4] and Conselho Nacional de Desenvolvimento Científico e Tecnológico [CNPq grant numbers 431264/2016-3, 310679/2015-0].

Ethical approval: All procedures performed in human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards (protocol number 48753115.0.0000.5417).

Informed consent: Informed consent was obtained from all participants included in the study.

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Tables

Table 1. Composition of dentifrices and pH of slurries under study.

Dentifrices	Composition	Batch number	pH of Slurries
REGENERATE™ ENAMEL SCIENCE France	Glycerin, Calcium Silicate, PEG-8, Hydrated Silica, Trisodium Phosphate, Sodium Phosphate, Aqua, PEG-60, Sodium Lauryl Sulfate, Sodium Monofluorphosphate (1450 ppm F^-), Aroma/Flavour, Synthetic Fluorphlogopite, Sodium Saccharin, Polyacrylic Acid, Tin Oxide, Limonene, CI77891	L:53078OB	9.60
Elmex® EROSION PROTECTION Austria	Aqua, Hydrated Silica, Glycerin, Sorbitol, Hydroxyethylcellulose, Aroma, Cocamidopropyl Betaine, Olaflur (700 ppm F^- from amine fluoride), Sodium Gluconate, Stannous Chloride (3500 ppm Sn^{2+}), Alumina, Chitosan (0,5%), Sodium Saccharin, Sodium Fluoride (700 ppm F^-), Potassium Hydroxide, Hydrochloric Acid, CI 77891	6052GB3411	4.54
Colgate Tripla Ação Brazil	Aqua, Calcium Carbonate, Sorbitol, Sodium Lauryl Sulfate, Sodium Monofluorphosphate (1450 ppm F^-), Aroma, Cellulose Gum, Tetrasodium Pyrophosphate, Sodium Bicarbonate, Benzyl Alcohol, Sodium Saccharin, Xantan Gum, Sodium Hydroxide, CI 74260, CI 75160, Limonene	5003BR122I	9.15

Table 2. Means and standard deviations of enamel loss (μm) according to the groups.

Groups	**Calcium silicate, sodium phosphate and sodium monofluorophosphate**		**Stannous chloride, amine fluoride and sodium fluoride**		**Sodium monofluorophosphate**		**Deionized Water - Negative Control**	
	ERO	**ABR**	**ERO**	**ABR**	**ERO**	**ABR**	**ERO**	**ABR**
Enamel loss	4.7 (±3.1) a	7.7 (±3.8) e	4.8 (±2.5) ab	5.4 (±1.5) abc	4.8 (±1.4) ab	6.2 (±1.7) cd	5.8 (±2.5) bcd	6.9 (±2.0) de

* ERO corresponds to erosive challenge and ABR corresponds to erosive plus abrasive challenge. Different letters show significant differences among the groups (two-way ANOVA and Fisher's Exact test, $p < 0.05$).

3 Discussion

3 Discussion

The discussion was divided into methodology, results and clinical relevance to better elucidate the ideas.

3.1. Methodology

The present study proposed to evaluate possible preventive measures for dental erosion in two distinguished aspects. Initially, when the acid is in contact with the tooth surface it can promotes loss of structural integrity and mechanical strength, corresponding to enamel softening which allows mineral redeposition (HUYSMANS; CHEW; ELLWOOD, 2011; SHELLIS et al., 2014). Whether the softening events are repeated the irreversible loss of dental hard tissues occurs, what is known as erosive tooth wear (HUYSMANS; CHEW; ELLWOOD, 2011; SHELLIS et al., 2014). The initial aspect of dental erosion (enamel softening) was evaluated in article 1 and thus, the study protocol differs from the other articles that evaluated the loss of dental hard tissue (erosive tooth wear).

The article 1 required an in vitro methodology since some of the vegetable oils assessed had not been previously evaluated regarding their anti-erosive potential. In vitro studies are widely used for evaluation of dental erosion, although some disadvantages are present because they are not able to resemble the biological characteristics of the oral cavity, such as the presence of human saliva and the formation of AEP. In vitro formed AEP has some differences from natural AEP, as it is known that the natural pellicle is more hydrophobic than in vitro ones (VAN DER MEI et al., 2002). Thus, a combined in situ/in vitro protocol was chosen in this study to allow the physiological formation of the AEP in situ prior to the in vitro application of the vegetable oils. The enamel blocks were protected by an orthodontic wire to avoid the mechanical impacts of the tongue (JORDÃO et al., 2017; MENDONÇA et al., 2017; SANTOS et al., 2018). A single short-term erosive challenge with citric acid was performed to more precisely evaluate the protective ability of the AEP modified by the

studied oils against initial enamel erosion. The response variable adopted in this study was percentage of surface hardness loss. Previous studies have considered hardness test as an adequate method to evaluate the initial softening of enamel surface (HARA; ZERO, 2008; STENHAGEN et al., 2010; YOUNG; TENUTA, 2011).

The articles 2, 3 and 4, adopted an in situ/ ex vivo methodology, which have the advantage of controlling erosive challenge while exposing the samples to the oral environment (YOUNG; TENUTA, 2011). Besides, the response variable was enamel loss analyzed by profilometry because it is considered an adequate method for in vitro analysis of erosive tooth wear (PAEPEGAEY et al., 2013; ATTIN; WEGEHAUPT, 2014; SCHLUETER et al., 2011). In those studies (2, 3 and 4), the enamel blocks were not protected by an orthodontic wire because in pilot studies it induced higher oil retention (article 2) and accumulated dental plaque (articles 3 and 4).

