DÉBORA DRUMMOND HAUSS MONTEIRO

EFEITO DO FLUXO SALIVAR NA RUGOSIDADE ECONTEÚDO MINERAL DO ESMALTE CLAREADO:

ESTUDO IN SITU E IN VITRO

Faculdade de Odontologia
Universidade Federal de Minas Gerais
Belo Horizonte
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Tese apresentada ao Colegiado de Pós-graduação em Odontologia da Faculdade de Odontologia da Universidade Federal deMinas Gerais, como requisito parcial à obtenção do grau de doutor em Odontologia, área de concentração em Clínica Odontológica.

Orientadora: Profa. Dra. Cláudia Silami de Magalhães

Coorientador: Prof. Dr. Allyson Nogueira Moreira

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FOLHA DE APROVAÇÃO

EFEITO DO FLUXO SALIVAR NA RUGOSIDADE E NO CONTEÚDO MINERAL DO ESMALTE CLAREADO: ESTUDO *IN SITU* E *IN VITRO*

DÉBORA DRUMMOND HAUSS MONTEIRO

Tese submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Odontologia, como requisito para obtenção do grau de Doutor, área de concentração Clínica Odontológica.

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RESUMO

Este estudo avaliou o efeito do contato com a saliva *in vitro* e do fluxo salivar normal e reduzido *in situ* na rugosidade e na composição de cálcio e fósforo do esmalte, após clareamento com peróxido de hidrogênio 35%. Espécimes obtidos de terceiros molares (5 x 5 mm) foram divididos em 5 grupos (n=15), G1: não clareado e não exposto à saliva; G2: clareado e não exposto à saliva; G3: clareado e mantido em saliva natural *in vitro*, G4: clareado e mantido em saliva humana *in situ* em voluntários com fluxo salivar normal, G5: clareado e mantido em saliva humana *in situ* em voluntários com baixo fluxo salivar. A rugosidade (*Ra*, *Rz*), a proporção cálcio/fósforo e as porcentagens de cálcio e fósforo do esmalte foram avaliadas, respectivamente, por perfilometria a laser 3D e espectroscopia de energia dispersiva por raios X, antes do clareamento (T1), após o clareamento (T2) e após contato com saliva (T3). A saliva dos participantes foi coletada e o fluxo salivar foi medido para alocação nos grupos 4 e 5. O pH salivar e a capacidade tampão foram avaliados por fitas medidoras.As concentrações de cálcio e fósforo salivar foram determinadas por espectrofotometria de absorbância. Os dados foram analisados por testes não paramétricos para análise entre grupos e entre os tempos. Um modelo de regressão linear foi ajustado para a variável dependente rugosidade do esmalte dos grupos 4 e 5 em T3, considerando as covariáveis fluxo salivar, pH, capacidade tampão e concentração de cálcio e fósforo salivar ($p<0,05$). Não houve diferença de rugosidade (*Ra*, *Rz*) entre grupos em T1 ($p>0,05$). Em T2, G1 diferiu de todos os grupos. Em T3, G5=G2>G3=G4=G1. Para G1, a rugosidade de T1=T2=T3. Para G2 e G5 T1<T2=T3. Para G3, T1<T3<T2; para G4, T1=T3<T2. Esses achados mostraram um aumento da rugosidade com o clareamento e diminuição com o contato com a saliva, exceto quando houve exposição ao baixo fluxo salivar. A proporção cálcio/fósforo e as porcentagens isoladas de cálcio e fósforo no esmalte não foram alteradas com o clareamento e nem com o contato com a saliva, não havendo diferença estatisticamente significativa entre os grupos ($p=0,514$) e nem entre T1, T2 e T3 ($p>0,05$). A rugosidade Ra foi em média 0,14 menor no grupo de fluxo salivar normal comparado ao grupo de fluxo reduzido, enquanto Rz foi em média 1,95 menor no grupo fluxo salivar normal. Conclui-se que o contato com a saliva humana *in vitro* e com o fluxo salivar normal *in situ* restabeleceu a rugosidade do esmalte. O baixo fluxo salivar *in situ* não restabeleceu a rugosidade inicial. O clareamento dentário e o contato com a saliva humana não alteram a proporção cálcio/fósforo e nem suas porcentagens isoladas no esmalte. A recuperação da rugosidade do esmalte clareado foi maior em fluxo salivar normal que em baixo fluxo salivar, independentemente do pH salivar e de sua capacidade tampão.

Palavras-Chave: Clareamento dental. Esmalte dentário. Espectrofotometria. Peróxido de hidrogênio. Saliva.

ABSTRACT

Effect of salivary flow on bleached enamel roughness and mineral content: an *in vitro* and *in situ* study.

This study evaluated the effect of saliva *in vitro* and regular or low salivary flow *in situ* on roughness, calcium/phosphorus ratio and calcium and phosphorus percentages of the enamel bleached with 35% hydrogen peroxide. Seventy-five specimens of third molars were divided in 5 groups, G1: Not bleached and not exposed to saliva; G2: Bleached and not exposed to saliva; G3: Bleached and stored in natural saliva *in vitro*, G4: Bleached and exposed to human saliva *in situ* in normal salivary flow participants, G5: Bleached and exposed to human saliva *in situ* in low salivary flow participants. Roughness (*Ra*, *Rz*) was evaluated with a 3D laser non-contact profilometer. Calcium/phosphorus ratio and calcium and phosphorus percentages were determined with energy- dispersive X-Ray spectrophotometry. These evaluations were performed before bleaching (T1), after bleaching (T2) and after the contact with saliva (T3). Participants saliva was collected and salivary flow was measured for their allocation in groups 4 and 5. Salivary pH and buffering capacity were evaluated with measuring tapes. Salivary calcium and phosphorus concentrations were determined by absorbance spectrophotometry. Data were analyzed by nonparametric tests for the analysis between groups and times. A model of linear regression was adjusted for the dependent variable enamel roughness of groups 4 and 5 in T3, considering the covariables salivary flow, pH, buffering capacity and salivary calcium and phosphorus concentration ($p<0.05$). Roughness was similar among groups in T1. In T2, G1 differed from all groups. In T3, G5=G2>G3=G4=G1. For G1, roughness of T1=T2=T3. For G2 and G5 T1<T2=T3. For G3, T1<T3<T2; for G4, T1=T3<T2. Considering the calcium/phosphorus ratio and percentages of calcium and phosphorus in enamel, there was not a statistically significant difference between groups ($p>0.05$), nor between the moments of evaluation T1, T2 and T3 ($p>0.05$). *Ra* and *Rz* were, respectively, 0.14 and 1.95 lower with normal salivary flow than reduced salivary flow. It was concluded that the contact with the human saliva *in vitro* and normal salivary flow *in situ* reestablished the enamel roughness to the original values, but the low salivary flow did not. Dental bleaching and the contact with human saliva cannot modify calcium/phosphorus ratio nor their isolated percentages. Recovery of bleached enamel roughness was higher in normal salivary flow than low salivary flow, regardless of saliva pH and buffering capacity.

Key Words: Dental bleaching. Dental enamel. Hydrogen peroxide. Saliva. Spectrophotometry.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

G1	Grupo 1
G2	Grupo 2
G3	Grupo 3
G4	Grupo 4
G5	Grupo 5
T1	Primeira avaliação, no tempo 1 (inicial)
T2	Segunda avaliação, no tempo 2 (após clareamento)
T3	Terceira avaliação, no tempo 3 (após contato com a saliva)
°C	Graus Celcius
(#)	Número
Ca	Cálcio
P	Fósforo
FC	Fator de Calibração
<	Menor
>	Maior
UFMG	Universidade Federal de Minas Gerais
CDTN	Centro de Desenvolvimento da Tecnologia Nuclear

SUMÁRIO

1 CONSIDERAÇÕES INICIAIS

Agentes clareadores com baixo pH podem induzir alterações deletérias na superfície dentária (SA *et al.*, 2012), e mesmo em pH neutro, elevadas concentrações de peróxido podem promover essas alterações (LLENA *et al*., 2018). Embora o pH não influencie a estabilidade de cor após clareamento dentário (BERSEZIO *et al*., 2019), os clareadores com maior concentração de peróxido podem induzir efeitos negativos na superfície do esmalte (CAVALLI *et al*., 2018; DE ABREU *et al*., 2011; USPENSKAYA *et al*., 2018; WANG *et al*., 2012) como a desmineralização (CAVALLI *et al*., 2018; DE ABREU *et al*., 2011; DOMINGUEZ *et al*., 2012; JUSTINO *et al*., 2004), com aumento da porosidade, depressões e irregularidades de superfície (FERREIRA *et al*., 2011; HAUSS MONTEIRO *et al*., 2019; LLENA *et al*., 2018; USPENSKAYA *et al*., 2018), acarretando maior susceptibilidade ao manchamento com a ingestão de corantes (AZER *et al*., 2011; GOPINATH *et al*., 2013; HAUSS MONTEIRO *et al*., 2019; KARADAS *et al*., 2014; KIM *et al*., 2011; PÚBLIO *et al*., 2013).

A película salivar desempenha um importante papel na proteção do esmalte contra a desmineralização (ALKATTAN *et al*., 2018), conferindo segurança ao uso de agentes clareadores. Ela pode evitar a perda de cálcio (JUSTINO *et al*., 2004; LI *et al*., 2010; SA *et al*., 2012) e promover a restauração dos níveis de cálcio e fosfato no esmalte clareado (FUJIKAWA *et al*., 2008; JUSTINO *et al*., 2004; MORI *et al*., 2015; SA *et al*., 2012; ZECZKOWSKI *et al*., 2015), dependendo da condição erosiva inicial do dente e da proporção de cálcio/fosfato na saliva (KARLINSEY *et al*., 2012). Quanto maior o fluxo salivar, maior é a concentração de cálcio e menor a de fosfato na saliva, o que leva à menor susceptibilidade da hidroxiapatita à erosão (Jager *et al*., 2011), ou seja, mais efetiva é a saliva na redução da desmineralização e na promoção da remineralização dentária.

