UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

CAMILA SIQUEIRA SILVA COELHO

FLUORETO QUIMICAMENTE SOLÚVEL EM DENTIFRÍCIOS À BASE DE MFP/$CaCO_3$ COMO PREDITOR DA BIODISPONIBILIDADE DE FLUORETO NA SALIVA

CHEMICALLY SOLUBLE FLUORIDE IN MPF/$CaCO_3$-BASED TOOTHPASTES AS A PREDICTOR OF FLUORIDE BIOAVAILABILITY IN SALIVA

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestra em Odontologia, na Área de Cariologia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Dentistry, in Cariology area.

Orientadora: Profa. Dra. Cinthia Pereira Machado Tabchoury
Coorientador: Prof. Dr. Jaime Aparecido Cury

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELA ALUNA CAMILA SIQUEIRA SILVA COELHO E ORIENTADA PELO PROFA. DRA. CINTHIA PEREIRA MACHADO TABCHOURY

Piracicaba
2019

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

C65f

Coelho, Camila Siqueira Silva, 1994-
Fluoreto quimicamente solúvel em dentifrícios à base de MFP/$CaCO_3$ como preditor da biodisponibilidade de fluoreto na saliva / Camila Siqueira Silva Coelho. – Piracicaba, SP : [s.n.], 2019.

Orientador: Cinthia Pereira Machado Tabchoury.
Coorientador: Jaime Aparecido Cury.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Fluoretos. 2. Dentifrícios. 3. Saliva. 4. Biodisponibilidade. I. Tabchoury, Cinthia Pereira Machado, 1969-. II. Cury, Jaime Aparecido, 1947-. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

Informações para Biblioteca Digital

Título em outro idioma: Chemically soluble fluoride in MFP/$CaCO_3$-based toothpaste as a predictor of fluoride bioavailability in saliva
Palavras-chave em inglês:
Fluorides
Dentifrices
Saliva
Bioavailability
Área de concentração: Cariologia
Titulação: Mestra em Odontologia
Banca examinadora:
Cinthia Pereira Machado Tabchoury [Orientador]
Paulo Frazão São Pedro
Fernando Neves Nogueira
Data de defesa: 27-02-2019
Programa de Pós-Graduação: Odontologia

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0002-4315-3854
- Currículo Lattes do autor: http://lattes.cnpq.br/0312530501959047

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Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de Mestrado, em sessão pública realizada em 27 de Fevereiro de 2019, considerou a candidata CAMILA SIQUEIRA SILVA COELHO aprovada.

PROFª. DRª. CINTHIA PEREIRA MACHADO TABCHOURY

PROF. DR. PAULO FRAZÃO SÃO PEDRO

PROF. DR. FERNANDO NEVES NOGUEIRA

A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

DEDICATÓRIA

Àqueles que estiveram comigo do início ao final dessa trajetória, que nos momentos de dificuldade estiveram ao meu lado e nas vitórias comemoram cada uma delas comigo, **minha família**.

AGRADECIMENTOS ESPECIAIS

"A vida é luta renhida: Viver é lutar.
A vida é combate, que os fracos abate,
Que os fortes, os bravos, só pode exaltar."
(Antônio Gonçalves Dias)

Ao final desta trajetória o que fica em minha mente são os bons momentos vividos, as amizades feitas, os desafios superados, os conhecimentos adquiridos, os erros cometidos e as críticas recebidas. E com o término dessa etapa, mestrado, agradeço os eternos presentes que levarei sempre comigo, serei eternamente grata.

Inicialmente, gostaria de agradecer aos **meus pais** por serem o ponto de apoio, no qual poderei retornar e sempre descansar. Muito obrigado por permitirem e sempre estarem ao meu lado quando decidi sair de casa para estudar, por apoiarem a minha escolha do mestrado e do doutorado e me ajudarem durante essa caminhada. A minha mãe, **Francisca Maria Siqueira Silva Coelho**, meu espelho como profissional, mas acima de tudo como pessoa. A senhora esteve sempre ao meu lado, guiando e apoiando nas minhas decisões, seja com palavras ou ações, muito obrigada. Ao meu pai, **Carlos Alberto Castelo Branco Coelho**, por nunca medir esforços para que eu e minha irmã estivéssemos bem, o senhor é minha inspiração para conseguir forças quando nem eu mesma sabia que podia, é meu companheiro de filmes e de coleta de água para pesquisa.

Quando saímos cedo de casa, deixamos muito mais que conforto do lar, ficam por lá, pessoas queridas e que são fundamentais em cada nova conquista. E entre essas pessoas gostaria de agradecer a **minha irmã**, **Lara Siqueira Silva Coelho**, pela compreensão da minha ausência em muitos momentos. Durante esses dois anos, as conversas divididas, os abraços foram combustíveis para que eu chegasse até o final de mais essa jornada. Além deles, agradeço a duas pessoas que estiveram ao meu lado desde os primeiros passos, **meus avós**, **Francisco de Assis Pacheco da Silva** e **Raimunda de Siqueira Silva**, vocês me ensinaram a sempre acreditar na minha capacidade de ir atrás dos meus sonhos e nunca desistir deles. Obrigada por, em meio a toda dificuldade estarem sempre presente em minha vida.

AGRADECIMENTOS

À **Universidade Estadual de Campinas - UNICAMP**, na pessoa do Magnífico Reitor, Prof. Dr. **Marcelo Knobel** e à **Faculdade de Odontologia de Piracicaba – FOP/UNICAMP**, na pessoa do seu diretor Prof. Dr. **Francisco Haiter Neto**.

À Prof. Dra. **Karina Gonzales Silvério Ruiz**, coordenadora dos Cursos de Pós-graduação da Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas.

À Prof. Dra. **Michelle Franz Montan Braga Leite**, coordenadora do Programa de Pós-graduação em Odontologia da Faculdade de Odontologia de Piracicaba.

O presente trabalho foi realizado com apoio da **Coordenação de Aperfeiçoamento de Pessoal de Nível Superior** – Brasil (CAPES) – Código de Financiamento 001.

À Profa Dra. **Cinthia Pereira Machado Tabchoury**, minha orientadora durante o mestrado, muito obrigada pela orientação durante esses dois anos de trabalho e por me instigar a sempre buscar fazer o melhor. A senhora é um exemplo de dedicação, organização e ética, características que fazem da senhora uma professora exemplo para todos que estão ao seu redor. Com a senhora aprendi que em meio a tantas obrigações, podemos e devemos fazer tudo com excelência, que sempre devemos está buscando ser profissionais melhores e nunca deixar nos abalar com um não que aparecer.

Ao Prof. Dr**. Jaime Aparecido Cury**, mais que um coorientador, o senhor é um exemplo de profissional, seja como professor ou pesquisador. Muito obrigada pelas oportunidades de aprender com o senhor, em sala de aula, no laboratório ou em reuniões, são momentos os quais pude crescer.

À Prof. Dra. **Altair Antoninha Del Bel Cury,** pela condução das disciplinas e toda dedicação dispensada a área de Cariologia neste período.

Ao Prof. Dr. **Antônio Pedro Ricomini Filho,** por todos os ensinamentos repassados e por toda disponibilidade em ajudar em diversos momentos durante esses dois anos.

À Prof. Dra. **Lívia Maria Andaló Tenuta,** pela orientação durante o período que fui estagiária no Laboratório de Bioquímica Oral (PROCAD - Capes) durante minha graduação, período que foi fundamental para a decisão de tentar a seleção do mestrado, assim como pelos conhecimentos repassados durante o mestrado.

Aos professores do **Programa de Pós-Graduação em Odontologia** da **Faculdade de Odontologia de Piracicaba**, em especial ao Prof. Dr. **Pedro Luiz Rosalen,** pela disponibilidade em ensinar, assim como nos mostrar que não é necessário estarmos restrito a apenas uma área, e sim, que podemos ser sempre mais.

Aos técnicos do Laboratório de Bioquímica da Oral, **Waldomiro Vieira Filho** e **José Alfredo da Silva**, pela disponibilidade no dia-a-dia, assim como pelas conversas nesse tempo de convivência.

Aos professores do **curso de Odontologia** da **Universidade Federal do Piauí**, obrigada pelos conselhos, ensinamentos, amizade e confiança. Sou eternamente grata por ter feito parte desta instituição e poder ter aprendido com cada um.

Aos Prof. Dra. **Ana Cristina Vasconcelos Fialho**, Prof. Dr. **Alexandre Henrique de Melo Simplício**, Prof. Dra. **Lúcia de Fátima Almeida de Deus Moura**, Prof. Dra. **Marina de Deus Moura de Lima**, Prof. Dra. **Mila Oliveira Santos Viana**, Prof. Dr. **Raimundo Rosendo Prado Junior** e Prof. Dra. **Regina Ferraz Mendes,** que sempre me incentivaram desde a época da graduação, acreditando no meu potencial e me aconselhando a seguir em frente no curso de pós-graduação. Obrigada pelos conselhos, ensinamentos, amizade e confiança. Vocês foram fundamentais nas minhas escolhas e eu agradeço imensamente.

À Prof. Dra. **Marcoeli Silva de Moura**, minha eterna orientadora, amiga e grande incentivadora, muito obrigada pela oportunidade de ter convivido com a senhora durante as iniciações científicas, monitoria e extensão, foram momentos de muito aprendizado. A senhora é um exemplo de professora, dentista, mas acima de tudo de pessoa, alguém que queremos sempre ao nosso lado.