One difficulty of in situ studies is volunteers' protocol compliance (ZERO et al., 1995). In order to obtain greater commitment to the study protocol, the volunteers selected were dentistry undergraduate and graduate students. Also, in situ daily protocol only involved working hours (8AM to 12PM and 14PM to 18PM) and days (Monday to Friday) (SANTOS et al., 2018). Even though, two volunteers from study 2 and one from study 3 had to be excluded because they did not correctly follow the protocol.

Besides erosive challenges, the abrasion procedures were added to studies 2 and 4. Appropriate toothbrushing is recommended as twice daily for approximately 2 minutes (GANSS et al., 2009), however any tooth surface might be contacted for a maximum of 10 to 15 seconds twice daily. Thus, the contact time for each specimen to toothbrushing filament was defined in 15 seconds twice daily (30 seconds/day) (GANSS et al., 2011; GANSS et al., 2012; SCHLUETER et al., 2017; GANSS; MÖLLERS; SCHLUETER, 2017) and it was conducted ex vivo. In attempt to decrease the inter and intra volunteer variation, they were trained just to touch the toothbrush filament on enamel block trying not to perform additional force, since power toothbrush was used. Ideally, the abrasive procedure should be performed with the appliance in the mouth of the volunteers. However, in a pilot study, the volunteers were unable to distinguish the blocks that should be brushed (erosion+abrasion groups) from those that should not (erosion groups). In addition, in order to prevent injuries to the

participants teeth's the erosive challenge was also performed ex vivo (JORDÃO et al., 2017; MENDONÇA et al., 2017).

There are many erosive and abrasive cycling protocols in the literature and it can impair the comparison of the data among the studies, which demonstrates the need for standardization. However, there is no established protocol regarding this aspect. In the present study, we opted to perform an erosive and abrasive challenge design that would better resemble everyday life with four acid exposure and twice toothbrushing daily. The acid challenge used in studies 2 and 4 was based in other studies that tested oral health products and adopted citric acid 0.5% in natural pH for 2 minutes and brushing for 15 seconds (GANSS et al., 2012; DA SILVA et al., 2017). The study 3 was also conducted with citric acid exposure (extrinsic challenge), with a concentration of 0.03M. The measure unit was transformed to facilitate the comparison with 0.01M hydrochloric acid (intrinsic challenge). Experimental studies simulating intrinsic erosion causes, also adopted 0.01M hydrochloric acid in this pH range (2.2 to 2.3) (HOVE et al., 2006; HOVE et al., 2007; HOVE et al., 2008; YOUNG; TENUTA, 2011; OLIVEIRA et al., 2015; OLIVEIRA et al., 2017). Different acidic concentrations of citric and hydrochloric acids can influence the comparison of the data, however previous study has found that concentration and the amount of titratable acid are of minor importance to determine the erosive potential when compared to pH and type of acid (HANNIG; HAMKENS; BECKER, 2005). Each erosive challenge was set in 120 seconds as previous studies (HOVE et al., 2007; HOVE et al., 2008; GANSS et al., 2012; OLIVEIRA et al., 2015) since it was demonstrated that the pH on tooth surfaces stays low during this time after exposition to an acid until the salivary clearance (MILLWARD et al., 1997; ORR et al., 2003). The 2-hour period for in situ exposure between challenges was determined because previous studies have shown that acquired pellicle formed up to 2 hours offers the maximum protection against erosive demineralization without significantly improving with longer maturation periods (AMAECHI et al., 1999; HANNIG et al., 2003; WETTON et al., 2006; MENDONÇA et al., 2016; MENDONÇA et al., 2017). Thus, 4 acid exposures were done daily, to allow the 2 hours interval of salivary exposition between them. In the in vitro study (article 1), the acid challenge was also performed with the citric acid and natural pH. However, it was a short-time single exposure of 30 seconds. The time of exposure was determined in a pilot study aiming to result in enamel softening without loss of enamel surface; the

initial hardness indentation was still present at this time and after longer acid exposures, it was not possible to identify the initial indentation. Due to the absence of saliva, the extraoral procedures can be considered a limitation of the study.

Another limitation of all the four studies is the use of bovine enamel, because they have some chemical and structural differences compared to human teeth (MEURMAN; FRANK, 1991; RIOS et al., 2006; ATTIN et al., 2007; TURSSI et al., 2010; YASSEN; PLATT; HARA, 2011; ORTIZ-RUIZ et al., 2018) which must be taken into account, when projecting the results to clinical practice. However, bovine teeth are widely used as a substrate for in vitro and in situ enamel erosion studies because it is easier to obtain in large quantities with a more uniform structure compared to human teeth (MEURMAN; FRANK, 1991; RIOS et al., 2006; ATTIN et al., 2007; TURSSI et al., 2010; YASSEN; PLATT; HARA, 2011; ORTIZ-RUIZ et al., 2018).