A redução do fluxo salivar pode advir de radioterapia em região de cabeça e pescoço (BARDOW & VISSINK, 2017), síndrome de Sjögren (BARDOW & VISSINK, 2017; BERMAN *et al*., 2019), envelhecimento (AFFOO *et al*., 2015), hipotireoidismo (MURALIDHARAN *et al*., 2013) ou mesmo do efeito colateral de medicamentos (JOHNSSON *et al*., 2016), e está relacionada à capacidade tampão e ao pH intra-oral (LOKE *et al*., 2016). A xerostomia é o efeito colateral mais prevalente entre

todas as classes de medicamentos (COCKBURN *Et al*., 2017). Ainda não foram investigados os efeitos de agentes clareadores sobre o esmalte de indivíduos que apresentam alterações no fluxo salivar. Questiona-se, se a recuperação da rugosidade e das alterações estruturais do esmalte clareado ocorre também em condições de fluxo salivar reduzido. Adicionalmente, em caso de recuperação, ela estará relacionada apenas às concentrações de cálcio (Ca) e fósforo (P) na saliva, independentemente do fluxo e da capacidade tampão salivares? A originalidade e relevância desse trabalho estão baseadas nesta lacuna. Essas respostas serão importantes para avaliar o efeito do clareamento com peróxido de hidrogênio 35% na rugosidade e estrutura do esmalte, fornecendo ao cirurgião dentista maior segurança na indicação do clareamento dentário para todos os pacientes que o desejam, considerando seu fluxo salivar.

O objetivo desse estudo experimental foi avaliar o efeito do contato com a saliva humana *in vitro* e do fluxo salivar normal e reduzido *in situ* na rugosidade, proporção de cálcio e fósforo e suas porcentagens isoladas no esmalte após clareamento dentário com peróxido de hidrogênio a 35%. Pretende-se também avaliar os efeitos do fluxo, pH, da capacidade tampão e das concentrações de cálcio e fósforo salivares na recuperação da rugosidade do esmalte clareado.

2 OBJETIVOS

2.1 Objetivo geral

Avaliar o efeito do contato com a saliva humana e do fluxo salivar normal e reduzido na rugosidade e no conteúdo mineral do esmalte humano após clareamento dentário com peróxido de hidrogênio a 35%.

2.2 Objetivos específicos

- Avaliar o efeito da saliva humana *in vitro* e do fluxo salivar normal e reduzido *in situ*, nos parâmetros de rugosidade de superfície (Ra e Rz) do esmalte humano, após clareamento dentário com peróxido de hidrogênio a 35%;
- Avaliar o efeito da saliva humana *in vitro* e do fluxo salivar normal e reduzido *in situ*, na proporção de cálcio e fósforo e nas porcentagens isoladas de cálcio e fósforo no esmalte humano, após clareamento dentário com peróxido de hidrogênioa 35%;
- Avaliar a taxa de fluxo, o pH, as concentrações de cálcio e fósforo e a capacidade tampão salivares dos participantes com fluxo salivar normal e reduzido;
- Avaliar os efeitos do fluxo, pH, da capacidade tampão e das concentrações de cálcio e fósforo salivares na recuperação da rugosidade do esmalte humano, *in situ*, após clareamento dentário com peróxido de hidrogênio a 35%.

3 HIPÓTESES

3.1 Hipótese nula primária

A rugosidade de superfície e o conteúdo mineral do esmalte clareado não são diferentes em contato com saliva humana *in vitro* ou nas condições de fluxo normal e baixo fluxo salivar *in situ*.

3.2 Hipótese nula secundária

O pH, a capacidade tampão e as concentrações de cálcio e fósforo salivares não diferem nas condições de fluxo normal e baixo fluxo salivar e não afetam a recuperação de rugosidade do esmalte clareado, *in situ*.

4 METODOLOGIA EXPANDIDA

4.1 Desenho do estudo

Trata-se de estudo experimental cujas unidades amostrais foram espécimes de esmalte dentário humano clareados com Peróxido de Hidrogênio 35% submetidos ao efeito da saliva humana *in vitro* e *in situ*. Os controles negativo e positivo foram, respectivamente, o esmalte não clareado e o esmalte clareado, armazenados em água destilada. O fator em estudo foi a condição da saliva humana: *in vitro ou in situ* com fluxo salivar normal ou reduzido. O pH, a capacidade tampão e as concentrações de cálcio e fósforo na saliva dos participantes também foram avaliados. As variáveis dependentes foram: a rugosidade superficial medida por perfilometria tridimensional a laser (Ra e Rz), a proporção de cálcio e fósforo e o percentual de cálcio e fósforo no esmalte, medidas antes do clareamento (T1), após o clareamento (T2) e após exposição à saliva por 7 dias. O tamanho amostral mínimo (n=15) foi calculado com o software G-Power versão 3.1.7, considerando os parâmetros α=0.05, β=0.20, e tamanho de efeito=0.20, utilizando-se as médias de avaliações de rugosidade obtidas em estudo prévio (Hauss Monteiro *et al*., 2019).

Esse estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade Federal de Minas Gerais (COEP: CAAE-69736817.8.0000.5149, ID 2.129.538) (Anexo 1).

4.2 Obtenção e preparo dos espécimes

Terceiros molares provenientes do Biobanco de Dentes Humanos da Faculdade de Odontologia da UFMG tiveram suas superfícies coronárias vestibular e lingual examinadas em lupa estereoscópica com aumento de 8 vezes para verificar a ausência de trincas ou defeitos de superfície. Os dentes selecionados foram seccionados para obter espécimes de aproximadamente 25mm^2 no terço médio da superfície vestibular ou lingual. A área experimental de 5mm de diâmetro foi delimitada com uma fita adesiva afixada na superfície do esmalte e contornada

usando broca esférica tamanho ½ (2801FG.1/2 KG Sorensen, Medical Burs Ind. E Com. de Pontas e Brocas Cirúrgicas Ltda, Cotia, Brasil). Planificação e polimento da superfície não foram realizados, para não remover a camada externa mais mineralizada do esmalte. Os espécimes foram identificados, esterilizados por raios gama (25kGy), submetidos à primeira avaliação de rugosidade, e então, divididos aleatoriamente em 5 grupos:

Grupo 1 (G1): Não clareado e não exposto à saliva, imerso em 30ml de água destilada, e mantido em estufa a 37 C, por 7 dias – (controle negativo);

Grupo 2 (G2): Clareado e não exposto à saliva, imerso em 30ml de água destilada, e mantido em estufa a 37º C, por 7 dias – para avaliar o efeito do clareamento (controle positivo).

Grupo 3 (G3): Clareado e exposto à saliva humana (30ml) *in vitro*, em estufa a 37 C,por 7 dias – para avaliar o efeito da saliva humana, sem considerar o fluxo salivar;

Grupo 4 (G4): Clareado e exposto à saliva humana *in situ*, por 7 dias, em voluntários com fluxo salivar normal – para avaliar o efeito do fluxo salivar normal;

Grupo 5 (G5): Clareado e exposto à saliva humana *in situ*, por 7 dias, em voluntários com fluxo salivar reduzido – para avaliar o efeito do fluxo salivar reduzido.

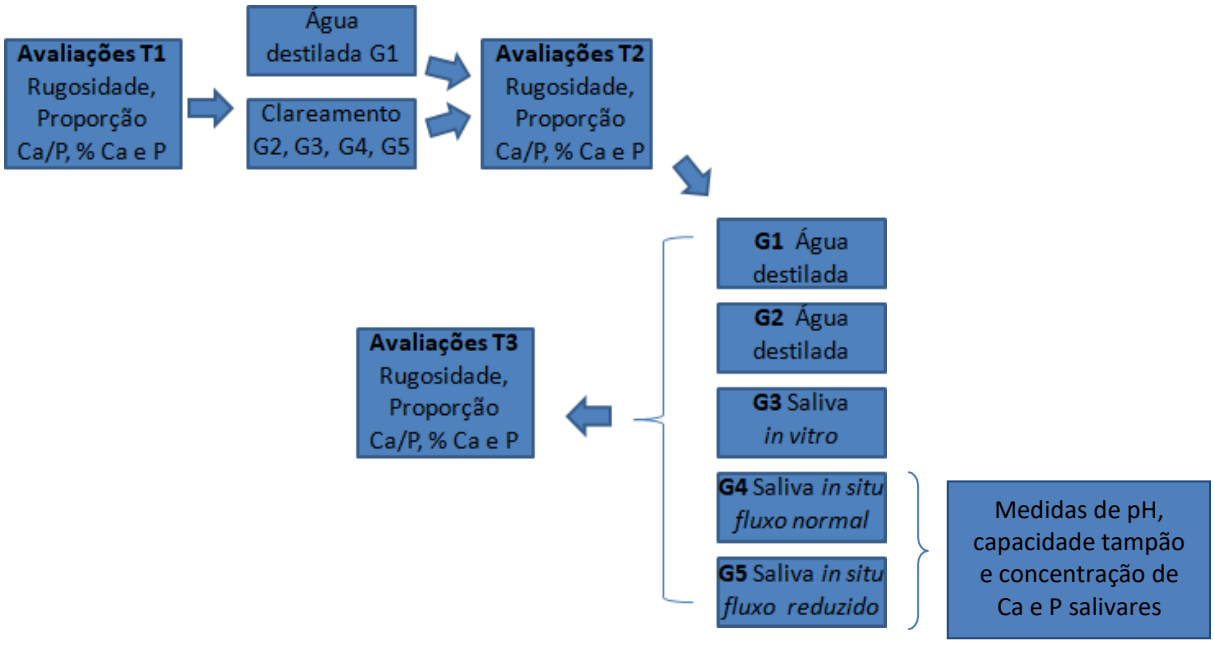
Para G3, a saliva utilizada *in vitro* foi coletada de um único voluntário de fluxo salivar normal (0,33ml/min). Para avaliação do efeito do fluxo salivar *in situ*, em G4 e G5 participaram 30 voluntários (n=15) que receberam um espécime dentário fixado com resina composta na face vestibular do 1º molar superior direito, e mantido por 7 dias (1 espécime por voluntário).