Aos amigos da área **Cariologia FOP Unicamp** pela boa convivência, amizade e momentos compartilhados.

Às amigas que partilharam comigo dessa jornada, **Alejandra Brenes** e **Maria Gerusa Brito Aragão**, vocês são eternas joias raras em minha vida. Obrigada pelas conversas, conselhos, risadas, pelas ajudas antes dos seminários, pelos momentos compartilhados no laboratório ou fora das paredes da FOP, e que graças a cada um deles esses dois anos passaram de maneira mais tranquila.

Aos amigos de caminhada **Luma Almeida, Robson Sousa** e **Matheus Kury** muito obrigada pela amizade que surgiu em meio à correria de disciplinas e que se fortificaram com tempo, e por de alguma forma terem contribuído durante esses dois anos.

Aos amigos **Luanne Mara** e **Josué Pierote**, que se tornaram minha família em Piracicaba, muito obrigada pelo cuidado e pelos gestos de carinho.

Aos amigos da **92ª Turma de Odontologia da UFPI** por me apoiarem, estarem sempre presente e me incentivar desde o início. Obrigada pelos bons momentos e pela amizade.

Aos amigos **Aila Cipriano, Ana Victoria, Francisco Costa Junior**, **Helly Karinny, Ingred Bida, Isnayra Pacheco, Keila Naiara, Natiele Carvalho e Taina Castelo Branco** meus eternos companheiros de batalha desde a graduação. Muito obrigada por todo apoio e incentivo desde o início, e mesmo com a distância vocês estiveram sempre presente.

Ao **Felipe Brancati** por ser meu companheiro em todos os momentos, participando e incentivando a sempre melhorar. Você esteve presente durante a escrita desse trabalho, com palavras de carinho e gestos que trouxeram tranquilidade nos momentos de maior dificuldade, sendo meu grande apoio.

Aos **voluntários** que participaram do estudo, pela colaboração, cooperação e dedicação indispensáveis para a realização desta pesquisa.

À todos que contribuíram de alguma forma para que este trabalho fosse concluído,

Muito Obrigada!

RESUMO

Já que não há protocolo validado de determinação de fluoreto nos dentifrícios que mostre relação entre a concentração de flúor solúvel encontrada quimicamente no dentifrício e aqueles presentes na saliva após a escovação foi realizado um estudo in vivo, cego e cruzado com 5 fases experimentais. Dez participantes adultos foram submetidos aos seguintes grupos de tratamentos: grupo I- Sorriso Dentes Brancos® (MFP/$CaCO_3$, 1450 ppm F) recém-formulado; grupos II a IV - Sorriso Dentes Brancos® submetidos a envelhecimento acelerado, em estufa a 55º C, apresentando concentração de fluoreto solúvel total (FST) de 1160, 900 e 597 ppm F (correspondendo a 20, 40 e 60% de fluoreto insolúvel, respectivamente) e grupo V- dentifrício placebo de fluoreto. Os dentifrícios foram dosados quanto à concentração de fluoreto total (FT) e fluoreto solúvel total (FST), seguindo o protocolo estabelecido por Cury et al. (2010). Em cada fase, os participantes escovaram os dentes por 1 min com 0,7 g do respectivo dentifrício, expectoraram num frasco os resíduos da escovação e lavaram a boca com 15 mL de água purificada por 15 s. Saliva não estimulada foi coletada antes da escovação e nos tempos de 3, 6, 9, 15, 30, 45, 60 e 120 min após a escovação. As concentrações de FT e FST foram determinadas nos resíduos de escovação e nas amostras de saliva. Dentifrício, resíduo da escovação e saliva foram analisados com eletrodo íon específico acoplado a analisador de íon, previamente calibrado com padrões conhecidos de fluoreto. A biodisponibilidade do fluoreto na saliva foi avaliada pela área sob a curva (ASC), calculada tanto com a concentração de FT quanto de FST na saliva versus tempo (baseline a 15 min). Os valores de ASC e as concentrações de fluoreto nos resíduos da escovação e na saliva foram analisados estatisticamente por ANOVA, seguido de teste Tukey. Foram realizadas análises de correlação entre o FT nos dentifrícios e a ASC para FT na saliva e entre o FST nos dentifrícios e a ASC para FST. Nível de significância em α= 0,05 foi adotado em todas as análises. Quanto à análise de ASC para FT foram encontrados os seguintes resultados (p<0,0001): I.3,7±1,7^{B}; II.32,4±17,1^{A}; III.34,0±17,1^{A}; IV.32,1±14,7^{A}; V.34,2±18,2^{A} e para FST (p<0,0001): I.1,3±0,4^{C}; II.31,0±14,5^{A}; III.29,5±14,2^{A}; IV.24,6±9,8^{AB}; V.16,1±7,7^{B}. Em relação à concentração de F nos resíduos da escovação, as concentrações de FT encontradas foram (p<0,0001): I.10,5±1,4^{B}; II.196,6±47,5^{A}; III.199,0±35,2^{A}; IV.188,3±22,7^{A}; V.178,0±13,5^{A} e de FST (p<0,0001): I.1,3±1,6^{D}; II.206,1±59,0^{A}; III.168,7±31,9^{AB}; IV.113,6±24,8^{B}; V.59,1±13,1^{C}. Uma correlação significativa (r= 0,445; p=0,004) foi observada entre a concentração de FST no dentifrício e a ASC para FST, mas entre a concentração de FT no dentifrício e a ASC para FT na saliva não foi observado correlação (r=-0,018; p=0,911). Assim, os resultados sugerem que a concentração de fluoreto quimicamente solúvel presente no dentifrício à base de

MFP/$CaCO_3$ encontrada na dosagem química é um indicador do fluoreto disponibilizado na saliva pela escovação dentária.

Palavras-Chaves: fluoretos, dentifrícios, saliva, biodisponibilidade

ABSTRACT

Since there is no validated fluoride determination protocol in the toothpaste that shows a relationship between the concentration of soluble fluoride found chemically in the toothpaste and those present in the saliva after the brushing, this an in vivo, blind and crossed study with 5 experimental phases was performed. Ten adult participants were submitted to the following treatment groups I- fresh samples of Sorriso Dentes Brancos® (MFP/$CaCO_3$, 1450 µg F/g); Groups II to IV- Sorriso Dentes Brancos®, submitted to accelerated aging and presenting total soluble fluoride (TSF) concentrations of 1160, 900 and 597 ppm F (20, 40 and 60% of insoluble fluoride, respectively): V- non-F placebo toothpaste. The toothpaste were analyzed for the concentration of total fluoride (TF) and total soluble fluoride (TSF), following the protocol established by Cury et al. (2010). At each phase, participants brushed their teeth for 1 min with 0.7 g of the respective toothpaste, expectorated in a flask the brushing residues and rinsed the mouth with 15 mL of purified water for 15 s. Unstimulated saliva was collected before brushing and at times of 3, 6, 9, 15, 30, 45, 60 and 120 min after brushing. Concentrations of TF and TSF were determined on brushing residues and saliva samples. Dentifrice, brushing residue and saliva were analyzed with a specific ion electrode coupled to an ion analyzer, previously calibrated with known fluoride standards. Fluoride bioavailability in saliva was evaluated by the area under the curve (AUC), calculated either with the concentration of TF or TSF in saliva versus time (baseline at 15 min). The AUC values and the fluoride concentrations in the brushing residues and saliva were statistically analyzed by ANOVA, followed by Tukey's test. Correlation analyzes were performed between the FT in the dentifrices and the ASC for FT in saliva and between the FST in the dentifrices and the ASC for FST. A significance level of $\alpha = 0.05$ was adopted in all analyses. With regard to the analysis of AUC for TF, the following results were found ($p<0.0001$): I.3.7±1.7^{B}; II.32.4±17.1^{A}; III.34.0±17.1^{A}; IV.32.1±14.7^{A}; V.34.2±18.2^{A} and for TSF ($p<0.0001$): I.1.3±0.4^{C}; II.31.0±14.5^{A}; III.29.5±14.2^{A}; IV.24.6±9.8^{AB}; V.16.1±7.7^{B}. In relation to the F concentration in the brushing residues, the concentrations of TF found were ($p<0.0001$): I.10.5±1.4^{B}; II.196.6±47.5^{A}; III.199.0±35.2^{A}; IV.188.3±22.7^{A}; V.178.0±13.5^{A} and for TSF ($p<0.0001$): I.1.3±1.6D; II.206.1±59.0A; III.168.7±31.9^{AB}; IV.113.6±24.8^{B}; V.59.1±13.1^{C}. A significant correlation (r=0.445, p=0.004) was observed between the concentration of FST in the toothpaste and the ASC for FST, but between the concentration of FT in the toothpaste and the ASC for FT in saliva, no correlation was observed (r=0.018, p=0.911). Thus, the results suggest that the concentration of chemically soluble fluoride present in the MFP/$CaCO_3$-based toothpaste found in the chemical analysis is an indicator of the fluoride bioavailable in saliva by toothbrushing.