3.2 Results

The prevention of erosive tooth wear can be challenging since it is a multifactorial condition in which patient-related and nutritional factors are involved (LUSSI; CARVALHO, 2014; BUZALAF; MAGALHÃES; RIOS, 2018). Specific anti-erosive products could be useful when is not possible to intercept and reverse these causal factors. The present study evaluated the effectiveness of possible preventive measures for dental erosion.

Acquired pellicle can protect the dental hard tissue against acids by modulating the demineralization/remineralization process; nonetheless, it does not prevent the acid diffusion completely (HANNIG; BALZ, 1999; HANNIG; HANNIG, 2014). Lipid-rich pellicles seems to be more resistant to acids (KENSCHE et al., 2013) thus it could reduce dental erosion. Previous studies have shown that the outer layer of the AEP absorbs lipids micelles when teeth are exposed to vegetable oils (DAS; ADHIKARY; BHATTACHARYYA, 1976; HANNIG et al., 2012). Vegetable oils are commonly used in foods, cosmetics, and medical products (KENSCHE et al., 2013). However, the protective effect of vegetable oils against acid challenge remains unclear, because only a few evidence-based researches are available in the literature (BUCHALLA et al., 2003; WIEGAND; GUTSCHE; ATTIN, 2007; HANNIG et al., 2012; KENSCHE et al., 2013) and their anti-erosive potential might be different according to their composition, including the types of fatty acids and other components. To date,

three of the vegetable oils (sunflower, coconut and palm oils) evaluated on this thesis have not been previously reported regarding the preventive effect against dental erosion. Thus, after conducting a pilot study, we opted to compare these oils with olive and safflower oil in 5% and 100% concentration. The concentration of 5% was chosen because it was higher than the concentration previously analyzed of 2% (WIEGAND; GUTSCHE; ATTIN, 2007) and also because at this concentration it is possible to added the oil into a mouthwash. The effect of safflower oil on the protective properties of the AEP formed in situ against the exposure to hydrochloric acid for 2 min was previously described (HANNIG et al., 2012). By transmission electron microscopy, the authors showed a lipid-rich AEP surface, but with no substantial integration of the lipids to the basal layer. In addition, the AEP modified by safflower oil rinsing was more susceptible to acid degradation than the in situ physiological AEP (HANNIG et al., 2012). Olive oil-based emulsions (100%, 2%, and 2% associated with mouthwash) had been previously evaluated regarding the preventive effect against erosive enamel wear being subjected to erosive cycle protocol and assessed using profilometry analysis (WIEGAND; GUTSCHE; ATTIN, 2007). The results have showed that pure olive oil did not offer protection and 2% olive-oil emulsion or 2% olive-oil containing mouthrinse offered some protection against dental erosion, but in a lower extent than the positive control (250 ppm acidic fluoride solution) (WIEGAND; GUTSCHE; ATTIN, 2007). Different from these findings, our results of study 1 showed that pure palm oil was able to protect enamel against initial erosion demineralization, but the same was not found for the 5% palm oil emulsion. No protective effect was observed to 5% emulsion and pure form of coconut, safflower, sunflower, and olive oils. The application of palm oil prior to a single short-term acid exposure resulted in 7.8% of enamel surface hardness loss compared to 23.7% of the Sn-containing solution (positive control) and 28.1% of the deionized water (negative control).

Considering the promising results of palm oil in study 1, an *in situ* study that exposes the enamel samples to biological factors and to mechanical forces from tongue and abrasion by an erosive-abrasive cycling could better represent the process that occurs on erosive enamel wear in vivo. Thus, study 2 evaluated the effect of palm oil alone or associated with a Sn-containing commercial solution on enamel loss prevention after 5 days of erosive and abrasive cycling regimen. Both studied treatments (palm oil and palm oil + Sn-containing solution) were able to protect against

erosive and abrasive enamel wear compared to deionized water. Nonetheless, it was not significantly different from the effect of Sn-containing solution. Palm oil presents low production cost and rich nutritional content and is the second largest produced and consumed vegetable oil in the world (SUNDRAM; SAMBANTHAMURTHI, 2003). It is rich in tocotrienols that have presented health benefits (AHSAN; AHAD; SIDDIQUI, 2015). The antioxidant effect has been attributed to the ability of tocotrienols to diffuse in the lipid layer of the cell membrane (AHSAN; AHAD; SIDDIQUI, 2015; KAMAT et al., 1997). Thus, despite the ultrastructure of the AEP not being analyzed, it could be inferred that palm oil might have been distributed into the lipids of the AEP basal layer retarding its disintegration. The possible alteration of the AEP ultrastructure by palm oil should be assessed in the future, to better understand its mechanism of action. The results of study 2 also showed a slight increase in enamel wear in groups subjected to erosion and abrasion by toothbrushing compared to the groups subjected to erosion only; around 4% for palm oil, 4% for palm oil+ Sn-containing solution, 21% for Sn-containing solution and 19% for deionized water. Besides no statistical difference found between erosion and erosion+abrasion challenges, it seems that the lubricating properties of the oil may play a role in protecting against abrasive toothbrushing, which can indicate a new possibility of research for palm oil-containing dentifrices. One possible hypothesis for the statistical similarity between erosion and erosion+abrasion is the enamel exposure to tongue friction, since the tongue can produces an abrasive effect on enamel softened by erosion (GREGG et al., 2004).