4.3 Clareamento

Foi utilizado um agente clareador à base de peróxido de hidrogênio a 35%

(Whiteness HP, FGM, Joinville, Brasil). Foram feitas 3 aplicações sequenciais de 15 minutos do clareador nos espécimes dos grupos 2, 3, 4 e 5, para simular uma sessão clínica de clareamento sem a aplicação de luz. Os espécimes foram lavados com água destilada por 30 segundos após cada aplicação. O pH do agente clareador foi medido em potenciômetro (Metrohm 827 pH lab, Metrohm Pensalab Instrumentação Analítica Ltda, São Paulo, Brasil), o qual foi previamente calibrado com solução tampão de pH 4 e pH 7 (CertiPUR, Merck KGaA, Darmstadt. Alemanha). Em 15 minutos, o pH da solução clareadora foi de 5,4. Os espécimes foram mantidos em ambiente úmido, em caixa de isopor, e levados imediatamente para a segunda avaliação da rugosidade.

Figura 1. Fluxograma da sequência metodológica.



Fonte: Elaborado pelo autor.

4.4 Seleção dos participantes

Os participantes foram selecionados por conveniência e informados sobre os aspectos relacionados à metodologia do estudo. Assinaram um Termo de Consentimento Livre e Esclarecido e responderam a um questionário (Anexo 2) em entrevista semi-estruturada sobre idade, gênero, saúde geral, hábitos deletérios,

dieta, auto-percepção do estado emocional mais frequente para definir a inclusão e exclusão, equilibrando a composição dos grupos conforme as características dos voluntários. Os seguintes critérios de inclusão foram considerados para os grupos 4 e 5: homens e mulheres, idade entre 20 e 60 anos, ingestão diária de 1 a 3 copos de água no mínimo, hábito de ingerir leite e seus derivados diariamente. Os critérios de exclusão foram: tabagismo, uso de próteses dentárias removíveis, hábito diário de usar soluções para bochecho e aqueles em tratamento de diálise. Os participantes receberam instruções para padronização da higiene bucal e um conjunto contendo uma escova macia, dentifrício contendo 1450ppm de fluoreto de sódio (Colgate Total 12, Colgate-Palmolive Company) e fio dental. Todas as orientações foram verbalizadas e entregues por escrito. Os grupos 4 e 5 foram uniformizados quanto ao gênero e idade dos voluntários.

4.5 Avaliação do fluxo salivar, pH e capacidade tampão da saliva

O fluxo salivar total não estimulado foi medido antes de qualquer procedimento de higiene bucal e da primeira refeição do dia (ZECZKOWSKI *et al*., 2015), de 6:30 até 8h da manhã, para diminuir a influência do círculo circadiano (FLINK *et al*., 2005). As coletas foram realizadas em ambiente clínico ou em laboratório (VARONI *et al*., 2016). Todos os participantes foram instruídos a engolir no tempo zero (INOUE *et al*. 2006), enxaguar a boca com água por 1 min e, depois, aguardou-se 1 minuto para o início da coleta. Nesse tempo de espera, os participantes foram orientados a não executar movimentos de mastigação, deglutição e fala, bem como durante a coleta. Os participantes ficaram em ambiente com iluminação artificial, sentados com o tronco voltado para frente, e depositaram a saliva acumulada na boca em um frasco, por 15 minutos (BARDOW & VISSINK, 2017; HAYASHIDA *et al*., 2015). A saliva foi pesada em balança de precisão e transportada em caixa de isopor com gelo (LIU *et al*., 2012). Considerando que a densidade da saliva é igual a 1,005g/cm3, foi utilizada a seguinte fórmula (PYATI *et al*., 2018) para o cálculo do fluxo salivar (ml/min):

$$\frac{\text{Peso final do frasco - Peso inicial do frasco}}{}$$

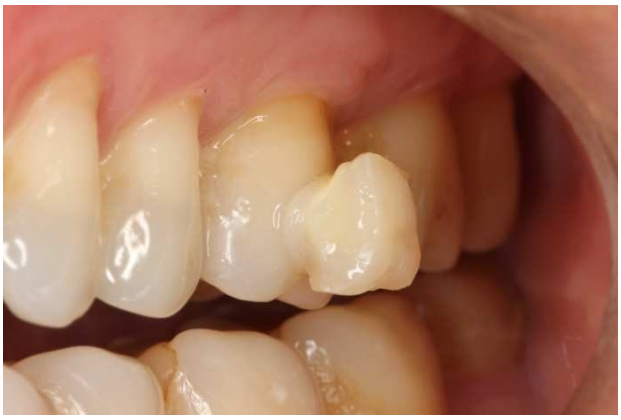
Tempo de coleta (15 minutos)

Após o cálculo, os voluntários foram alocados nos grupos 4 e 5, segundo o fluxo salivar. Quando maior que 0,1ml/min indicou atividade normal das glândulas salivares, ou seja, fluxo salivar normal (FLINK *et al*., 2005). O pH e a capacidade tampão da saliva foram avaliados por meio de fitas colorimétricas (Saliva-check buffer, GC America Inc., Alsip, EUA). A saliva coletada foi armazenada em freezer a -80 C para posterior análise da concentração de cálcio˚ e fósforo (SANTOS *et al*., 2018.

4.6 Fixação dos espécimes de esmalte dentário

A superfície dentária foi condicionada com ácido fosfórico 37% (Condac, FGM Produtos Odontológicos, Joinville, Brasil) e, em seguida, foi aplicada uma camadado sistema adesivo Adper Single Bond 2 (3M Espe, St Paul, USA), a qual foi fotoativada por 20 segundos com aparelho fotoativador (Bluephase, Ivoclar Vivadent Ltda, Barueri, Brasil). Um espécime foi fixado na superfície vestibular do 1º molar superior direito de cada voluntário (Fig. 2) com resina fotopolimerizável (Tetric Ceram, Ivoclar Vivadent Ltda, Barueri, Brasil) fotoativada por 20 segundos. Após 7 dias, os espécimes foram removidos com alicate ortodôntico (Alicate ortodôntico saca resina 193, Quinelato Instrumentos Cirúrgicos, Schobell Industrial Ltda, Rio Claro, Brasil) e a superfície vestibular do dente foi polida com ponta multilaminada em baixa rotação (número 283, 12 lâminas FG, Microdont Microusinagem de Precisão Ltda. São Paulo, Brasil) e borrachas abrasivas (Eve Diapol, W17Dg, W17Dmf, W17D. Eve Ernst Vetter GmbH, Keltern, Alemanha). Em seguida, foi realizada a terceira avaliação da rugosidade superficial. Os dentifrícios dados aos voluntários foram requisitados para a avaliação visual do conteúdo e verificação do seu uso durante o intervalo de tempo de 7 dias.

Figura 2. Espécime fixado na superfície vestibular do 1º molar.



Fonte: Débora Drummond Hauss Monteiro

4.7 Medida da rugosidade superficial

Os parâmetros “Ra” (µm) e “Rz” (µm) foram obtidos por leitura em perfilômetro de não contato a laser 3D (NewView 1700, Zygo Corporation, Connecticut, EUA). Os parâmetros de leitura considerados foram 0,001% para a modulação mínima e 7 pixels para a área mínima. Os valores obtidos na primeira leitura da rugosidade superficial (T_1 - inicial) e após cada uma das leituras subsequentes (T_2 após o clareamento e T_3 após 7 dias de exposição à saliva ou água destilada) foram registrados para cada grupo experimental (Fig. 1).

4.8 Análise da proporção de cálcio e fósforo e seus percentuais isolados no esmalte

Três espécimes de cada grupo experimental foram selecionados aleatoriamente e avaliados quanto à proporção de cálcio/fósforo e aos percentuais isolados de cálcio e fósforo do esmalte, por meio de espectroscopia de energia dispersiva por raios X (EDX) (X Flash 410-M, Bruker Nano GmbH, Berlim,

Alemanha). O sistema é controlado pelo software ESPRIT (Bruker Nano GmbH, Berlim, Alemanha) que realiza análises pontuais, varreduras em linha e obtém mapas de distribuição de elementos. As avaliações foram realizadas antes (T1) e após o clareamento (T2) e após a exposição à saliva humana ou água destilada, por 7 dias (T3). Os elementos de interesse, Ca e P, foram pesquisados em 4 aferições para cada espécime. A amostra foi irradiada por Raios X e a quantificação dos elementos Ca e P foram obtidas através da proporcionalidade Ca/P e também da porcentagem de Ca e P, separadamente.

4.9 Análise das concentrações de cálcio e fósforo da saliva dos participantes

As concentrações de cálcio e fósforo da saliva foram determinadas por espectrofotometria de absorbância (Evolution 160 UV-Vis, Thermo Fisher Scientific, Waltham, USA) usando padrões comerciais Bioclin® (Quibasa Química Básica Ltda, Belo Horizonte, Brasil). As soluções branco e padrão foram obtidas conforme as orientações do fabricante. A intensidade de cor produzida pelo composto formado com o cálcio e o fósforo foi avaliada por meio das leituras de absorbância das soluções branco e padrão (controles) e das amostras salivares.

A saliva total não estimulada de cada participante foi centrifugada em velocidade de 13000 RPM por 7 minutos e o sobrenadante coletado. Desse sobrenadante, 100µl foi diluído em 900µl de água destilada. Dessa diluição, 100µl foimisturado com 1000µl do reagente 1 do kit. Essa mistura foi colocada em uma cubeta para a leitura da absorbância e determinação das concentrações de cálcio e fósforo, referentes a cada participante.