Key-words: fluorides, dentifrices, saliva, bioavailability

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é uma doença biofilme-açúcar dependente, que leva à dissolução progressiva do mineral da estrutura dental (desmineralização) devido aos ácidos produzidos por bactérias do biofilme, quando essas são expostas constantemente a carboidratos fermentáveis (Fejerskov e Kidd, 2018). A presença do biofilme dental é um fator necessário para o desenvolvimento da cárie, contudo, os açúcares da dieta são considerados o fator determinante negativo dessa doença (Tenuta e Cury, 2010). Devido, então, à sua etiologia, medidas para o controle da cárie dentária deveriam concentrar-se em ações de aconselhamento dietético, assim como na remoção ou desorganização regular do biofilme dental (Tenuta e Cury, 2010). Entretanto, essas medidas têm mostrado ação limitada, uma vez que 2,5 bilhões de pessoas no mundo ainda apresentam cárie dentária não tratada (Kassebaum et al., 2017).

Visto isso, sabe-se que atualmente o método preventivo capaz de atuar no controle da cárie dentária é o fluoreto (F) (Cury e Tenuta, 2008). E para que isso aconteça, esse íon deve ser encontrado no local certo (fluido do biofilme e saliva) e no momento ideal (quando o biofilme é exposto a açúcares e logo após a remoção do biofilme) para que possa interferir nos eventos de desmineralização e remineralização, uma vez que seu mecanismo de ação é local (Cury e Tenuta, 2008; Cury e Tenuta, 2009). O uso do fluoreto para o controle da doença cárie é efetivo, mesmo que ele não atue no acúmulo de biofilme (fator necessário) ou na produção de ácidos a partir dos açúcares (fator determinante), pois é capaz de contrabalancear as perdas minerais por meio da redução da desmineralização e da ativação da reposição mineral na forma de fluorapatita na estrutura dentária (Tenuta e Cury, 2010).

Dentre os meios de utilização de fluoretos, o dentifrício fluoretado é considerado o mais racional, uma vez que combina a remoção/desorganização do biofilme dental (efeito mecânico) com o aumento da concentração desse íon na cavidade bucal (efeito físico-químico) (Cury e Tenuta, 2008). Os dentifrícios são produtos que apresentam uma composição complexa, na qual, tipicamente, um abrasivo ou mistura deste é suspenso em uma fase aquosa e nela são adicionados os demais ingredientes, como por exemplo, agentes terapêuticos, compostos aromatizantes, corantes e conservantes (Lippert, 2013). Atenção deve ser dada aos componentes presentes nesses produtos, pois há a necessidade de uma combinação adequada entre o agente terapêutico fluoretado e o abrasivo utilizado, de forma a garantir a solubilidade do fluoreto (Lippert, 2013). Além disso, é necessário que os dentifrícios apresentem uma concentração mínima de 1.000 µg F/g para que tenham efeito anticárie (Walsh et al., 2010; Santos et al., 2013) e que esse fluoreto encontre-se em sua forma iônica (solúvel) (Cury e Tenuta, 2008).

Uma adequada combinação deve garantir que os dentifrícios apresentarão o fluoreto na sua forma ideal, solúvel, para atuar no processo da cárie dentária, uma vez que abrasivos à base de cálcio interagem com o fluoreto livre, ainda na bisnaga de dentifrício, tornando-o insolúvel e ineficaz (Lippert, 2013; Tenuta e Cury, 2013). De fato, as primeiras formulações de dentifrícios fluoretados, preparadas com sais de flúor do tipo fluoreto de sódio (NaF) e abrasivos à base de cálcio, foram incapazes de demonstrar um efeito anticárie significativo em ensaios clínicos (Stookey, 1985). Isso porque o NaF é altamente solúvel e, assim, o fluoreto iônico liberado reage com os íons cálcio presentes no abrasivo, formando sais de baixa solubilidade (Lippert, 2013).

Para que seja fosse possível a incorporação de abrasivos à base de cálcio, permitindo uma formulação de dentifrício que possua qualidade e ação anticárie, o monofluorfosfato de sódio é um sal compatível (Cury e Tenuta, 2014). Dentifrícios que apresentem esse sal podem ser formulados com abrasivos à base de cálcio, uma vez que o fluoreto presente encontra-se ligado covalentemente a um grupamento fosfato (FPO^{2-}_3) (Lippert, 2013; Tenuta e Cury, 2013). O íon monofluorfosfato liberará fluoreto solúvel na cavidade oral, devido à ação de fosfatases orais inespecíficas (Pearce e Jenkins, 1977). Contudo, a disponibilidade química do fluoreto nessas formulações precisa ser monitorada, uma vez que haverá a formação de sais insolúveis ao longo do tempo. Mesmo que a inativação do fluoreto não ocorra de forma significativa e imediata, haverá com o tempo a formação de sais insolúveis, pois a ligação entre o fluoreto e o fosfato é hidrolisada (Cury e Tenuta, 2014). Assim, uma redução significativa na concentração de fluoreto solúvel é observada com o tempo (Conde et al., 2003; Hashizume et al., 2003; Ricomini-Filho et al., 2012; Cury et al., 2015).

Sabe-se que uma concentração mínima de fluoreto solúvel para os dentifrícios apresentarem efeito anticárie é necessária. Assim, as legislações que regulamentam esses produtos deveriam garantir que esses mantivessem a concentração mínima durante o prazo de validade. Contudo, é observado que algumas legislações, como da União Europeia e do Mercosul, determinam apenas que os dentifrícios apresentem uma concentração máxima de fluoreto que não exceda 0,15% (1.500 µg F/g) (Mercosul, 2002; União Europeia, 2008). Por outro lado, a legislação americana, além de estabelecer o valor máximo de fluoreto total que os dentifrícios devem conter, exige que tenham uma determinada concentração mínima de fluoreto solúvel (US Food and Drug Administration, 2013; Bureau des Normes de Madagascar, 2017). Recentemente a Federação Odontológica Mundial (FDI) defendeu que os dentifrícios devem apresentar concentração total de fluoreto entre 1.000 e 1.500 µg F/g, mantendo um mínimo de 800 µg F/g de fluoreto biodisponível (FDI, 2018). A preocupação com a estabilidade do fluoreto

nos dentifrícios que utilizam cálcio como abrasivo é válida, uma vez que estudos já demonstraram que a concentração de fluoreto solúvel nesses produtos reduz com o tempo, de forma que, os valores de fluoreto podem atingir níveis que não apresentem efeito anticárie (Hashizume et al., 2003; Ricomini-Filho et al., 2012; Marín et al., 2017).

A dosagem química, utilizando o eletrodo íon específico (EIE) de fluoreto para a determinação de fluoreto total e (bio) disponível em dentifrícios é considerada o método mais comumente utilizado e simples (Martinez-Mier et al., 2018). Para que seja possível a dosagem de fluoreto utilizando o EIE, o fluoreto deve estar em sua forma iônica. Portanto em dentifrícios à base de MFP, na qual o fluoreto é comumente adicionado aos dentifrícios na forma ionizável (íon MFP - ainda não iônica), é necessário que uma hidrólise prévia seja realizada (Martinez-Mier et al., 2018). Por conta disso, uma técnica padronizada (Cury et al., 2010), a qual foi adaptada de Pearce (1974), é utilizada há quase 40 anos no laboratório de Bioquímica Oral da Faculdade de Odontologia de Piracicaba, Brasil. Nessa técnica é realizada a hidrólise ácida prévia, e assim, é possível a quantificação de fluoreto total e solúvel total, em dentifrícios à base de MFP, sendo isso demonstrado em diversos estudos de dosagem de dentifrícios de todo o mundo (Cury et al., 1981; Sarmiento et al., 1994; Hashizume et al., 2003; Cury et al., 2010; Carrera et al., 2012; Giacaman et al., 2013; Soysa et al., 2015). Com essa técnica, é possível estimar o fluoreto total, fluoreto solúvel total (iônico e ionizável, separadamente) e fluoreto insolúvel em dentifrícios (Cury et al., 2010).

Na literatura são relatadas diversas técnicas para dosagem de fluoreto em dentifrícios e, com o objetivo de reunir e avaliar essas técnicas, a Organização Europeia para Pesquisa de Cárie (ORCA) realizou em 2015 um workshop que discutiu detalhadamente as questões acerca da análise de fluoreto em dentifrícios, a fim de chegar a um consenso sobre a terminologia e as melhores práticas, sempre que as evidências disponíveis permitissem (Martinez-Mier et al., 2018). Dentre as necessidades discutidas nesse workshop, foi considerado o desenvolvimento de métodos para a avaliação do fluoreto (bio)disponível de forma a verificar se existe correlação entre a concentração de fluoreto solúvel total quimicamente determinado em um dentifrício e a concentração encontrada na cavidade oral durante e após a escovação dentária (Martinez-Mier et al., 2018).

Como a relação entre solubilidade do fluoreto no produto e biodisponibilidade bucal durante a escovação dental não tem sido estudada, o presente estudo teve como objetivo avaliar a relação entre a concentração de fluoreto quimicamente solúvel em dentifrício à base de MFP/$CaCO_3$ e fluoreto solúvel (biodisponível) na saliva por escovação de dentes.