Another substance that can contributes to reduction on erosive tooth wear is aspartame (RIOS et al., 2009; RIOS et al., 2011). It is a dipeptide formed by the reaction of aspartic acid with phenylalanine, which can be released in contact with saliva. Phenylalanine could present potent antioxidant effect protecting against inflammatory diseases (GRAFF, 1992); also, it has showed to attenuate ulcerogenic parameters and to improve the gastric hemorrhagic erosion in acid-irrigated stomachs of rats (HUNG; HUNG, 1999). Light cola presents aspartame in its composition and demonstrated less erosive potential than regular cola (RIOS et al., 2009; RIOS et al., 2011). Our results of study 3 showed that the experimental aspartame mouthwash prior to acid challenge resulted in a reduction of only 16% of enamel loss in comparison with deionized water, without statistical differences. Sn-containing solution promoted less enamel loss than deionized water and aspartame solution. Light cola soft drink

has pH around 3 and thus, the protons of the acid might be captured by the carboxylic (COO-) or aminic (NH2) groups of phenylalanine, diminishing deleterious action on the tooth enamel (RIOS et al., 2009; RIOS et al., 2011). The pH of aspartame solution was 5.6, which could have impaired its anti-erosive effect. Different results might be obtained in solutions with lower pH (<4.0) which should be tested in future studies. Additionally, the results also showed that hydrochloric acid promotes higher enamel loss than citric acid for all tested solution. Hydrochloric acid is a strong monovalent acid (pKa = −6.3 – HCl) that can dissolve and remove the mineral surface more quickly than weaker polyvalent acids, such as citric acid (pKa1 = 3.15; pKa2 = 4.77; pKa3 = 6.40) (SCHLUETER; KLIMEK; GANSS, 2013). Thus, tooth erosion from intrinsic acids seems to be more severe than from extrinsic acids (BARTLETT; EVANS; SMITH, 1996; MOAZZEZ; BARTLETT, 2014), which is in accordance with the results from study 3.

Stannous solution was able to diminish erosive tooth wear in studies 2 and 3. However, it did not present any protective effect against initial erosive lesion in study 1. The mechanism of action of stannous ions, such as stannous chloride, is based on the formation of an acid-resistant layer on the surface of teeth (ELLINGSEN 1986; GANSS et al., 2008). This might increase the substrate resistance due to incorporation of ions into the enamel (SCHLUETER; KLIMEK; GANSS, 2009) and to the high reactivity to hydroxyapatite (YU et al., 2010). When it is associated with fluoride ions, the acquired enamel pellicle can be quantitatively and qualitatively modified (ALGARNI et al., 2015), forming a more stable and acid-resistant precipitates (GANSS et al., 2008). This mouthwash solution contains 800 ppm of Sn2+ from stannous chloride, 125 ppm of F- from amine fluoride, and 375 ppm of F- from sodium fluoride. It was adopted as positive control in studies 1, 2 and 3 since it has showed preventive effect against tooth erosion (ALGARNI et al., 2015; OLIVEIRA et al., 2015; DA SILVA; NAZELLO; DE FREITAS, 2017).

Patient-related factors, such as the performance of daily oral hygiene and products used to this purpose, also present an impact on dental erosion process (WIEGAND; SCHLUETER, 2014; HELLWIG; LUSSI, 2014). A new dentifrice containing calcium silicate, sodium phosphate, and fluoride has now been available with the proposal to regenerate the enamel lost due to erosive acids. Greater rehardening capacity of eroded enamel and formation of hydroxyapatite on tooth

surface have been previously demonstrated by dentifrices containing calcium silicate, sodium phosphate, and fluoride than conventional fluoride dentifrices (WANG et al., 2012; HORNBY et al., 2014; JOINER et al., 2014; JONES et al., 2014). The main purpose of study 4 was to determine the best preventive product to better orient patients. The new dentifrice containing calcium silicate, sodium phosphate, and fluoride was evaluated and compared to two other commercial dentifrices, one specifically indicated for dental erosion and the other for general use. Limited protective action against erosion, reducing enamel loss by approximately 18% after erosive challenges was demonstrated to the calcium silicate/phosphate and fluoride dentifrice, which was better than deionized water but did not differ from the other two dentifrices analyzed. It is hypothesized that the tissue loss occurred because the erosive challenge applied was able to demineralize the hydroxyapatite both from the calcium silicate dentifrice and the enamel. Also, the calcium silicate dentifrice resulted in higher enamel loss than the other evaluated dentifrices, when abrasion episodes were added to erosion. The effect of this dentifrice was similar to deionized water, showing no protective role against erosion associated to abrasion. It seems like the supposedly hydroxyapatite layer formed by the dentifrice may be easily removed by abrasive forces. Furthermore, short 60-second contact between enamel blocks and the dentifrice slurry could have influenced on the results. Better erosion protection were obtained by previous studies with 1 to 3 minutes application of calcium silicate dentifrice slurry (HORNBY et al., 2014; JOINER et al., 2014; JONES et al., 2014) Perhaps, a more resistant layer of hydroxyapatite could be formed with longer exposure of the enamel to the dentifrice.