O padrão foi feito com a mistura de 10µl do reagente 2 e 1000µl do reagente 1. O outro controle, chamado branco, foi 1000µl do reagente 1 puro, sem nenhuma mistura, conforme orientações do fabricante do kit utilizado.

A primeira avaliação de espectrofotometria de absorbância foi feita com a solução do branco inserida na cubeta do compartimento de referência e, também na cubeta do compartimento de amostra, para zerar o aparelho. Trata-se de uma solução livre de cálcio e fósforo, que terá absorbância zero e transmitância 100%,em que a luz incidente não é absorvida pelo líquido.

A segunda avaliação de espectrofotometria de absorbância foi feita com a solução do branco inserida na cubeta do compartimento de referência, e o padrão na cubeta do compartimento de amostra. Esta terá absorbância 100% e transmitância zero. A primeira e segunda avaliações foram realizadas para ajuste da transmitância e absorbância do espectrofotômetro.

A terceira medida foi feita com a solução do branco inserida na cubeta do compartimento de referência, e a primeira amostra de saliva na cubeta do compartimento de amostra. Assim sucessivamente, as amostras de saliva de todos os participantes foram avaliadas.

A determinação do Ca foi realizada utilizando-se o reagente Cálcio Arsenazo III, que reage com o Ca salivar formando um complexo de cor azul a violeta. A determinação do P foi realizada com o reagente Verde de Malaquita, que reage com o P. A intensidade de cor é proporcional à concentração na saliva. A intensidade de energia eletromagnética que atravessa o meio diminui exponencialmente segundo sua concentração iônica. Quanto maior a concentração do íon na amostra, maior será a absorbância (VOGEL *et al*., 2002).

Para a avaliação do Ca e do P, o comprimento de onda do espectrofotômetro foi ajustado em 650nm e 340nm respectivamente. Para obter a concentração final decálcio e fósforo em mg/dl, foi utilizada a lei de Lambert-Beer, utilizando o fator de calibração (FC):

$$FC = \frac{\text{concentração padrão}}{\text{absorbância do padrão}}$$

A concentração padrão considerada foi a fornecida pelo fabricante do kit, sendo 4mg/dl para o fósforo e 10mg/dl para o cálcio.

Após o cálculo do fator de calibração, a concentração final (mg/dl) de cálcio e fósforo foi obtida através da equação:

$$\text{Concentração final (mg/dl)} = \text{absorbância da amostra} \times FC$$

4.10 Análise do pH e capacidade tampão da saliva dos participantes

O pH da saliva dos voluntários foi avaliado por meio de fitas colorimétricas

(pH-Fix 0-14, Macherey-Nagel GmbH & Co., Düren, Deutschland), que foram inseridas por 15 segundos dentro da saliva coletada de cada voluntário. A capacidade tampão da saliva dos participantes foi avaliada também com fitas medidoras (Saliva-check buffer kit, GC Corporation, Tokyo, Japan), colocando-se 10µl de saliva sobre cada espaço de cor da fita. As alterações de cor visualizadas foram identificadas nas tabelas contidas nos kits e os valores de capacidade tampão foram transformados em escores conforme sugerido na bula do Saliva-check buffer: 0 a 5 capacidade tampão muito baixa; 6 a 9 baixa; 10 a 12 normal/alta.

4.11 Análise estatística

Os dados obtidos foram tabulados e as medidas de tendência central e de variabilidade foram calculadas. Os testes Kolmogorov Smirnov e Levene mostraram que os dados de Ra e Rz não atenderam aos pressupostos de normalidade ($p<0,001$ em Ra e Rz) e de homogeneidade de variâncias ($p<0,001$ em Ra e Rz). Os efeitos do fluxo salivar sobre a rugosidade e a proporção cálcio/fósforo e porcentagens isoladas no esmalte clareado, nos 3 momentos de avaliação, foram analisados utilizando os testes estatísticos Kruskal-Wallis e post hoc de Mann Whitney para análise entre grupos, e Friedman e post hoc de Wilcoxon para análise entre os tempos. A comparação das concentrações de cálcio e fósforo na saliva dos grupos 4 e 5 foi realizada por meio de teste estatístico não paramétrico de Mann Whitney. Foi realizada análise entre os valores de pH e capacidade tampão da saliva dos voluntários com fluxo salivar normal e reduzido, com o teste de Wilcoxon. Um modelo de regressão linear foi ajustado para a variável dependente rugosidade do esmalte (Ra e Rz) dos grupos G4 e G5, em T3, considerando as covariáveis fluxo salivar, pH, capacidade tampão e concentração de cálcio e de fósforo da saliva. Após o ajuste, foi analisada a distribuição dos resíduos, homocedasticidade dos resíduos e colinearidade entre as variáveis independentes. O nível de significância adotado em todos os testes foi de 5%. Foi utilizado o software SPSS versão 17.0 (Statistical Product and Service Solutions, IBM, Nova York, EUA).

5 ARTIGO 1

EFFECT OF SALIVARY FLOW ON BLEACHED ENAMEL ROUGHNESS AND MINERAL CONTENT: AN *IN VITRO* AND *IN SITU* STUDY

ABSTRACT

OBJECTIVES

To evaluate the effect of saliva *in vitro* and salivary flow *in situ* on roughness and mineral content of bleached enamel.

MATERIALS AND METHODS

Dental specimens were divided in five groups (n=15): not bleached (G1); bleached (35% hydrogen peroxide) and exposed to: distilled water (G2), saliva *in vitro* (G3), *in situ* normal salivary flow (NSF) (G4) and low salivary flow (LSF) (G5). Enamel roughness (*Ra*, *Rz*) and calcium/phosphorus contents were evaluated with laser profilometry and energy-dispersive spectroscopy, in *baseline* (T1), after bleaching (T2) and after 7 days (T3). Salivary pH and buffering capacity were evaluated with strips. Salivary calcium and phosphorus with absorbance spectrophotometry. Data were analyzed with non-parametric tests and linear regression ($p<0.05$).

RESULTS

Roughness was similar among groups in T1. In T2, G1 differed from all

groups. In T3, G5=G2>G3=G4=G1. For G1, roughness of T1=T2=T3. For G2 and G5 T1<T2=T3. For G3, T1<T3<T2; for G4, T1=T3<T2. Enamel calcium/phosphorus content did not change with bleaching nor saliva contact ($p>0.05$). Buffering capacity and calcium concentration did not differ between LSF and NSF. Phosphorus was higher and pH was lower in LSF. *Ra* and *Rz* were, respectively, 0.14 and 1.95 lower with NSF than LSF.

CONCLUSIONS

NSF *in situ* and saliva *in vitro* recovered original enamel roughness. Experimental conditions did not affect enamel mineral content. Recovery of bleached enamel roughness was higher in NSF than LSF, regardless of salivary pH, calcium and phosphorus concentration and buffering capacity. **Clinical Relevance** Measuring salivary flow before tooth bleaching can estimate the potential of recovery enamel roughness and guarantee safe approaches.

KEY WORDS

Tooth Bleaching. Dental Enamel. Profilometry. Spectrophotometry. Hydrogen Peroxide. Saliva.

INTRODUCTION

Bleaching agents with low pH may induce deletary alterations on dental surfaces [1] and even with a neutral pH these alterations can be observed with high concentrations peroxides [2]. Bleaching agents with high peroxide concentrations may affect enamel surface [3-6], causing demineralization [3,4,7], with porosity increase, depressions and surface irregularities [2,5,8,9], resulting in higher

susceptibility to staining after dyes ingestion [8,10].

Subjects with none or very little activity of enamel erosion seem to have a more favourable physicochemical saliva composition with higher concentrations of calcium and phosphate [11]. Salivary film plays an important role on the enamel protection against demineralization [12], making the use of bleaching agents safe. Salivary role may avoid calcium loss [1] and restore calcium and phosphate levels on bleached enamel [1,13,14]. However, the mineral recovering will depend on the initial erosive condition of the dental surface and calcium/phosphate ratio of saliva [15]. The higher the salivary flow, the higher the calcium concentration [16], the less the phosphorus concentration [17] and the less the susceptibility of hydroxiapatite to erosion [16], that is, more effective is saliva on reducing dental desmineralization and promoting remineralization.

Low salivary flow may be a consequence of head and neck radiotherapy [18,19], Sjögren syndrome [18,20], aging [21], hypothyroidism [22] or even a medicine side effect [23], and is related to low intraoral pH and buffering capacity [24]. The bleaching agent effect on the enamel of individuals with salivary flow alterations has not yet been investigated. The research question is if the enamel structural alterations recovery also occurs in low salivary flow patients. Additionally, in case of complete recovery, would it be related only to salivary calcium and phosphorus concentrations, regardless the flow, pH and buffering capacity of saliva? The originality and relevance of this study are based on this gap. The objective of this study was to evaluate the effect of the contact with human saliva *in vitro* and normal and low salivary flow *in situ* on enamel roughness and calcium and phosphorus contents after tooth bleaching with 35% hydrogen peroxide. We also evaluated the salivary pH, buffering capacity and calcium and phosphorus concentrations of individuals with normal and low salivary flow, and estimate their association with the enamel roughness alteration. The primary null hypothesis is that the surface roughness and mineral content of bleached enamel are not different in contact with human saliva *in vitro* or with low and normal salivary flow *in situ*. The secondary null hypothesis is that salivary calcium and phosphorus concentrations, pH and buffering capacity do not differ in normal and low salivary flow patients and are not associated with enamel roughness recovery.