2 ARTIGO

Chemically soluble fluoride in MFP/$CaCO_3$-based toothpaste as an indicator of fluoride bioavailable in saliva during and after toothbrushing

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Short title: Toothbrushing with F-toothpaste and bioavailable fluoride in saliva

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Key words: fluorides, toothpastes, saliva

Abstract

The relationship between the concentration of chemically soluble fluoride found in toothpaste and that present in saliva during and after brushing was evaluated as indicator of potentially bioavailable fluoride in toothpaste. Ten adult participants brushed their teeth with the assigned toothpastes: I fresh sample of a fluoride toothpaste: MFP/$CaCO_3$, 1,450 µg F/g of total fluoride (TF) and 1,378 of total soluble fluoride (TSF); Groups II to IV - aged samples of toothpaste presenting TSF concentrations of 1160, 900 and 597 µg F/g, respectively; V - non-F placebo toothpaste. The volunteers brushed their teeth for 1 min with 0.7 g of the toothpaste, all toothbrushing residues produced (TR) were collected, the mouth was washed and saliva samples were collected up to 120 min. TF and TSF concentrations were determined in TR and in saliva samples with specific electrode. TSF concentration (µg F/mL) in TR was determined as indicator of fluoride bioavailability during toothbrushing and the areas under curves of saliva fluoride concentration vs. time (AUC_{after}= µg F/mL x min) were calculated as indicator of fluoride bioavailability after toothbrushing. A significant correlation was found between the TSF concentrations in the toothpastes and the variables TR (r=0.850; p=0.0001) and AUC_{after} (r=0.445; p=0.004). For TF no significant correlation was found for TR (r=-0.099; p=0.542) and AUC_{after} (r=-0.018; p=0.912). The findings suggest that TSF concentration chemically found in MFP/$CaCO_3$-based toothpaste could estimate how much fluoride would be bioavailable in saliva when the teeth are brushed.

Introduction

The use of fluoride toothpaste to control caries is strongly based in evidence [Marinho et al., 2003] and the effect is concentration dependent, either for permanent [Walsh et al., 2019] or deciduous teeth [dos Santos et al., 2013]. Furthermore, it is well known from clinical trials conducted in the past that fluoride should be chemically soluble in the toothpaste formulation to be effective to prevent caries [Stookey, 1985]. The necessity to have soluble fluoride in the formulation is indeed very clear considering the mechanism of action of fluoride toothpaste on caries control [Tenuta and Cury, 2013].

Therefore, to guarantee that fluoride remains chemically soluble in the formulation during its expiry time after manufacturing [Cury et al., 2004], a toothpaste containing calcium in the abrasive ($CaCO_3$ or $CaH_2PO_4.2H_2O$) should not be formulated with NaF, SnF_2 or AmF salts [Lippert, 2013]. To formulate a toothpaste containing calcium in the abrasive, sodium monofluorophosphate (MFP) has been the fluoride salt used because MFP ion (PO_3F^{2-}) does not react immediately with calcium ion (Ca^{2+}) from the abrasive [Tenuta and Cury, 2013]. Moreover, toothpaste formulations containing MFP/$CaCO_3$ are considered affordable choices given that $CaCO_3$ is less expensive than silica as an abrasive and are mainly used in developing countries [Cury et al., 2004; Kikwilu et al., 2008; Cury et al., 2010; Benzian et al., 2012; Ricomini-Filho et al., 2012; Veeresh and Wadgave, 2014; Cury et al., 2016; Fernandéz et al., 2017; Soysa et al., 2018]. In addition, they are among the most consumed toothpastes [Cury et al., 2004; Ricomini-Filho et al., 2012]. However, MFP/Ca-based toothpastes are not chemically stable during the whole storage, because MFP ion is hydrolyzed, releasing fluoride ion that forms insoluble salts with Ca^{2+} into the toothpaste tube [Tenuta and Cury, 2013]. A toothpaste with MFP/Ca is usually formulated with 1450 μg F/g, close to the total maximum fluoride (0.15%) allowed for most legislations of the world [Mercosul, 2002; European Union, 2008]. As MFP/Ca toothpaste may not maintain during its expiry time enough chemically soluble fluoride to be effective on caries reduction, it is necessary to have a methodology to estimate if fluoride is bioavailable in the toothpaste formulation [Martinez-Mier et al., 2019].

Analytical laboratorial methods are used to determine total fluoride (soluble + insoluble) and total soluble fluoride (F ion + MFP ion) in toothpastes [Martinez-Mier et al., 2019]. The determination of total fluoride (TF) concentration in a toothpaste is important to check if it is in agreement with the local legislation of each country, but in terms of its therapeutic anti-caries effect the most important determination is the total soluble fluoride (TSF) concentration. Unfortunately, not all legislation require how much soluble fluoride a toothpaste should contain [US Food and Drug Administration, 2013; Bureau des Normes de

Madagascar, 2017]. Although there are protocols to determine TSF concentration in toothpastes [Cury et al., 1981; ADA, 2005; van Loveren et al., 2005; Cury et al., 2010], they were not validated to estimate how much fluoride is biocompatible to be released in the oral cavity during toothbrushing. A method of analysis that besides valid is feasible, reliable and inexpensive could encourage the adoption of TSF determination in toothpastes to guarantee its anti-caries benefit [FDI, 2018].

Cury et al. [1981] have used ion selective electrode (ISE) by the direct technique to determine TF and TSF in toothpastes. The protocol is feasible, reliable and inexpensive, and we hypothesized that the chemical determination of TSF in MFP/$CaCO_3$-based toothpaste, using this protocol described by Cury et al. [2010], may estimate the local bioavailability of fluoride in saliva when the teeth are brushed. This hypothesis was supported by the fact that it has been demonstrated that the gastrointestinal absorption (systemic effect) of fluoride from MFP/$CaCO_3$-based toothpaste depends on how much fluoride was chemically soluble in the formulation [Falcão et al., 2013]. Thus, the aim of the present study was to evaluate the relationship between the concentration of chemically soluble fluoride found in a toothpaste determined by Cury et al. protocol and the soluble fluoride (bioavailable) in saliva during and after toothbrushing.

Materials and Methods

Ethical Considerations

This study was approved by the Ethics Committee of Piracicaba Dental School (protocol No. 70493717.0.0000.5418), conducted according to the guidelines of the Helsinki Declaration, and registered as a clinical trial [ReBEC, ensaiosclinicos.gov.br/rg/RBR-9hspyy/, RBR-9hspyy]. All volunteers signed informed consent forms.

Experimental design and participants

Ten healthy volunteers (5 men and 5 women; 23-34 years old) were recruited to participate in the experiment. The volunteers had normal salivary flow (unstimulated: >0.35 mL/min) and lived in Piracicaba, São Paulo, Brazil, a city with optimally fluoridated water (0.6-0.8 mg F/L).

The study was single blind (with respect to the volunteer) with a crossover design and consisted of five experimental phases, with the following treatments: Group I - fresh samples of Sorriso Dentes Brancos® (MFP/$CaCO_3$, 1450 µg F/g of TF and 1378 µg F/g of TSF; manufactured and donated by Colgate-Palmolive; batch 8198BR122J; expiration date

July/2020); Groups II to IV - aged samples of Sorriso Dentes Brancos® presenting total soluble fluoride (TSF=F ion+MFP ion) concentrations of 1160, 900 and 597 µg F/g [20, 40 and 60% of insoluble fluoride (InsF), respectively]; Group V - - non-F placebo toothpaste (manufactured and donated by Colgate-Palmolive from Brazil). In each phase, all volunteers were subjected to one of the treatments, whose sequence was: I, II, III, V and IV. The volunteers brushed their teeth for 1 min with 0.7 g of the respective toothpaste and the toothbrushing residues (toothpaste+saliva slurry; TR) were collected. Then, they rinsed their mouth with 15 mL of purified water for 15 s and expectorated [Serra and Cury, 1992]. Unstimulated whole saliva samples were collected before brushing (baseline) and at 3, 6, 9, 15, 30, 45, 60 and 120 min after brushing. Concentrations of TF (TSF + InsF) and TSF were determined on toothbrushing residues and saliva samples with ion specific electrode (ISE). The response variables were: TF and TSF (µg F/mL) in saliva and in the TR and AUC (µg F/mL in saliva vs. time) for TF and TSF. The null hypothesis tested (p<0.05) was that the chemical concentration of TSF in MFP/$CaCO_3$-based toothpaste would not correlate with the fluoride bioavailability in saliva during and after toothbrushing. Correlation tests were used as: 1) indicator of fluoride bioavailability during toothbrushing through the correlation between TF and TSF concentrations in the brushing residues and the TF and TSF concentrations in the toothpastes. 2) Indicator of fluoride bioavailability of toothbrushing through the correlation between the AUC (F vs. time) of TF and TSF in saliva and the TF and TSF concentrations in the toothpastes.

Toothpastes and Accelerated Aging

Fresh samples of the toothpaste Sorriso Dentes Brancos® [MFP/$CaCO_3$, 1450 µg F/g of TF; 1378 µg F/g of TSF (5% InsF)] were submitted to accelerated aging at 55°C [Tabchoury and Cury, 1994] to obtain samples with different percentages of TSF required for the study. After 10, 34 and 78 days, the toothpastes reached 1160, 900 and 597 µg F/g of TSF, respectively. When the desired TSF concentration was reached, the respective tubes were removed from the incubator and within three days they were used in the experiment.