Sn-containing dentifrice was adopted as positive control. Besides the promising effect of Sn-containing solution against dental erosion, the effect of Sn-containing dentifrices has not been well studied (HUYSMANS et al., 2011; GANNS et al., 2012; CARVALHO; LUSSI, 2014; GANNS et al., 2016). Sn-ions can be adsorbed by silica reducing its availability on the mouth (GANNS et al., 2012; GANNS et al., 2016). On erosive challenge, the results of study 4 showed that Sn-containing dentifrice promoted a borderline behavior, presenting similar enamel loss than calcium silicate denfrice but also to conventional fluoride dentifrice and deionized water. At the same time, regarding erosive plus abrasive challenges, the Sn dentifrice was the only one to result less enamel wear than negative control. In this way, the Sn-containing

layer formed by this dentifrice seems to present higher mechanical stability against toothbrushing abrasion when compared to the other dentifrices tested. In accordance with the 21% reduction of enamel loss in comparison with deionized water observed in the present study, one in situ study have tested two Sn-containing dentifrices against erosive and abrasive challenges (3×/day 0.05 M citric acid, pH 2.3 for 5 min and 2×/day application of slurry for 2 min and brushing 10 strokes with 150 g) and found 24 and 35% increased protection compared to water (HUYSMANS et al., 2011). Conventional dentifrices used in daily oral care usually have 1100-1450 ppm monovalent fluoride (HUYSMANS; YOUNG; GANSS, 2014). Limited or no protection to enamel and dentin erosion of conventional fluoride formulations with 1000-1500 ppm NaF have been demonstrated by some studies, especially in situ ones (ATTIN; ZIRKEL; HELLWIG, 1998; TURSSI et al., 2004; LUSSI et al., 2008; RIOS et al., 2008). In the study 4, conventional fluoride dentifrices resulted in 10% reduction on erosive and abrasive wear when compared with deionized water. Two in situ studies found in the literature did not show any protective effect (TURSSI et al., 2004; RIOS et al., 2008), one study showed 7% protection (HUYSMANS et al., 2011), and two studies showed around 21% protection (MAGALHÃES et al., 2007; SCHLUETER; KLIMEK; GANSS, 2013). The erosive and abrasive cycling protocols are extremely variable among studies; methodology standardization is required for effective comparisons of results.

3.3 Clinical Relevance

Stannous fluoride has demonstrated preventive effects against tooth erosion (ALGARNI et al., 2015; OLIVEIRA et al., 2015; DA SILVA; NAZELLO; DE FREITAS, 2017; DA SILVA et al., 2017). However, there is no ideal therapy established for the prevention and inhibition of erosive dental wear (HUYSMANS; YOUNG; GANSS, 2014; BUZALAF; MAGALHÃES; WIEGAND, 2014). Articles 1, 2 and 3 can be considered as the first steps for the development of intraoral products to prevent against ETW. While article 4 is helpful to guide patients about dentifrices available in the market regarding erosive enamel wear.

In this view, vegetable oils could be an interesting option since it is a natural, edible, low-cost and worldwide accessible source (SUNDRAM; SAMBANTHAMURTHI; TAN, 2003). In articles 1 and 2, palm oil showed promising

results for the prevention of enamel erosion and abrasion. Thus, strategies to enhance this effect should be developed.

Aspartame could be the responsible for the lower erosive potential of light cola drink (RIOS et al., 2009; RIOS et al., 2011). Despite no statistical difference regarding the prevention of enamel loss between aspartame and deionized water, the results of the article 3 showed a numerically reduction of 16% of enamel loss when aspartame mouthwash was performed prior to intrinsic or extrinsic erosive challenges. The aspartame solution should be improved regarding concentration and pH and tested in future studies, in order to develop a low-cost product to dental erosion prevention. Also, it seems that intrinsic causes of erosion, such as hydrochloric acid exposition, results in higher enamel loss than the extrinsic causes, such as citric acid exposition. Thus, it should be important that health professionals dealing with eating disorders and gastroesophageal reflux guide their patients to seek for oral health care.

Dentifrices specifically indicated for the prevention and treatment of dental erosion are now available in the market. However, patients with erosive tooth wear are advised to use any fluoride toothpaste. Thus, we aimed to determine the best preventive product and better orient patients. In article 4 it was elucidated that the tested anti erosive dentifrice with calcium silicate, sodium phosphate and fluoride was unable to protect enamel, when erosion was associated to toothbrushing abrasion. Better preventive effect was showed for stannous-containing dentifrice against erosive and abrasive challenges.

4 Conclusions

4 Conclusions

Considering the hypothesis raised before, it is possible to conclude that:

- Article 1: Only pure palm oil demonstrated preventive effect against initial erosive demineralization among all types and concentrations of vegetable oils evaluated.
- Article 2: Palm oil and Sn-containing solution, alone or combined, presented a preventive effect against erosive and erosive plus abrasive challenge when compared to deionized water.
- Article 3: The aspartame solution did not inhibit enamel loss when used before erosive challenge by hydrochloric and citric acids. Sn-solution promoted less enamel loss than deionized water and aspartame solution. Higher enamel loss was induced by simulated intrinsic (hydrochloric) acid than extrinsic (citric) acid.
- Article 4: The dentifrice containing calcium silicate, sodium phosphate, and fluoride was able to reduce enamel loss after erosive acid challenge. Nevertheless, when toothbrushing abrasion was combined, the application of this dentifrice resulted in higher enamel wear than the other dentifrices and did not differ from water. The Sn-containing dentifrice had the best preventive potential against erosive and abrasive challenges.