MATERIALS AND METHODS STUDY DESIGN

In this experimental study the specimens were dental human enamel bleached with 35% hydrogen peroxide exposed to the human saliva *in vitro* and *in situ*. The study factors were the salivary conditions: *in vitro*, *in situ* with normal or low salivary flow. The negative and positive controls were, respectively, non bleached enamel and bleached enamel, both stored in distilled water. The dependent variables were: surface roughness measured with non-contact laser profilometry (Ra and Rz), calcium/phosphorus ratio and the percentages of calcium and phosphorus on enamel, which were evaluated on baseline (T1), after bleaching (T2) and after 7-days saliva exposuring (T3). The minimal sample size (n=15) was estimated with the software G-Power version 3.1.7, considering the parameters α=0.05, β=0.20, and effect size=0.20, using roughness means from a previous study [8]. This study was approved by the local Research Ethics Comitee (CAAE-69736817.8.0000.5149, ID 2.129.538).

SPECIMENS PREPARATION

Human third molars from the teeth biobank of the Faculty of Dentistry (Federal University of Minas Gerais, Brazil) were obtained. The buccal and lingual coronary surfaces were examined in 8 x- stereoscopic magnifying glass to verify the absence of cracks or surface defects. The teeth were sectioned to obtain specimens of approximately 25mm^2 on the coronary middle third. An experimental area of 5mm-diameter was delimited with an adhesive tape fixed on the enamel surface and outlined using a spherical bur size ½ (2801FG. ½ KG Sorensen, Cotia, Brazil). The specimens were identified, sterilized with gamma ray (25kGy) and subjected to the 1st evaluation of roughness (T1). Then they were randomly divided in 5 groups:

Group 1 (G1): Not bleached, and stored in 30ml of distilled water at 37 C (negative control)

Group 2 (G2): Bleached and stored in 30ml of distilled water at 37 C (positive control)

Group 3 (G3): Bleached and stored in 30ml of human saliva *in vitro* at 37 C

Group 4 (G4): Bleached and exposed to human saliva *in situ* in normal salivary flow participants Group 5 (G5): Bleached and exposed to human saliva *in situ* in low salivary flow participants

For G3, the saliva *in vitro* was collected of a participant with normal salivary flow (0.33ml/min). For G1, G2 and G3, distilled water or saliva were daily changed. For the evaluation of the effect of salivary flow *in situ,* 30 participants were allocated in G4 and G5 (n=15), with 1 specimen/participant.

BLEACHING

Three sequential applications (15 minutes each) of 35% hydrogen peroxide (Whiteness HP, FGM, Joinville, Brazil) were performed on specimens of groups 2, 3, 4 and 5, simulating a clinical session of bleaching without light application. The bleaching agent pH was 5.45 measured with a potentiometer (Metrohm 827 pH lab, Metrohm Pensalab Instrumentação Analítica Ltda, São Paulo, Brazil). The specimens were washed with distilled water for 30 seconds and submitted to the 2nd roughness evaluation (T_2).

PARTICIPANTS SELECTION

The participants were selected by convenience and were informed about the study method. They signed an informed consent form. Groups 4 and 5 were balanced regarding participants gender and age, following the inclusion criteria: male and female, 20 to 60 years old, minimum daily ingestion of 1 to 3 glasses of water,

daily habit to ingest milk and its derivatives. The exclusion criteria were: smoking, dental prosthesis wearing, daily habit to use mouthwash solutions, doing dialysis. The participants received instructions for standardization of oral hygiene and a set containing one soft toothbrush, a dentifrice containing 1450ppm of sodium fluoride (Colgate Total 12, Colgate-Palmolive Company) and a dental floss. All orientations were verbalized and given written.

EVALUATION OF THE SALIVARY FLOW, PH AND BUFFERING CAPACITY OF SALIVA

Non stimulated salivary flow was measured before breakfast and any oral hygiene [14], from 6:30 a.m. until 8:00 a.m, to decrease the influence of the circadian cycle [25,26]. All participants were instructed to seat in a clinical environment, swallow on time zero [27, 28], rinse the mouth with water for 1 minute and wait 1 minute to initiate the collection. While waiting and also during the collection, participants were instructed not to chew, deglute or speak. The participants deposited the accumulated saliva in a falcon tube for 15 minutes [18,29]. Saliva was weighed in a precision scale [30] and considering saliva density 1.005g/cm^3, the following formula was applied [31] to calculate salivary flow rate (ml/min):

$$\frac{\text{Tube final weight} - \text{Tube initial weight}}{\text{Collection time (15 minutes)}}$$

Salivary flow rates higher than 0.1ml/min indicated normal activity of the salivary glands [26]. The participants were allocated in groups 4 and 5 according to salivary flow. The salivary pH and buffering capacity were evaluated with colorimetric strips (Saliva-check buffer, GC America Inc., Alsip, USA). Collected saliva was stored in a freezer at -80ºC for posterior analysis of calcium and phosphorus concentration [32,33].

FIXATION OF DENTAL ENAMEL SPECIMENS *IN SITU* (G4 AND G5)

One specimen was fixed with composite resin (Tetric Ceram, Ivoclar Vivadent Ltda, Barueri, Brazil) on the buccal surface of the 1st right superior molar of each participant. Dental surface was conditioned with 37% phosphoric acid (Condac, FGM Produtos Odontológicos, Joinville, Brazil), followed by adhesive (Adper Single Bond 2, 3M Espe, St Paul, USA) and composite resin applications and lightcuring (20 seconds, Bluephase, Ivoclar Vivadent Ltda, Barueri, Brazil).

After 7 days the specimens were removed with an orthodontic plier (Orthodontic plier 193, Quinelato Instrumentos Cirúrgicos, Schobell Industrial Ltda, Rio Claro, Brazil). The buccal surface of tooth was polished with a multiblade bur in low rotation (number 283, 12 blades FG, Microdont microusinagem de precisão ltda. São Paulo, Brazil) and rubber polishers (Eve Diapol, W17Dg, W17Dmf, W17D. Eve Ernst Vetter GmbH, Keltern, Germany). Then the 3rd roughness evaluation (T_3) was performed. The dentifrices given to the participants were requested and their use was assured by visual inspection.

SURFACE ROUGHNESS MEASUREMENT

Ra (µm) and *Rz* (µm) parameters were assessed with a 3D non-contact laser profilometer (NewView 1700, Zygo Corporation, Connecticut, USA). The reading parameters were: 0.001% for minimal modulation and 7 pixels for minimal area. The values of the 1st surface roughness assessment (T_1) and subsequent assessments (T_2, after bleaching and T_3, after 7 days of exposure to saliva or distilled water) were registered for each experimental group.

MINERAL CONTENT OF ENAMEL SPECIMENS

Three specimens of each group were randomly selected and evaluated with energy-dispersive X-Ray spectroscopy (EDX) (XFlash 410-M, Bruker Nano GmbH, Berlin, Germany). The system is controlled by the software ESPRIT (Bruker Nano GmbH, Berlin, Germany) that provides maps of elements distribution. The elements of interest (Ca and P) were assessed 4 times for each specimen. The specimens were irradiated with X-Ray and the Ca/P ratio and the percentages of Ca and P separately, were achieved. The specimens were evaluated before (T1) and after bleaching (T2) and after 7 days of exposure to human saliva or distilled water (T3).

CALCIUM AND PHOSPHORUS CONCENTRATIONS IN PARTICIPANTS SALIVA

Calcium and phosphorus concentrations in the saliva of the participants were assessed with absorbance spectrophotometer (Evolution 160 UV-Vis, Thermo Fisher Scientific, Waltham, USA) and using the comercial standard Bioclin® (Quibasa Química Básica Ltda, Belo Horizonte, Brazil). The intensity of color produced by the compound formed with calcium and phosphorus was evaluated with the absorbance readings of blank and standard solutions and saliva samples. Blank and standard solutions were obtained according to manufacturer recommendations.

Non stimulated saliva from each participant was centrifuged with 13000 RPM for 7 minutes and the supernatant was collected. From this supernatant, 100µl was diluted in 900µl of distilled water. From this dilution, 100µl was mixed with 1000µl of reagent 1 and this mixture was assessed for calcium and phosphorus concentrations of each sample. Calcium was assessed using the reagent calcium arsenazo III, which reacts with the salivary calcium forming a blue/violet complex. Phosphorus was assessed with the reagent malachite Green. The color intensity is proportional to the concentration in saliva. The intensity of electromagnetic energy that penetrate the medium decreases exponentially according to its ionic concentration. The higher the ion concentration on the sample, the higher is the absorbance.

For calcium and phosphorus evaluation, the wavelength of the spectrophotometer was adjusted to 650nm and 340nm, respectively. The final concentration of calcium and phosphorus in mg/dl, was calculated according to the Lambert-Beer Law:

$$\text{Calibration Factor (CF)} = \frac{\text{Pattern concentration}}{\text{Pattern absorbance}}$$

Final concentration (mg/dl) = sample absorbance x CF

The standard concentration considered was 10mg/dl for calcium and 4mg/dl for phosphorus, which were provided by manufacturer.

EVALUATION OF PH AND BUFFERING CAPACITY OF PARTICIPANTS SALIVA

The salivary pH was assessed with the insertion of colorimetric strips (pH-Fix 0-14, Macherey-Nagel GmbH & Co., Düren, Germany) in the saliva for 15 seconds. Buffering capacity was assessed placing 10µl of saliva on each color space of the colorimetric tape (Saliva-check buffer kit, GC Corporation, Tokyo, Japan). The alterations of color were identified on each kit table and the values of pH and buffering capacity were registered for each participant and recorded as scores.

STATISTICAL ANALYSIS

The data normal distribution and homogeneity of variance were analyzed with Kolmogorov Smirnov ($p<0.001$ for *Ra* and *Rz*) and Levene test ($p<0.001$ for *Ra* and *Rz*). The effects of salivary flow on roughness, calcium/phosphorus ratio and their isolated percentages on the bleached enamel, in T1, T2 and T3, were analyzed with Kruskal-Wallis and Mann Whitney post hoc for analysis among groups, and Friedman and Wilcoxon post hoc for analysis among times. The comparison of the salivary calcium and phosphorus concentrations of G4 and G5 was performed with Mann Whitney test. Saliva pH and buffering capacity were analyzed with Wilcoxon

test. A regression linear model was adjusted for the depedent variable enamel roughness (*Ra* and *Rz*) of G4 and G5, in T3, considering the covariables salivary flow, pH, buffering capacity and calcium and phosphorus salivary concentration. After adjustment, the residuals distribution and homocedasticity and colinearity of the independent variables were analyzed. The software SPSS version 17.0 (Statistical Product and Service Solutions, IBM, New York, USA) was used, and the confidence level of all tests was 5%.