Determination of fluoride concentration in toothpastes

The analysis was made according to the protocol used since 1980 in the Laboratory of Oral Biochemistry at Piracicaba Dental School as described by Cury et al. [2010]. An amount from 90 to 110 mg of toothpaste was weighed (±0.01 mg), homogenized in 10.0 mL of purified water and duplicates of 0.25 mL of the suspension were transferred to test tubes for TF analysis. The remaining of the suspension was centrifuged (3,000 g, 10 min, room temperature) to

remove insoluble fluoride (InsF) bound to the abrasive and abrasive particles. Duplicates of 0.25 mL of the supernatant were transferred to plastic assay tubes to determine TSF. For the TF and TSF tubes, 0.25 mL of 2.0 M HCl was added and, after 1 h at 45 ºC, the samples were neutralized with 0.5 mL of 1.0 M NaOH and buffered with 1.0 mL of TISAB II (1.0 M acetate buffer, pH 5.0, containing 1.0 M NaCl and 0.4% CDTA). The analyses were carried out using ISE (Orion 96-06; Orion Research Inc., Boston, MA, USA) coupled to an ion analyzer (Orion Star A214; Orion Research Inc.), previously calibrated with F standards containing 0.0625 to 4.0 µg F/mL, 0.25 M HCl, 0.25 M NaOH and TISAB II 50% (v/v). F concentration in the samples was determined from linear regression of the logarithm of F concentrations of the standards with the respective mV values (r^2=0.9999), using Excel spreadsheet (Microsoft®). The InsF concentration was estimated from the difference between TF and TSF concentration found in the analyses [Cury et al., 2010]. The average variation coefficients of the repeated determination (duplicate) was of 1.1% (n=58).

Volunteers' sample size determination

The sample size was calculated from data of a pilot study, which indicated that eight volunteers would allow a power of 80% and an α-value of 5% to differentiate by AUC the effect of toothpastes containing 950 and 580 µg F/g of TSF. A 20% increase in sample size was considered due to the possibility of eventual losses during the study.

***In vivo* study**

The experiment was always conducted in the morning and at least 2 h after meals and oral hygiene. Unstimulated whole saliva was collected for 2 min before starting the experiment (baseline data). Subsequently, each volunteer brushed their teeth with 0.7 g of the assigned toothpastes for 1 min in their habitual way. All saliva and toothpaste slurry produced (named toothbrushing residues, TR) was collected by expectoration to determine fluoride concentration generated into the mouth during the toothbrushing. The volunteers rinsed the oral cavity with 15 mL of purified water for 15 s [Serra and Cury, 1992] and this residue was discarded. Unstimulated whole saliva was then collected for 2 min at 3, 6, 9, 15, 30, 45, 60 and 120 min after the end of the brushing, as indicator of fluoride bioavailability after toothbrushing. During the collection of saliva samples, the volunteers were instructed to refrain from talking during the first 15 min, period of time when depuration of salivary fluoride is observed, and from eating or drinking during the experimental period. This recommendation was followed to avoid salivary stimulus.

A lead-in and washout periods of at least 3 days [Fernández et al., 2015], when the volunteers brushed their teeth with the non-F placebo toothpaste, was established between each phase and, during this period, it was also recommended for the volunteers to refrain from consuming black tea, a high-fluoride drink.

Fluoride determination in brushing residues and saliva

The toothbrushing residues (TR) produced during toothbrushing and saliva samples collected after were agitated and aliquots of 0.1 and 0.2 mL were collected for TF and TSF determinations, respectively. For TSF, the aliquots were centrifuged at 16,000 *g* for 10 min and 0.1 mL of the supernatants were transferred to plastic assay tubes. To TF and TSF tubes, 0.1 mL of 2.0 M HCl was added and, after 1 h at 45 °C, the samples were buffered and neutralized with 0.2 mL of TISAB II containing 1.0 M NaOH. The F concentrations were determined using the ISE (Orion 96-06; Orion Research Inc., Boston, MA, USA) coupled to an ion analyzer (Orion Star A214; Orion Research Inc.). The electrode was calibrated with standards F solutions, ranging from 0.0312 to 8 µg F/mL, mixed with 0.5 M HCl and TISAB II (containing 1 M NaOH) at 50% (v/v). The accuracy of the analyses was validated using internal standards (coefficient of variation of 1.3% of triplicates). A linear regression between F concentration in the standards and mV values (r^2=0.9995) was constructed with Microsoft Excel software and used to calculate the F concentration (µg/mL) in each sample. The area under the curve (AUC) for TF and TSF concentration in saliva vs. time (from baseline up to 15 min) was calculated (Microsoft® Office Excel, 2013). The cutoff point at 15 min was established because the fluoride toothpaste groups did not differ ($p>0.05$) on fluoride concentrations in saliva collected at 30, 60, 90 and 120 min.

Statistical analysis

The normality distribution of data was checked for each response variable using the Kolmogorov-Smirnov test. The TSF in toothbrushing residues did not present parametric data and was transformed into log10. The AUC (TF and TSF concentration vs. time) and TF and TSF concentrations in the toothbrushing residues and saliva were compared using analysis of variance (ANOVA). Tukey's test was used as a post hoc test. Correlation tests were performed between 1) the concentrations of TF and TSF in TR and the respective concentrations of TF and TSF in the toothpastes and 2) the AUC_{after} (F vs. time) of TF and TSF in the saliva and the concentrations of TF and TSF in the toothpastes. The level of significance was set at 5% and

analyzes were performed on SPSS® Statistic 21.0 software (SPSS for Windows, version 21.0, SPSS Inc., Chicago, IL, USA).

Results

All participants completed the study. Table 1 shows the concentrations of TF, TSF and InsF in the fresh and aged samples of the toothpastes used in the experiment. The toothpastes of the treatment groups I, II, III and IV presented the same concentration of TF and distinct concentrations of TSF, suitable to test the hypothesis under study.

Table 1. Concentrations (µg F/g) of total fluoride (TF), total soluble fluoride (TSF) and insoluble fluoride (InsF) in the fresh and aged samples of toothpaste Sorriso Dentes Brancos® (mean±SD of 3 tubes)

Fluoride Forms (µg F/g)	Formulations				
	Fresh Sample Group I	Aged Samples			Non-F Placebo Group V
		Group II	Group III	Group IV	
TF	1437.8±21.7	1449.9±6.0	1480.0±15.0	1454.3±33.7	81.6±0.5
TSF	1378.2±24.2	1160.9±15.7	900.0±13.1	597.2±32.5	<7.5±1.0*
InsF	59.6±11.3	289.0±18.4	580.0±12.7	857.1±53.4	74.1±0.8

*Below the detection limit of the electrode

Figure 1 shows TF concentration (a) and TSF (b) in the toothbrushing residues (TR) collected during toothbrushing. In terms of TF or TSF, all fluoride groups (I-IV) differed statistically from the placebo group (p<0.0001). However, while the difference among the fluoride groups was not statistically significant for TF (Figure 1a), they differed for TSF (Figure 1b). Furthermore, the fluoride group presenting the lowest TSF concentration in the toothpaste (Group IV) also showed the lowest TSF in the residues of toothbrushing (Figure 1b). A significant correlation was found between the TSF concentrations in the toothpastes and in TR (r=0.850; p=0.0001), but not for TF (r=-0.099; p=0.542).