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Appendixes

APPENDIX A - DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We declare to be aware that the study entitled **Effect of vegetable oils applied over acquired enamel pellicle on initial erosion** will be included in the Thesis of the student Franciny Querobim Ionta and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, April 5th, 2017.

Franciny Querobim Ionta

Catarina Ribeiro Barros de Alencar

Poliana Pacífico Val

Ana Paula Boteon

Maísa Camillo Jordão

Heitor Marques Honório

Marília Afonso Rabelo Buzalaf

Daniela Rios

APPENDIX B - DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We declare to be aware that the study entitled **Effect of palm oil alone or associated to stannous solution on enamel erosive-abrasive wear: a randomized in situ/ex vivo study** will be included in the Thesis of the student Franciny Querobim Ionta and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, April 5th, 2017.

Franciny Querobim Ionta

Catarina Ribeiro Barros de Alencar

Natália Mello dos Santos

Bianca Tozi Portaluppe Bergantin

Poliana Pacifico Val

Heitor Marques Honório

Thais Marchini de Oliveira

Daniela Rios

APPENDIX C - DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We declare to be aware that the study entitled **Effect of aspartame against erosive enamel wear by intrinsic and extrinsic acids: an in situ/ex vivo study** will be included in the Thesis of the student Franciny Querobim Ionta and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, April 5th, 2017.

Franciny Querobim Ionta

Marcela Bassoto de Azevedo

Natália Mello dos Santos

Heitor Marques Honório

Evandro José Dionísioa

Thiago Cruvinel

Daniela Rios

APPENDIX D - DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We declare to be aware that the study entitled **Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear? an in situ/ ex vivo study** will be included in the Thesis of the student Franciny Querobim Ionta and may not be used in other works of Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, April 5th, 2017.

Franciny Querobim Ionta

Natália Mello dos Santos

Isabela Maníglia Mesquita

Evandro José Dionísio

Thiago Cruvinel

Heitor Marques Honório

Daniela Rios

Annexes

ANNEX A – Ethics committee approval for articles 1 and 2

FACULDADE DE ODONTOLOGIA DE BAURU-USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: O efeito de lipídios na erosão e no desgaste dentário erosivo
Pesquisador: Catarina Ribeiro Barros de Alencar
Área Temática:
Versão: 2
CAAE: 45792415.8.0000.5417
Instituição Proponente: Universidade de Sao Paulo
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.173.522
Data da Relatoria: 29/07/2015

Apresentação do Projeto:
Idem ao parecer número 1.113.748 emitido em 17/06/2015

Objetivo da Pesquisa:
Idem ao parecer número 1.113.748 emitido em 17/06/2015

Avaliação dos Riscos e Benefícios:
Idem ao parecer número 1.113.748 emitido em 17/06/2015

Comentários e Considerações sobre a Pesquisa:
Idem ao parecer número 1.113.748 emitido em 17/06/2015

Considerações sobre os Termos de apresentação obrigatória:
Idem ao parecer número 1.113.748 emitido em 17/06/2015

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:
Segue a lista de inadequações:

a) A pesquisadora não informou no TCLE aos participantes da pesquisa o exame clínico mencionado no item 3.3.6., como também não informou da avaliação do fluxo salivar não

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Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.173.522

estimulado mencionado no item 3.3.6.1 e da avaliação do fluxo salivar estimulado mencionado no item 3.3.6.2. Seja corrigido.

Pendência ATENDIDA

b) A pesquisadora não informa o que fará com a saliva coletada depois de obtido o resultado dos testes de interesse da pesquisadora. Seja informado.

Pendência ATENDIDA

c) Quanto ao cronograma. O cronograma anexado no projeto gerado na PB está incorreto. Por aquele cronograma a pesquisa já estaria iniciada. Seja corrigido. Por outro lado o cronograma apresentado no projeto anexado na PB em Word está correto, só precisa que a pesquisadora informe que o mês 1 (um) do cronograma deve ser contado a partir da aprovação do projeto junto ao CEP. Por fim solicitamos a pesquisadora que nos apresente as correções solicitadas em destaque (de preferencia em vermelho).

Pendência ATENDIDA

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião extraordinária do CEP de 29.07.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.173.522

pertinentes.

BAURU, 06 de Agosto de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

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ANNEX B – Ethics committee approval for article 3

FACULDADE DE ODONTOLOGIA DE BAURU-USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Efeito do aspartame na prevenção do desgaste dentário erosivo
Pesquisador: Franciny Querobim Ionta
Área Temática:
Versão: 2
CAAE: 48729115.0.0000.5417
Instituição Proponente: Universidade de Sao Paulo
Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.335.392

Apresentação do Projeto:
Idem ao parecer 1.237.189.

Objetivo da Pesquisa:
Idem ao parecer 1.237.189.

Avaliação dos Riscos e Benefícios:
Idem ao parecer 1.237.189.

Comentários e Considerações sobre a Pesquisa:
Idem ao parecer 1.237.189.