RESULTS

Regarding the parameter *Ra*, there was a statistically significant difference among the times of evaluation (T1, T2 and T3) ($p<0.001$). G1 did not differ among T1, T2 and T3 ($p>0.05$). G2 and G5 roughness increased after bleaching and did not change after the contact with distilled water and low salivary flow, respectively. G3 and G4 roughness significatively increased after bleaching but decreased after the contact with saliva *in vitro* and normal salivary flow. G4 roughness in T1 and T3 did not change ($p=0.112$). Enamel roughness in T1 did not differ among groups G1 to G5 ($p=0.089$). In T2 (after bleaching), there was a significant difference among groups G1 to G5 ($p<0.001$). Roughness of G1<G2=G3=G4=G5 ($p<0.001$). In T3, there was also a significant difference among groups G1 to G5 ($p<0.001$). Roughness of G5=G2>G3=G4=G1 (table 1). Regarding the parameter *Rz*, there was a statistically significant difference among the times of evaluation (T1, T2 and T3) ($p<0.001$). The results were similar to *Ra*, except G3 that did not differ in T1 and T3 ($p=0.078$). G1 to G5 did not differ in T1 ($p=0.662$). In T2, G1<G2=G3=G4=G5 ($p<0.001$). In T3, G5=G2>G3=G4=G1 ($p<0.001$). *Rz* results
among groups were similar to *Ra* (table 1).

Table 1. Median (interquartilic range) of roughness values (*Ra*, *Rz*) on baseline (T1), after bleaching (T2) and after the contact with saliva (T3)

Experimental condition in *Ra*	n	Median(IR) T1	Median(IR) T2	Median(IR) T3
G1 Distilled water	15	0.17(0.07)Aa	0.16(0.06)Aa	0.15(0.06)Aa
G2 Bleaching+Distilled water	15	0.13(0.03)Aa	0.31(0.12)Bb	0.27(0.07)Bb
G3 Bleaching+Saliva *in vitro*	15	0.13(0.07)Aa	0.25(0.11)Bb	0.17(0.10)Ca
G4 Bleaching+Normal flow	15	0.12(0.03)Aa	0.35(0.07)Bb	0.15(0.07)Aa
G5 Bleaching+Low flow	15	0.14(0.06)Aa	0.39(0.11)Bb	0.35(0.13)Bb

Experimental condition in *Rz*	n	Median(IR) T1	Median(IR) T2	Median(IR) T3
G1 Distilled water	15	2.82(1.33)Aa	2.79(1.53)Aa	2.87(1.44)Aa
G2 Bleaching+Distilled water	15	2.27(1.19)Aa	3.96(1.56)Bb	3.80(1.29)Bb
G3 Bleaching+Saliva *in vitro*	15	2.49 (1.99)Aa	4.56(1.95)Bb	3.15(1.83)Aa
G4 Bleaching+Normal flow	15	2.10(1.12)Aa	4.68(1.16)Bb	2.34(1.20)Aa
G5 Bleaching+Low flow	15	2.58(0.89)Aa	5.28(1.71)Bb	5.00(2.02)Bb

Wilcoxon (p<0.05), Mann Whitney (p<0.05); Medians followed by equal letters did not differ statistically; uppercase letters compared between columns; lowercase letters compared between lines.

Enamel calcium/phosphorus ratio did not differ significantly among groups (p=0.514) nor among times ofevaluation T1, T2 and T3 (p>0.05) (table 2).

Table 2. Median (minimum-maximum) of Ca/P ratio on baseline (T1), after bleaching (T2) and after the contact with saliva (T3)

Experimental condition	n	Median (CI) T1	Median (CI) T2	Median (CI) T3
G1 Distilled water	3	2.52(2.49-2.55)Aa	2.53(2.26-2.81)Aa	2.50(2.37-2.66)Aa
G2 Bleach.+Distilled water	3	3.13(2.01-4.20)Aa	2.32(2.16-2.46)Aa	2.40(1.20-4.08)Aa
G3 Bleach.+Saliva *in vitro*	3	2.95(1.65-3.98)Aa	2.84(1.58-3.71)Aa	3.44(2.00-4.77)Aa
G4 Bleach.+Normal flow	3	2.81(2.10-3.58)Aa	2.31(2.08-2.61)Aa	3.18(1.89-4.18)Aa
G5 Bleach.+Low flow	3	2.96(1.78-4.39)Aa	2.31(2.11-2.61)Aa	2.80(2.35-3.13)Aa

Friedman (p>0.05); Kruskal Wallis (p>0.05). Medians followed by equal letters did not differ statistically; uppercase letters compared between columns; lowercase letters compared between lines.

The isolated percentage of enamel calcium and phosphorus did not statistically differ among groups (p=0.514) nor times of evaluation T1, T2 and T3 (p>0.05).

The concentrations of calcium (mg/dl) in saliva of G4 (normal flow) and G5 (low flow) was not statistically different (p=0.49). The concentrations of salivary phosphorus were higher in G5 than G4 (p=0.02) (table 3). The mean salivary pH of participants with normal flow was 7.2 (SD=0.41), while with low flow was 6.69 (SD=0.40), and this difference was statistically significant (p=0.006). The G4 (median 7.0; IR=6) and G5 (median 8.0; IR=6) saliva buffering capacity did not differ (p=0.714).

Table 3. Median (interquartilic range) of calcium and phosphorus concentrations (mg/dl) of saliva of groups 4 (normal flow) and 5 (low flow) participants

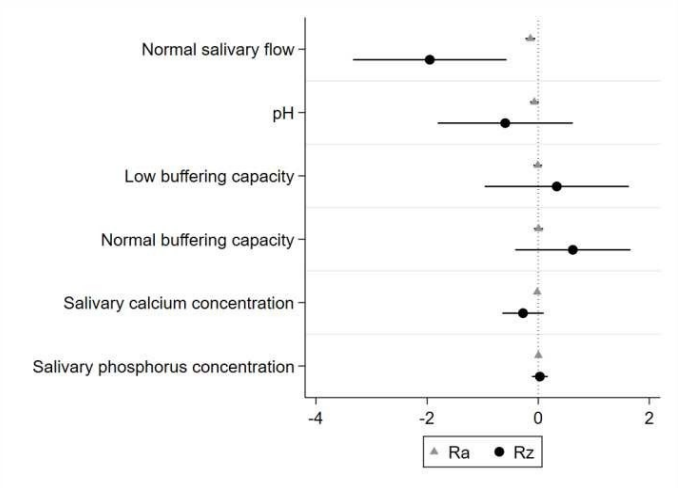
Groups – Salivary flow	n	Ca Concentration (IR)	P Concentration (IR)
G4 Normal salivary flow	15	1.74 (0.23) A	2.60 (0.46) A
G5 Low salivary flow	15	1.64 (0.28) A	2.73 (0.32) B

Mann Whitney ($p<0.05$); Medians followed by different letters are statistically different for comparison on the same column

The linear regression model showed that salivary flow was the only variable statistically associated with *Ra* and *Rz* parameters, considering the standardized *β* coefficients magnitude (Fig. 1). There was no colinearity between the independent variables. Model variables explained 57.4% of roughness variation. Figure 1 shows that normal salivary flow was associated to a lower *Ra* and *Rz*. For the other factors, the confidence interval crosses the zero line, showing that there was no difference. On X axis of the graph there are the *β* coefficients values, represented by the small ball, while the horizontal line is the magnitude of the confidence interval of the linear regression coefficient (minimum and maximum value of *β*). For this linear regression, *Ra β* coefficient was -0.14 and *Rz β* coefficient was -1.95, which shows that having normal salivary flow is associated on average to -0.14 on *Ra* value and to -1.95 on *Rz* value; this is the magnitude of response variation, considering the variation of the normal salivary flow. So, the *Ra* roughness is on average 0.14 lower on normal salivary flow group compared to low salivary flow, while *Rz* is on average 1.95 lower on normal salivary flow group.

The comparisons were performed according to each reference category when the variable was qualitative, and the reference category is not shown on the graph. The result of normal salivary flow was obtained in comparison with reduced salivary flow; pH was a quantitative variable; low and normal buffering capacity were compared to very low buffering capacity (according to the scores suggested by the manufacturer of the strips used); while salivary calcium and phosphorus concentrations were quantitative variables.

Fig. 1 *β* coefficients of linear regression



DISCUSSION

The results of this experimental study showed that after dental bleaching, enamel in contact with low salivary flow *in situ* had higher surface roughness than in contact with human saliva *in vitro* or normal salivary flow *in situ*, that did not differ from non bleached enamel. Calcium and phosphorus contents on enamel were not affected by experimental conditions evaluated. Calcium concentration did not differ between normal and low salivary flow groups, while phosphorus concentration was higher on low salivary flow group. The primary and secondary null hypotheses were rejected. These results are considered novel information as other studies showing the overall effect of human saliva, considering flow, pH, buffering capacity and mineral content, on enamel roughness and mineral content after bleaching were not found yet.