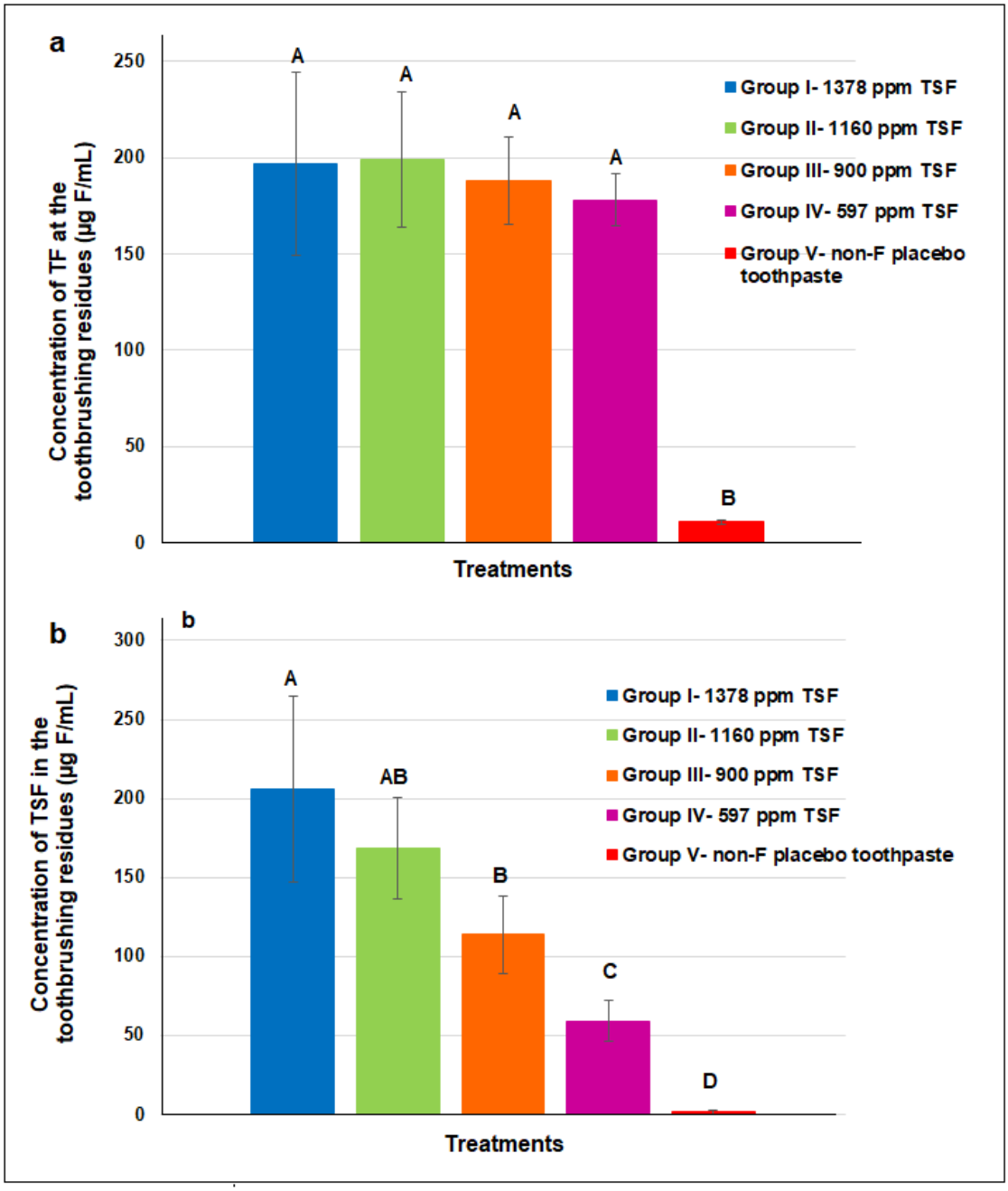

Fig. 1. Mean (±SD; n = 10) TF (a) and TSF (b) concentrations (µg F/mL) in the slurry toothpaste saliva produced during 1 min of toothbrushing, according to treatment groups. Distinct letters indicate statistically significant differences among the groups (p<0.0001).

Regarding fluoride concentration retained in saliva after toothbrushing, figure 2 shows the kinetics overtime and the respective values of AUC (µg F/mL vs. min) for TF (2a) and TSF (2b). The AUC for all fluoride groups (I-IV) was statistically different (p<0.0001) from the non-F placebo group, either for TF (Figure 2a) or TSF (Figure 2b). The difference among the fluoride groups in terms of AUC was also not statistically significant for TF (Figure 2a), but

they differed for TSF (Figure 2b). A significant correlation was found between the TSF concentrations in the toothpastes and AUC (r=0.445; p=0.004), but not for TF (r=-0.018; p=0.912).

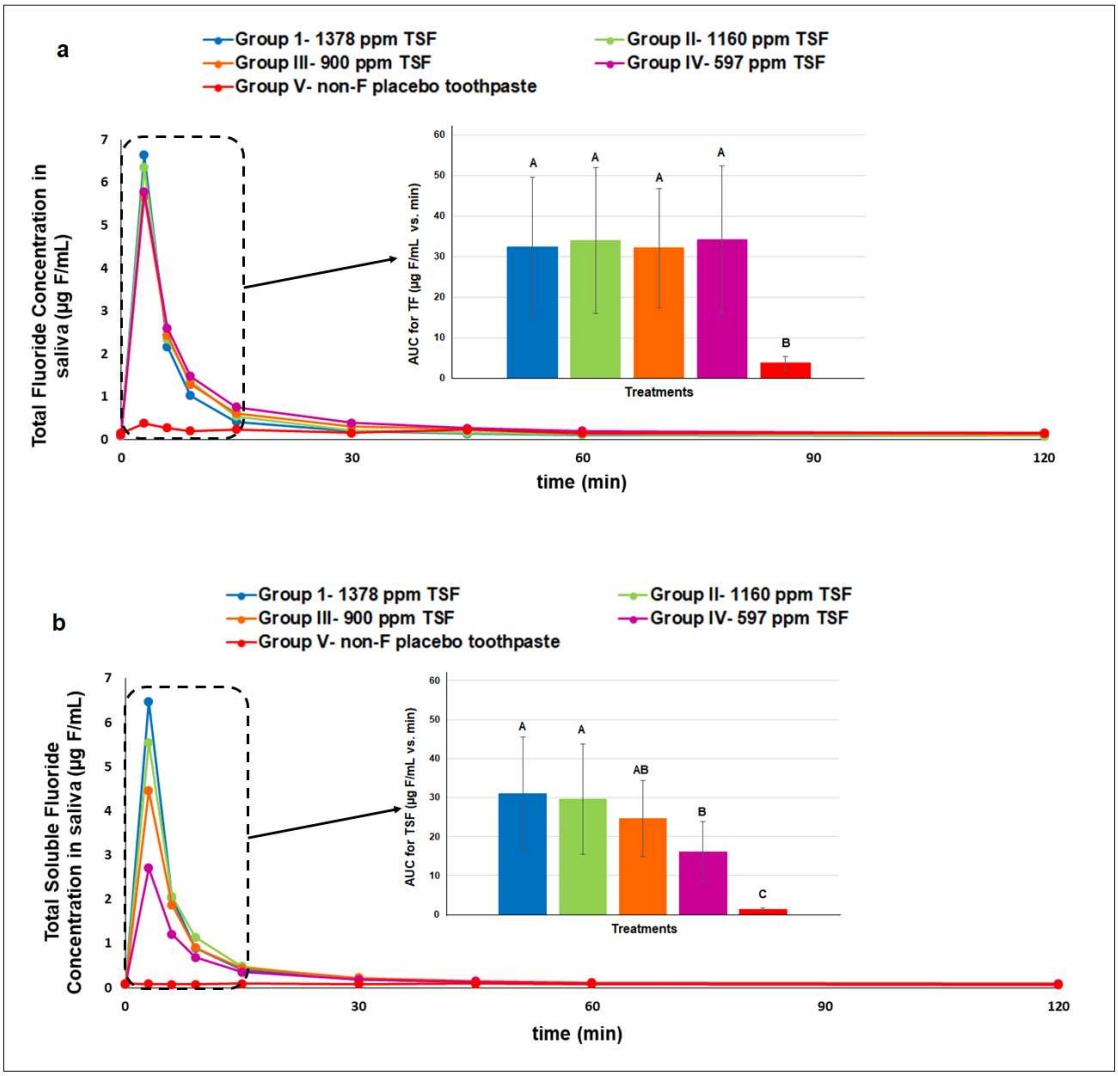

Figure. 2. Mean (± SD; n=10) of TF (a) and TSF (b) concentrations (µg F/mL) in saliva before brushing (baseline) and retained in the mouth overtime after the end of toothbrushing, the slurry generated has been expectorated and the mouth washed, according to the treatment groups. Bar graphics illustrate, the respective areas under the curve (AUC) of fluoride concentration in saliva versus time (µg F/mL vs. min); distinct letters indicate statistically significant differences among the groups (p<0.0001).

Discussion

Fluoride use is considered the backbone strategy to control caries at community, professional and individual level, and toothpaste is considered not only the most rational way

of fluoride use but the strongest one based on scientific evidence [Marinho et al., 2003]. However, fluoride must be chemically soluble in any toothpaste formulation to be released in the oral cavity during toothbrushing to interfere with the physicochemical process of caries, either reducing demineralization of sound enamel-dentine mineral structure or enhancing remineralization of incipient caries lesions [Cury and Tenuta, 2008; Cury and Tenuta, 2009]. Based on this premise and the necessity of research to evaluate the relationship between the concentration of soluble fluoride present in toothpaste and the concentration found in saliva during and after brushing [Martinez-Mier et al., 2019], this study was done with MFP/$CaCO_3$-based toothpaste.

For this research, the brand of toothpaste was fixed (Table 1) and the variable under study was the concentration of total soluble fluoride (TSF = ion MFP + ion F) in the toothpaste, which was obtained by standardized time of accelerated aging [Tabchoury and Cury, 1994]. Thus, the toothpaste used in each treatment group had the same TF concentration but distinct concentrations of TSF. In addition, a placebo non-F toothpaste was formulated and used as a negative control group (group V). The concentrations of TSF in the groups II, III and IV (Table 1) are usually found after approximately one, two and three years of manufacture of MFP/$CaCO_3$-based toothpastes formulated with 1,450 μg F/g as TF [Conde et al., 2003; Benzian et al., 2012; Carrera et al., 2012; Ricomini et al., 2012; Cury et al., 2015; Fernández et al., 2017; Marin et al., 2017].

The data showed clearly (Figures 1 and 2) that all fluoride toothpaste groups (I-IV) differed from the non-F placebo toothpaste (Group V) in terms of TF and TSF concentration in the toothpaste slurry produced during the 1-min of toothbrushing (Figure 1) and that retained in the mouth after toothbrushing residues (TR) have been expectorated and the mouth washed (Figure 2). Even the group IV, presenting the lowest TSF concentration (597.2 ppm F), was able to increase (Figure 1b) and maintain (Figure 2b, AUC data) statistically greater fluoride concentration in the mouth than the non-F group V. Considering that low fluoride toothpaste (500-600 ppm F) found in the market presents all fluoride chemically soluble, because they are NaF/silica-based formulations [Lippert, 2013], a MFP/$CaCO_3$-based toothpaste formulated with 1,450 μg F/g as TF would be after 3 years of fabrication equivalent in terms of benefits and risks to a NaF/silica-based toothpaste containing around 500-600 ppm F [Falcão et al., 2013; Oliveira et al., 2013]. The relevance of chemically soluble fluoride (potentially bioavailable) in a toothpaste formulation containing Ca in abrasive will be discussed further.