Considerações sobre os Termos de apresentação obrigatória:
Idem ao parecer 1.237.189.

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:
1- Adequar o cronograma do projeto de pesquisa com o da Plataforma Brasil, devem constar as mesmas informações.
PENDÊNCIA ATENDIDA.

2- No TCLE, substituir a palavra convidado por participante da pesquisa, e esclarecer se os

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Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.335.392

participantes da pesquisa serão alunos da pós-graduação da Faculdade de Odontologia de Bauru, pois no TCLE descreve "Cabe ressaltar que não haverá nenhum gasto para os convidados envolvidos na pesquisa, visto que esta pesquisa será realizada enquanto o convidado estiver presente na Faculdade, em horários que não interfiram em suas atividades curriculares", porém em nenhum momento esclarece que os participantes são alunos de pós-graduação da FOB. Caso sejam alunos de pós-graduação da FOB, o pesquisador responsável deverá providenciar autorização do setor responsável pela abordagem aos alunos, considerando a relação de dependência, conforme Res.466/12.
PENDÊNCIA ATENDIDA. FORAM ANEXADOS OS TERMOS DE AQUIESCÊNCIA DAS COMISSÕES DE PÓS E GRADUAÇÃO.

Considerações Finais a critério do CEP:
Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 18.11.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_556145.pdf	13/10/2015 15:13:05		Aceito
Outros	aquiescencia_voluntarios_comissao_de_graduacao.pdf	13/10/2015 15:12:32	Franciny Querobim Ionta	Aceito
Outros	aquiescencia_voluntarios_posgraduacao.pdf	13/10/2015 15:11:58	Franciny Querobim Ionta	Aceito
Outros	carta_resposta_CEP.pdf	13/10/2015 15:07:37	Franciny Querobim Ionta	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_Aspartame.pdf	13/10/2015 15:07:03	Franciny Querobim Ionta	Aceito
TCLE / Termos de Assentimento / Justificativa de	TCLE_aspartame.pdf	13/10/2015 15:06:26	Franciny Querobim Ionta	Aceito

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.335.392

Ausência	TCLE_aspartame.pdf	13/10/2015 15:06:26	Franciny Querobim Ionta	Aceito
Outros	Termo de aquiescencia - ciencias biologicas.pdf	11/08/2015 16:51:58		Aceito
Outros	QuestionarioTecnicoPesquisador.pdf	29/07/2015 21:34:47		Aceito
Outros	Declaração de Compromisso do Pesquisador com os Resultados.pdf	29/07/2015 21:32:28		Aceito
Outros	Termo de aquiescencia.pdf	29/07/2015 21:32:08		Aceito
Folha de Rosto	Folha de Rosto.pdf	29/07/2015 21:30:28		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

BAURU, 24 de Novembro de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

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ANNEX C – Ethics committee approval for article 4

FACULDADE DE ODONTOLOGIA DE BAURU-USP

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Avaliação in situ de um dentifrício contendo silicato de cálcio, fosfato e flúor na prevenção do desgaste erosivo e abrasivo

Pesquisador: Franciny Querobim Ionta

Área Temática:

Versão: 2

CAAE: 48753115.0.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.335.385

Apresentação do Projeto:

Idem ao parecer 1.235.615

Objetivo da Pesquisa:

Idem ao parecer 1.235.615

Avaliação dos Riscos e Benefícios:

Idem ao parecer 1.235.615

Comentários e Considerações sobre a Pesquisa:

Idem ao parecer 1.235.615

Considerações sobre os Termos de apresentação obrigatória:

Idem ao parecer 1.235.615

Recomendações:

Idem ao parecer 1.235.615

Conclusões ou Pendências e Lista de Inadequações:

Trata-se de uma pesquisa bem interessante que poderá contribuir na prevenção e controle do desgaste dentário erosivo uma vez que se estima que o dentifrício com silicato de cálcio possa ser

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.335.385

uma medida alternativa viável nessa prevenção e controle. A pesquisa está bem elaborada e bem descrita, não existindo problemas éticos que a torna inviável. Entretanto, algumas considerações devemos fazer:

1) As pesquisadoras não informam aonde pretendem recrutar os participantes da pesquisa.
PENDÊNCIA ATENDIDA. FOI ANEXADO O TERMO AQUIESCÊNCIA.

2) Quanto ao TCLE as pesquisadores devem fazer duas correções:
a) Informar ao participante da pesquisa que ele não terá gastos por participar da pesquisa e,
PENDÊNCIA ATENDIDA.

b) Esclareça o porquê a escovação deve ser feita com escova elétrica conforme se menciona no TCLE... "E deve escovar com escova elétrica e solução do dentifrício por 15 segundos..." O kit a ser entregue a cada participante não prevê o ganho de uma escova elétrica; tampouco o orçamento..., somente escovas da marca CURAPROX.
PENDÊNCIA ATENDIDA.ESCLARECIMENTO FEITOS EM RELAÇÃO AO USO DA ESCOVA ELETRICA SOMENTE NO ESMALTE BOVINO.

3) Esclareça qual será a participação da aluna Isabela Maníglia Mesquita na pesquisa, uma vez que a mesma figura como parte integrante da equipe de pesquisa.
PENDÊNCIA ATENDIDA.