Roughness parameters (*Ra*, *Rz*) measured before bleaching did not differ between experimental groups, showing the uniformity of sample in baseline. After bleaching, there was a change on enamel surface morphology with an increase of roughness, which was highlighted by the difference between non bleached control group and the other groups that were bleached with 35% hydrogen peroxide. Bleaching may induce alterations on the enamel morphology [2,4-6,8-10] and on

mineral content [3,4,7]. Bleached group that was maintained in distilled water had higher roughness than the non bleached control, showing the bleaching effect and that the medium was not able to recover enamel original condition. After staying for 7 days in the oral cavity of normal salivary flow participants, the bleached specimens showed similar roughness of non bleached control, emphasizing the significant roughness recovery. They differed from the bleached specimens that were maintained in distilled water. These findings suggest the remineralizing ability of normal salivary flow on bleached enamel, similarly to that demonstrated on *in situ* enamel recovery after phosphoric and hydrochloric acid microabrasion [34]. Otherwise, in low salivary flow condition, specimens did not have a significant roughness recovery, that differed from the control. The recovery induced by natural saliva may depend on salivary flow volume and remineralizing potential.

Specimens maintained in natural saliva *in vitro* had the same roughness recovery than those exposed to the normal salivary flow *in situ*. Besides, its roughness was lower than that of bleached specimens maintained in destilled water and similar to non bleached control. These findings show that the saliva *in vitro* was able to recovery the surface topography and validates previous *in vitro* studies with natural saliva. Dental enamel specimens exposed to human saliva *in vitro* behaved similar to those maintained *in situ* with normal flow. Natural saliva may be considered an effective storage medium for protection and recovery of the damages caused by in-office bleaching and suitable for *in vitro* studies [14]. Other authors found hardness alteration on *in vitro* bleached enamel kept in distilled water compared to *in situ* model with an increase of it when bleached enamel was exposed to natural saliva *in vitro* [35]. This result is similar to the one found with the exposure to remineralizing agents [36].

In the present study, natural saliva of one participant was used *in vitro* (G3), which was daily renewed and contained 3.79 mg/dl of calcium and 4.76 mg/dl of phosphorus, pH 7.0 and normal buffering capacity. The statistical difference between roughness of G3 and G5 showed that the roughness recovery was different between the specimens kept in natural saliva *in vitro* and the specimens kept in the oral cavity of low salivary flow participants during the same time. This strengthens the idea that the volume of natural saliva may interfere on dental enamel surface roughness alteration. The difference between G2 and G5 found in T3 showed that after being maintained in low salivary flow *in situ*, the specimens had some mineral

recovery which made its roughness different from the bleached specimens that were kept in destilled water. However, as a difference was found between G4 and G5, it seems that the mineral recovery is still higher on normal salivary flow participants. As the salivary flow gets higher, so does the saliva pH [16,37] as in this study, salivary pH of the participants with normal salivary flow was significantly higher. Furthermore, an increased biofilm pH increases the saturation status of all types of calcium phophates [25,38] and changes the ratio of phosphate that decreases the solubility of dental mineral content. Although the total content of phosphate decreases with the increase of salivary flow, the Ca^{2+} concentration gets higher [16], and remineralization is favoured with the increase of salivary flow. In the present study, for the normal salivary flow participants there was a higher roughness recovery when compared to the low flow, independently of saliva pH and buffering capacity. Besides that, the participants with low salivary flow presented higher phosphorus concentration in the saliva, although calcium concentration did not differ between the participants with normal and low salivary flow.

Calcium loss from enamel may be lower *in situ* than *in vitro* after erosive challenge [39]. However, in this study there was no statistically significant difference among groups for calcium/phosphorus ratio and the isolated percentages of calcium and phosphorus. These results corroborate with Alkattan *et al*. (2018) that used energy-dispersive X-ray spectroscopy and Llena *et al*. (2018) that used environmental scanning electron microscopy combined with a microanalysis system (ESEM + EDX) and both did not find significant differences comparing to control. It is possible that despite surface roughness alteration shown by profilometry, this was not enough to induce a statistically significant difference on calcium and phosphorus ratio of dental enamel among groups. However, Cavalli *et al*. (2018) found that the calcium/phosphorus ratio of sound enamel decreased after bleaching with 35% hydrogen peroxide using micro energy dispersive micro X-ray fluorescence spectrometer (μ-EDXRF) and Dourado Pinto *et al*. (2019) observed with enamel microbiopsy in colorimetry spectrophotometry the reduction of the enamel calcium and phosphorus isolated content after bleaching with 40% hydrogen peroxide, with 10% HP and with these concentrations combined. The method of EDX was chosen in this study to avoid the inclusion of artefacts, which could influence repeated measures over time, but the test run in triplicate may not have been enough to have a statistical difference.

In normal salivary flow participants, calcium and phosphorus concentration was inferior when compared with low salivary flow group, although the difference was significant only for phosphorus. Saliva is supersaturated with calcium in relation to hydroxyapatite, to prevent teeth dissolution when exposed to oral fluids, foods and acid diets in general [41]. Salivary flow rate may interfere on calcium concentration on human saliva, and this relation is directly proportional according to some authors [16] and inversely related according to others [42]. Other authors found that a high calcium level is related to a lower pH [17]. High concentrations of phosphate in saliva may result in a reduction of the protection of biofilm when facing an acid challenge, increasing hydroxyapatite erosion [16]. The most important biological function of saliva inorganic phosphate is the contribution to the solubility product in relation to calcium phosphate, for dental structure maintenance. In physiological pH, the major part of phosphate exists on monovalent and divalent inorganic forms, and it is strictly regulated with calcium [43]. A low phosphorus level is related to an increased salivary flow [17]. Phosphate is important for buffering capacity in low salivary flow. With the salivary flow increase, there is an increase on mineral content and on buffering capacity of saliva, which facilitates acid neutralization. A low salivary flow induces an increase of erosion

[16] and decrease of the intra-oral pH [24] and buffering capacity of saliva [16,24]. In this study, there was no statistical difference between saliva buffering capacity of participants with normal and low flow, but regarding saliva pH, participants with low flow had lower saliva pH than normal flow participants and this difference was statistically significant. The total salivary flow rate of a person should be compared only with data of another group of the same gender [44]. To minimize a possible bias between G4 and G5, the balance of age and gender of participants was performed as a lower total non stimulated salivary flow is found in women due to the smaller size of their salivary glands and size of their body [27]. Also, the aging process is associated with a reduction of salivary flow in the salivary glands and this reduction cannot be explained by the medications in use [21].

The strengths of this study design are the sample size estimative according variables range from previous study, the standardization of saliva collection methods and the maintenance of the original enamel surface without flattening to simulate the clinical condition and increase the external validity of the results,. Planification and polishing of the surface could affect the results [45], since

the most mineralized layer is removed exposing the subsurface of enamel, which is more reactive. Roughness of natural enamel becomes very different from that of polished enamel, when it is exposed to an erosive challenge [46]. Also, we have fixed each specimen on the same maxillary area of participants to minimize experimental bias.

Although using pH and buffer capacity strips are valid and clinically usefull methods, it can be a drawnback of the study as saliva gets exposed to atmosphere, which could result in CO_2 loss, and this could affect the pH, making it more alkaline and changing the buffering capacity measure [47].

Based on the results of the present study, it can be suggested that the dentists conduct a protocol of saliva collection with a graduated container to calculate the salivary flow by volume, before the beginning of bleaching, to infer the potential of salivary remineralization. The moment of evaluation of non stimulated total salivary flow largely influences the hypossalivation diagnosis. Tests should be ideally performed on a determined moment or in a limited time interval, early in the morning, to control the influence of the circadian rhythm of salivary flow on saliva collection [26].

CONCLUSIONS

After 35% hydrogen peroxide bleaching, low salivary flow was not able to recover enamel surface roughness to the original values, but the normal salivary flow was. The enamel contact with human saliva *in vitro* was sufficient to recover the original enamel surface roughness. However, enamel bleaching and the contact with human saliva *in vitro* or *in situ* did not affect the calcium/phosphorus ratio nor the isolated percentages of calcium and phosphorus.

COMPLIANCE WITH ETHICAL STANDARDS

ETHICAL APPROVAL

This research had the approval of the Local Research Ethics Comitee (CAAE-69736817.8.0000.5149, ID 2.129.538).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

INFORMED CONSENT

The participants of the study received and signed an informed consent form andanswered a questionnaire with a semi-structured interview before the inclusion in the study.

REFERENCES

1. Sa Y, Chen D, Liu Y, Wen W, Xu M, Jiang T *et al* (2012) Effects of two in-office bleaching agents with different pH values on enamel surface structure and color: an *in situ* vs. *in vitro* study. J Dent 40 Suppl 1:e26-34

2. Llena C, Esteve I, Forner L (2018) Effects of in-office blaching on human enamel and dentin. Morphological and mineral changes. Ann Anat 217:97-102

3. Cavalli V, Rosa DA, Silva DP, Kury M, Liporoni PCS, Soares LES, Martins AA (2018) Effects of experimental bleaching agents on the mineral content of sound and demineralized enamels. J Appl Oral Sci 26:e20170589, 1-11

4. de Abreu DR, Sasaki R, Amaral FLB, Florio FM, Basting RT (2011) Effect of home-use and in- office bleaching agents containing hydrogen peroxide associated with amorphous calcium phosphate on enamel microhardness and surface roughness. J Esthet Restor Dent 23(3):158-168

5. Uspenskaya AO, Trefilova OV (2018) Effect of bleaching on the biochemical composition of oral fluid and dental hard tissues structure. Stomatologiia 97(5):27-30

6. Wang J, Yang X, Sun K, Sun P, Pan J, Zhu W, *et al* (2012) Tooth enamel evaluation after tooth bleaching with hydrogen peroxide assisted by a DC nonthermal atmospheric-pressure plasma jet. IEEE Trans Plasma Sci 40(9):2157-2162

7. Dominguez JA, Bittencourt B, Michel M, Sabino N, Gomes JC, Gomes OM (2012) Ultrastructural evaluation of enamel after dental bleaching associated with fluoride. Microsc Res Tech 75(8):1093-1098

8. Hauss Monteiro DD, Valentim PT, Elias DC, Moreira AN, Machado Cornacchia TP, Magalhães CS (2019) Effect of surface treatments on staining and roughness of bleached enamel. Indian J Dent Res 30:393-398

9. Cunha AG, De Vasconcelos AA, Borges BC, Vitoriano JeO, Alves-Junior C, Machado CT *et al* (2012) Efficacy of in-office bleaching techniques combined with the application of a casein phosphopeptide-amorphous calcium phosphate paste at different moments and its influence on enamel surface properties. Microsc Res Tech 75(8):1019-1025

10. Gopinath S, James V, Vidhya S, Karthikeyan K, Kavitha S, Mahalaxmi S (2013) Effect of bleaching with two different concentrations of hydrogen peroxide containing sweet potato extract as an additive on human enamel: An *in vitro* spectrophotometric and scanning electron microscopy analysis. J Conserv Dent JCD 16(1):45-49

11. Bardow A, Lykkeaa J, Qvist V, Ekstrand K, Twetman S, Fiehn N (2014) Saliva composition in three selected groups with normal stimulated salivary flow rates, but yet major differences in caries experience and dental erosion. Acta Odontol Scand 72:466-473

12. Alkattan R, Lippert F, Tang Q, Eckert GJ, Ando M (2018) The influence of hardness and chemical composition on enamel demineralization and subsequent remineralization. J Dent 75:34-40.