When the F-toothpaste groups are compared in terms of fluoride concentration in the slurry produced in the mouth during the 1-min of toothbrushing (Figure 1), a linear dose-

response effect was found for TSF (r=0.850; *p*=0.0001), but not for TF (r=-0.099; *p*=0.542). The absence of correlation between TF concentration in the toothpastes and TF concentrations in toothbrushing residues (TR) was expected, because the toothpastes of the groups I-IV presented the same concentration of TF (around 1,450 ppm F). However, the amount of the TSF present in the toothpastes (fluoride chemically soluble) that would be released in the mouth during the toothbrushing (bioavailable fluoride) was unknow and the findings give support to our hypothesis because a statistically significant high and linear correlation was found between the variables under study.

When the groups I-IV are compared, the lowest bioavailability was found for group IV, whose TSF fluoride concentration in the toothpaste (597.2 ppm F) was from 1.5 to 2.3 lower than the other groups. Therefore, for toothpastes containing Ca in the abrasive the concentration of TF found chemically in formulation has no relevance in terms of the anticaries benefit of fluoride (local effect), because only the soluble fraction (TSF) is potentially available to be released in the oral cavity during toothbrushing. Our findings give support to the discussion that the legislations about F-toothpaste used in most countries of the world [Cury et al., 2015] must be changed, because they establish only the maximum concentration of TF (1500 ppm F) that a toothpaste should contain and not how much should be present as TSF to be anticaries effective. This change is necessary to guarantee that affordable toothpastes formulated with MFP/$CaCO_3$, which are the most consumed in developing countries, may benefit the most vulnerable population against caries [Cury et al., 2015]. Political decision in this direction was recently stated by FDI declaration [FDI, 2018] about how much soluble fluoride a toothpaste should contain and the present findings give strong support for this change. In addition, the data corroborate the discussion [Oliveira et al., 2013] about the systemic effect of fluoride when Ca-based toothpaste is ingested (risks), because the gastric and intestinal absorption also depends on the TSF present in the formulation and not the TF [Ekstrand and Ehrnebo, 1980; Roldi and Cury, 1986; Falcão et al., 2013].

The current findings, showing that fluoride released in the mouth during toothbrushing with MFP/$CaCO_3$ toothpaste (Figure 1) depends on TSF concentration chemically found but not on TF, were confirmed by the data of fluoride concentration retained in the mouth during certain time after the toothpaste slurry has been spat and the mouth has been washed (Figure 2). Figure 2a and 2b show that the kinetics of fluoride in saliva for TF and TSF follows the classical pattern described by Duckworth and Morgan [1991]. The data of AUC and the linear correlation found confirm that the concentration of TSF in the toothpastes, but not TF, must be the parameter considered to estimate the anticaries potential of toothpaste

Ca-based. Thus, it reinforces the importance of changes in toothpaste regulations, to emphasize TSF concentration.

Furthermore, when the data of TSF concentration in the slurry produced during toothbrushing are compared with the concentration in saliva after the toothbrushing (Figure 1b vs. 2b) the ratio was around 28 times greater for the former. Thus, in terms of mechanism of action of fluoride from toothpaste [Tenuta and Cury, 2013], the concentration of fluoride generated in the oral cavity during toothbrushing may be more important than that retained after toothbrushing. Thus, during the toothbrushing, total fluoride present in toothpaste is spread through saliva [Cury and Tenuta, 2008; Zamataro et al., 2008], but only the chemically soluble one is able to diffuse into the remaining biofilm [Cenci et al., 2008; Cury et al., 2010] and to react with clean tooth surfaces [Tenuta et al., 2009]. To positively affect the balance of de-remineralization towards mineral gain, fluoride must be chemically soluble in any toothpaste formulation [Tenuta and Cury, 2013].

Overall, the results give support to experimental and clinical data showing that there is dose-response effect of fluoride concentration in toothpaste and enamel demineralization reduction [Queiroz et al., 2008; Cury et al., 2010; Ortiz Ade et al., 2016] or caries prevention [dos Santos et al., 2013; Walsh et al., 2019]. For toothpastes containing silica as abrasive, the concentration of TF is equal TSF because insoluble fluoride is not formed [Cury et al., 2015], but for toothpastes containing Ca as abrasive ($CaCO_3$ or $CaH_2PO_4.2H_2O$) the concentration of TSF, and not TF, may be considered to estimate the anticaries benefit and the risk of this kind of formulation. The present findings were shown for toothpaste containing MFP/$CaCO_3$ and should be confirmed for MFP/$CaH_2PO_4.2H_2O$ formulation. Also, the differences found in salivary fluoride according to the concentration of TSF present in the MFP/$CaCO_3$ could be supported by data of enamel-dentine reduction of demineralization or enhance of remineralization, although is known for a long time that fluoride should be chemically soluble in a toothpaste formulation to be active against caries [Stookey, 1985].

In summary, this study shows for the first time that the concentration of soluble fluoride chemically determined in MFP/$CaCO_3$-based toothpaste can be used as indicator of fluoride post-toothbrushing bioavailability in the oral cavity. The findings have worldwide impact in the legislations about the anticaries quality of fluoride toothpaste market.

Acknowledgements

We thank the volunteers for their valuable participation. This study was partially supported by an agreement (4887.1) managed by FUNCAMP, the Coordenação de Aperfeiçoamento de

Pessoal de Nível Superior - Brazil (CAPES) (Finance Code 001) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Cnpq) (Finance 435955/2018-7). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Partial data of this study were presented at the 66th ORCA meeting.

Statement of Ethics

This study was approved by the Research Ethics Committee of the Piracicaba Dental School and conducted according to the guidelines of the Helsinki Declaration.

Declaration of Interests: The authors have no conflicts of interest to declare.

Author Contributions

Conceived and designed the experiment: JAC, CPMT; Performed the experiment: CSSC; Analyzed the data: CSSC, CPMT, JAC; Wrote the paper: CSSC, CPMT, JAC.

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3 CONCLUSÃO

Os resultados sugerem que a concentração de fluoreto quimicamente solúvel encontrada no dentifrício à base de MFP/$CaCO_3$, pelo protocolo de dosagem química descrito por Cury et al. (2010), é um indicador da biodisponibilidade do fluoreto na cavidade oral pós-escovação dentária.