4) As pesquisadoras não anexaram nenhum documento (carta de encaminhamento) na PB encaminhando o projeto para este Comitê analisar. Considerando essas observações a serem esclarecidas sou de parecer pela pendencia da pesquisa.
PENDÊNCIA ATENDIDA

Considerações Finais a critério do CEP:
Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 18.11.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores,

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.335.385

cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_569180.pdf	13/10/2015 15:53:57		Aceito
Outros	carta_resposta_CEP.pdf	13/10/2015 15:42:18	Franciny Querobim Ionta	Aceito
Outros	aquiescencia_voluntarios_graduacao.pdf	13/10/2015 15:41:13	Franciny Querobim Ionta	Aceito
Outros	aquiescencia_voluntarios_posgraduacao.pdf	13/10/2015 15:40:43	Franciny Querobim Ionta	Aceito
Outros	carta_de_encaminhamento.pdf	13/10/2015 15:39:52	Franciny Querobim Ionta	Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_REGENERATE.pdf	13/10/2015 15:38:26	Franciny Querobim Ionta	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE_regenerate.pdf	13/10/2015 15:38:12	Franciny Querobim Ionta	Aceito
Outros	questionario_tecnico_pesquisador_completo.pdf	25/08/2015 21:13:12	Franciny Querobim Ionta	Aceito
Outros	Declaracao_de_compromisso_com_os_resultados.pdf	25/08/2015 21:09:21	Franciny Querobim Ionta	Aceito
Declaração de Instituição e Infraestrutura	termo_uso_laboratorio.pdf	25/08/2015 21:07:45	Franciny Querobim Ionta	Aceito
Declaração de Instituição e Infraestrutura	termo_de_aquiescencia_departamento.pdf	25/08/2015 20:55:02	Franciny Querobim Ionta	Aceito
Folha de Rosto	folha_de_rosto.pdf	25/08/2015 20:50:21	Franciny Querobim Ionta	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.335.385

BAURU, 24 de Novembro de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
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ANNEX D – Manuscript submission letter confirmation from Archives of Oral Biology

De: **Archives of Oral Biology** eesserver@eesmail.elsevier.com
Assunto: Track your recent Co-Authored submission to AOB
Data: 9 de abril de 2018 3:37 PM
Para: francinyionta@hotmail.com

Dear Dr. Franciny Ionta,

You have been listed as a Co-Author of the following submission:

Journal: Archives of Oral Biology
Title: Effect of palm oil alone or associated to stannous solution on enamel erosive-abrasive wear: a randomized in situ/ex vivo study
Corresponding Author: Daniela Rios
Co-Authors: Franciny Q Ionta; Catarina R de Alencar; Natália M dos Santos; Bianca T Bergantin; Poliana P Val; Heitor M Honório; Thais M de Oliveira;

To be kept informed of the status of your submission, register or log in (if you already have an Elsevier profile).

Register here: https://ees.elsevier.com/aob/default.asp?

Or log in:

If you did not co-author this submission, please do not follow the above link but instead contact the Corresponding Author of this submission at daniriosop@yahoo.com.br;danirios@usp.br.

Thank you,

Archives of Oral Biology

ANNEX E - Manuscript submission letter confirmation from International Dental Journal

De: **International Dental Journal** onbehalfof@manuscriptcentral.com
Assunto: International Dental Journal - Account Created
Data: 20 de abril de 2018 9:08 AM
Para: francinyionta@hotmail.com

20-Apr-2018

Dear Ms. Ionta

A manuscript titled Effect of aspartame against erosive enamel wear by intrinsic and extrinsic acids: an in situ/ex vivo study (IDJ-Apr-18-OA-0164) has been submitted by Ms. Franciny Ionta to International Dental Journal.

You are listed as an author for this manuscript and so the online peer-review system, has automatically created a user account for you. Your International Dental Journal account information is as follows:

Site URL: https://mc.manuscriptcentral.com/idj
USER ID: francinyionta@hotmail.com
PASSWORD: For security reasons your password is not contained in this email. To set your password click the link below.

You can use the above USER ID and PASSWORD (once set) to log in to the site and check the status of papers you have authored/co-authored. You may log in to https://mc.manuscriptcentral.com/idj to check and update your account information via the edit account tab at the top right.

Thank you for your participation.

Kind regards,
International Dental Journal Editorial Office

ANNEX F - Manuscript submission letter confirmation from Clinical Oral Investigations

De: **Clinical Oral Investigations** em@editorialmanager.com
Assunto: Clinical Oral Investigations - Submission Notification to co-author
Data: 29 de setembro de 2017 3:55 PM
Para: Franciny Querobim Ionta francinyionta@hotmail.com

Re: "Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear: an in situ study?"
Full author list: Franciny Querobim Ionta; Natália Mello dos Santos; Isabela Maníglia Mesquita; Evandro José Dionísio; Thiago Cruvinel Silva; Heitor Marques Honório; Daniela Rios, Ph.D

Dear Miss Franciny Ionta,

We have received the submission entitled: "Is the dentifrice containing calcium silicate, sodium phosphate and fluoride able to protect enamel against chemical mechanical wear: an in situ study?" for possible publication in Clinical Oral Investigations, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Dra Daniela Rios who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Clinical Oral Investigations