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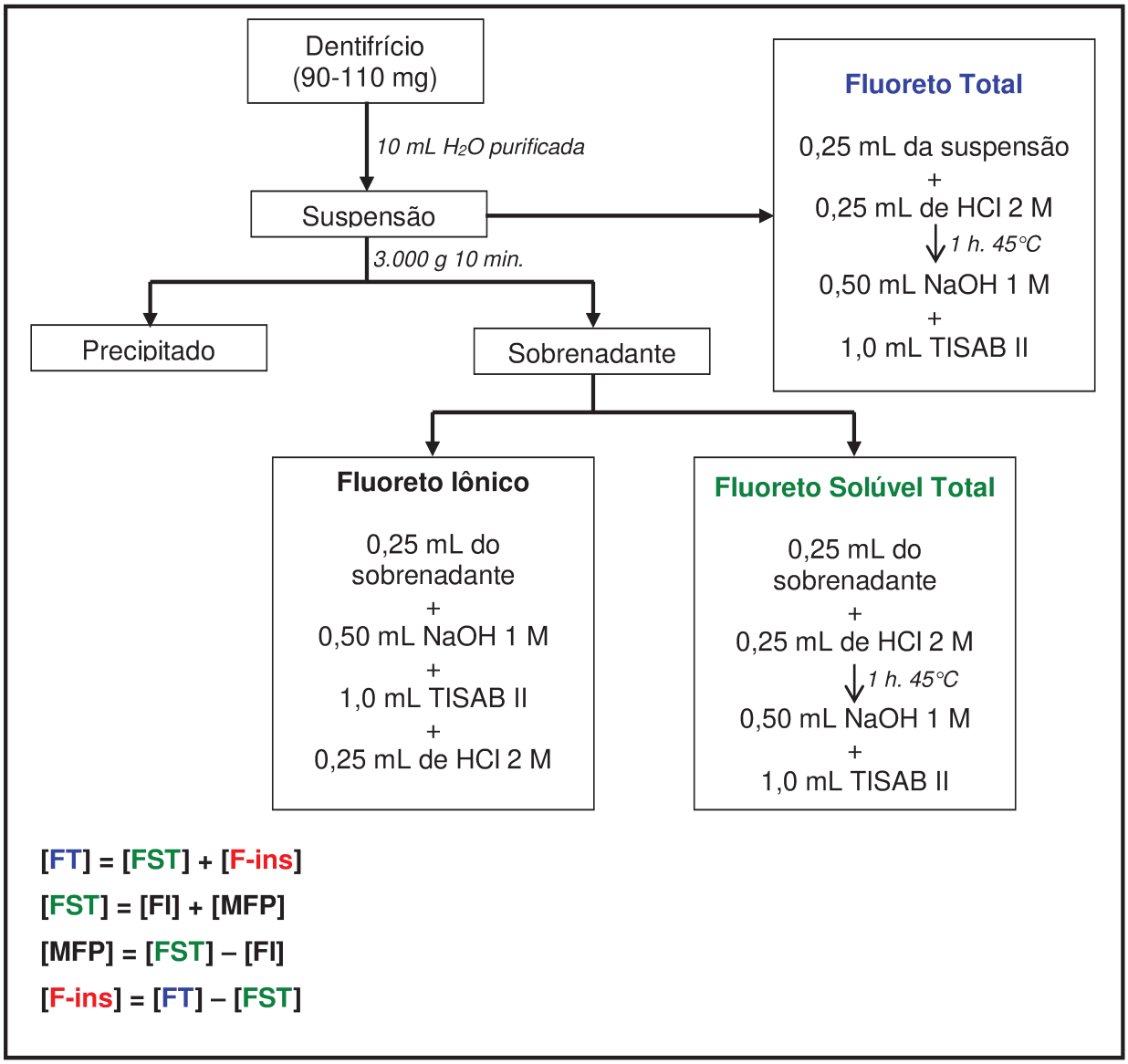
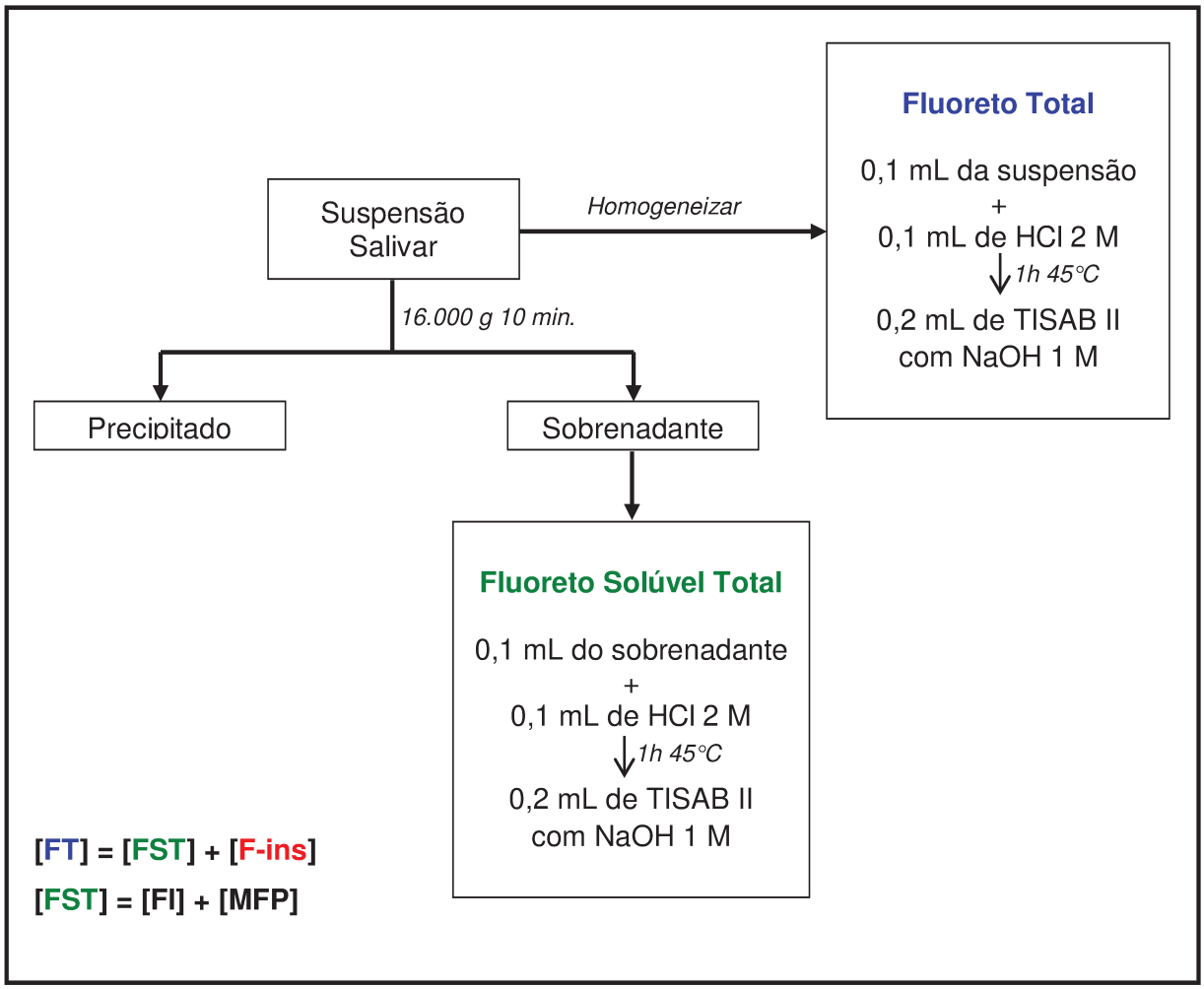
APÊNDICE 1 – Fluxograma da dosagem de fluoreto nos dentifrícios



APÊNDICE 2 – Fluxograma da dosagem de fluoreto na saliva



ANEXO

ANEXO 1 – Verificação de originalidade e prevenção de plágio

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ANEXO 2 – Certificado De aprovação do Comitê de Ética

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa "**Fluoreto quimicamente solúvel encontrado em creme dental como preditor da biodisponibilidade de fluoreto na saliva**", CAAE **70493717.0.0000.5418**, dos pesquisadores **Cínthia Pereira Machado Tabchoury, Camila Siqueira Silva Coelho** e **Jaime Aparecido Cury**, satisfaz as exigências das resoluções específicas sobre ética em pesquisa com seres humanos do Conselho Nacional de Saúde – Ministério da Saúde e foi aprovado por este comitê em sua versão original em 09/08/2017 e na versão emendada em 03/10/2017.

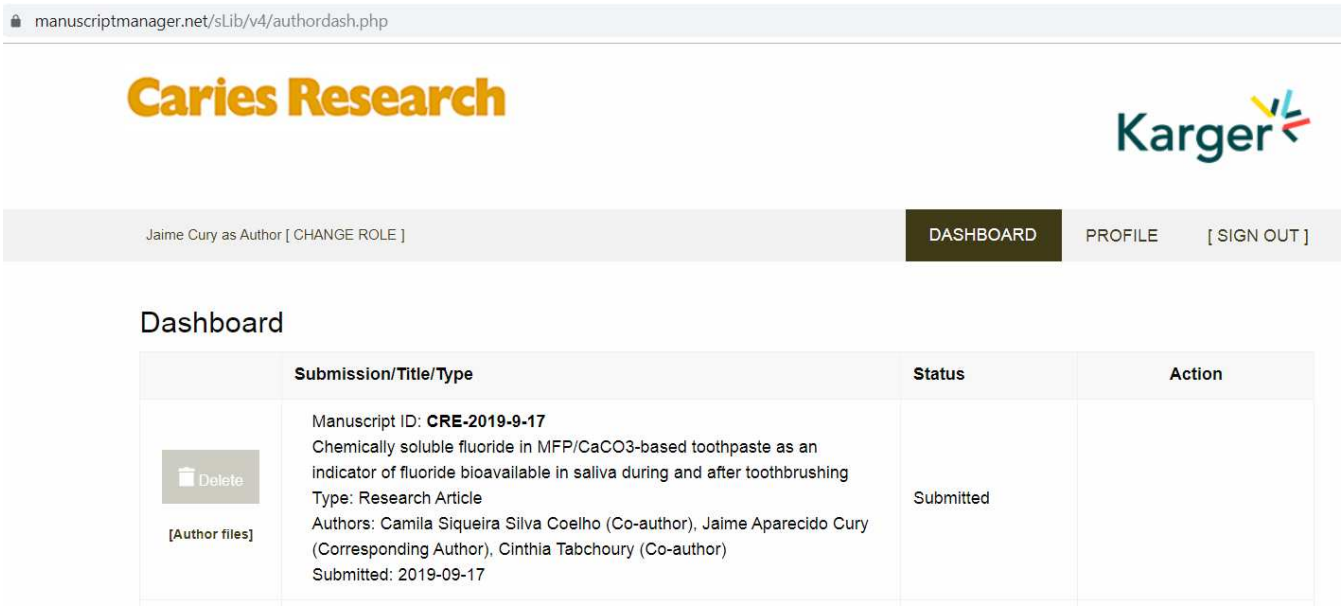
The Research Ethics Committee of the Piracicaba Dental School of the University of Campinas (FOP-UNICAMP) certifies that research project "**Chemically soluble fluoride found in toothpaste as a predictor of the bioavailability of fluoride in saliva**", CAAE **70493717.0.0000.5418**, of the researcher's **Cínthia Pereira Machado Tabchoury, Camila Siqueira Silva Coelho** and **Jaime Aparecido Cury**, meets the requirements of the specific resolutions on ethics in research with human beings of the National Health Council - Ministry of Health, and was approved by this committee on 9th of August of 2017 (original version) and 3rd of October of 2017 (amended version).

Profa. Fernanda Miori Pascon
Vice Coordenador
CEP/FOP/UNICAMP

Prof. Jacks Jorge Junior
Coordenador
CEP/FOP/UNICAMP

Nota: O título do protocolo e a lista de autores aparecem como fornecidos pelos pesquisadores, sem qualquer edição.
Notice: The title and the list of researchers of the project appears as provided by the authors, without editing.

ANEXO 3 - Certificado de submissão do artigo



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Delete [Author files]	Manuscript ID: **CRE-2019-9-17** Chemically soluble fluoride in MFP/CaCO3-based toothpaste as an indicator of fluoride bioavailable in saliva during and after toothbrushing Type: Research Article Authors: Camila Siqueira Silva Coelho (Co-author), Jaime Aparecido Cury (Corresponding Author), Cinthia Tabchoury (Co-author) Submitted: 2019-09-17	Submitted	