13. Mori AA, Lima FF, Benetti AR, Terada RSS, Fujimaki M, Pascotto RC (2016) Susceptibility to coffee staining during enamel remineralization following the in-office bleaching technique: an *in situ* assessment. J Esthet Restor Dent 28(1):S23-31

14. Zeczkowski M, Tenuta LMA, Ambrosano GMB, Aguiar FHB, Lima DANL (2015) Effect of different storage conditions on the physical properties of bleached

enamel: an *in vitro* vs. *in situ* study. J Dent 43:1154-1161.

15. Karlinsey RL, Mackey AC, Blanken DD, Schwandt CS (2012) Remineralization of eroded enamel lesions by simulated saliva *in vitro*. Open Dent J 6:170-176

16. Jager DHJ, Vieira AM, Ligtenberg AJM, Bronkhorst E, Huysmans MCDNJM, Vissink A (2011) Effect of salivary factors on the susceptibility of hydroxyapatite to early erosion. Caries Res 45:532–537

17. Kuchler EC, Pecharki GD, Castro ML, Ramos J, Barbosa Jr F, Brancher JA, Vieira AR, Gerlach RF, Trevilatto PC (2017) Genes involved in the enamel development are associated with calcium and phosphorous level in saliva. Caries Res 51(3):225-230

18. Bardow A, Vissink A (2017) Dental caries: the disease and its clinical management. In: Fejerskov O, Nyvad B, Kidd E. Dental caries – Pathophysiology and treatment. 3rd edn. Guanabara Koogan, Rio de Janeiro, pp 73-93

19. Daniel FI, Lima LD, Grando LJ, Castro R, Cordeiro EAK, Santos CR (2018) Salivary evaluation in radioactive I^{131} treated patients with thyroid carcinoma. Acta Odontol Scand 76(2):148-152

20. Berman N, Vivino F, Baker J, Dunham J, Pinto A (2019) Risk factors for caries development in primary Sjogren syndrome. Oral Surg Oral Med Oral Pathol Oral Radiol 128(2):117-122

21. Affoo RH, Foley N, Garrick R, Siqueira WL, Martin RE (2015) Meta-analysis of salivary flow rates in young and older adults. J Am Geriatrics Society 63(10): 2142–2151.

22. Muralidharan D, Fareed N, Pradeep PV, Margabandhu S, Ramalingam K, Ajith Kumar BV (2013) Qualitative and quantitative changes in saliva among patients with thyroid dysfunction prior to and following the treatment of the dysfunction. Oral Surg Oral Med Oral Pathol Oral Radiol 115:617-623

23. Johnsson M, Winder M, Zawia H, Lödöen I, Tobin G, Götrick B (2016) *In vivo* studies of effects of antidepressants on parotid salivary secretion in the rat. Arch Oral Biol 67:54-60

24. Loke C, Lee J, Sander S, Mei L, Farella M (2016) Factors affecting intra-oral pH – a review. J Oral Rehabil 43:778-785

25. Andrade MRTC, Salazar SLA, Sá LFR, Portela M, Ferreira-Pereira A, Soares RMA, Leão ATT, Primo LG (2015) Role of saliva in the caries experience and

calculus formation of young patients undergoing hemodialysis. Clin Oral Invest 19:1973-1980

26. Flink H, Tegelberg A, Lagerlöf F (2005) Influence of the time of measurement of unstimulated human whole saliva on the diagnosis of hyposalivation. Arch Oral Biol 50:553-559

27. Inoue H, Ono K, Masuda W, Morimoto Y, Tanaka T, Yokota M, Inenaga K (2006) Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. Arch Oral Biol 51:1055-1060

28. Varoni EM, Federighi V, Decani S, Carrassi A, Lodi G, Sardella A (2016) The effect of clinical setting on the unstimulated salivary flow rate. Arch Oral Biol 69:7-12

29. Hayashida JN,Minami S, Moriyama M, Toyoshima T, Shinozaki S, Tanaka A, Maehara T, Nakamura S (2015) Differences of stimulated and unstimulated salivary flow rates in patients with dry mouth. J Oral and Maxillofac Surg, Med and Pathol 27:96-101

30. Liu J, Duan Y (2012) Saliva: a potential media for disease diagnostics and monitoring. Oral Oncol 48(7):569–577

31. Pyati AS, Naveen Kumar R, Kumar V, Praveen Kumar NH, Parveen Reddy KM (2018) Salivaryflow rate, pH, buffering capacity, total protein, oxidative stress and antioxidant capacity in children with and without dental caries. J Clin Pediatr Dent 42(6):445-449

32. Santos DR, Souza RO, Dias LB, Ribas TB, Oliveira LCF, Sumida DH, Dornelles RCM, Nakamune ACMS, Chaves-Neto AH (2018) The effects of storage time and temperature on the stability of salivary phosphatases, transaminases and dehydrogenase. Arch Oral Biol 85:160-165

33. Daniel FI, Lima L, Santos CR (2016) Salivary calcium and phosphate stability in different time and temperature storage. Braz J Pharmac Sci 52(4):679-684

34. Pini NIP, Lima DANL, Sundfeld RH, Ambrosano GMB, Aguiar FHB, Lovadino JR (2016) Tooth enamel properties and morphology after microabrasion: an *in situ* study. J Invest Clin Dent 1–8

35. Henn-Donassollo S, Fabris C, Gagliola M, Kerber I, Caetano V, Carboni V, Salas MMS, Donassollo TA, Demarco FF (2016) *In situ* and *in vitro* effects of two bleaching treatments on human enamel hardness. Braz Dent J 27(1):56-59

36. Heshmat H, Ganjkar MH, Miri Y, Fard MJK (2016) The effect of two

remineralizing agents and natural saliva on bleached enamel hardness. Dent Res J 13:52-57

37. Dawes C, Pedersen AML, Villa A, Ekström J, Proctor GB, Vissink A, Aframian D, McGowan R, Aliko A, Narayana N *et al* (2015) The functions of human saliva: a review sponsored by the World Workshop on Oral Medicine VI. Arch Oral Biol 60:863-874

38. Dawes C, Wong DTW (2019) Role of saliva and salivary diagnostics in the advancement of oral health. J Dent Res 98(2):133-141

39. Batista GR, Torres CRG, Sener B, Attin T, Wiegand A (2016) Artificial saliva formulations versus human saliva pretreatment in dental erosion experiments. Caries Res 50(1):78-86

40. Dourado Pinto AV, Carlos NR, Amaral FLBD, França FMG, Turssi CP, Basting RT (2019) At- home, in-office and combined dental bleaching techniques using hydrogen peroxide: Randomized clinical trial evaluation of effectiveness, clinical parameters and enamel mineral content. Am J Dent 32(3):124-132

41. Carpenter GH (2013) The secretion, components, and properties of saliva. Annu Rev Food SciTechnol 4:267-276

42. Rabiei M, Asli HN, Mohamadi MH (2019) Comparison of salivary calcium level in dentulous and edentulous patients. Eur J Dent 13(1):36-41

43. Aps JKM, Martens LC (2005) Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int 150:119-131.

44. Yamamoto K, Kurihara M, Matsusue Y, Imanishi M, Tsuyuki M, Kirita T (2009) Whole saliva flow rate and body profile in healthy young adults. Arch Oral Biol 54:464-469

45. Mullan F, Austin RS, Parkinson CR, Bartlett DW (2018a) An *in-situ* pilot study to investigate the native clinical resistance of enamel to erosion. J Dent 70:124-128

46. Mullan F, Mylonas P, Parkinson C, Bartlett D, Austin RS (2018b) Precision of 655nm confocal laser profilometry for 3D surface texture characterisation of natural human enamel undergoing dietary acid mediated erosive wear. Dent Mater 34:531-537

47. Bardow A, Moe D, Nyvad B, Nauntofte B (2000) The buffer capacity and buffer systems of human whole saliva measured without loss of CO_2. Arch Oral Biol 45:1-12

6 ARTIGO 2

METALLIZATION AND AR-O PLASMA EFFECTS ON DENTAL ENAMEL ROUGHNESS EVALUATED WITH SEM AND MEX™ FOR 3D RECONSTRUCTION

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ABSTRACT

The MeX™ software is a useful tool for tridimensional data collection for surface evaluation and could be relevant to evaluate the same specimen in different phases of the study, assuming repeated measures of dental enamel roughness. The aim of this study was to evaluate the influence of sample metallization for dental enamel roughness analysis with 3D images reconstructed using MeX™ software from Scanning Electron Microscopy (SEM) images. The influence of 74.98% (%mol/mol) argonoxygen plasma for carbon layer removal on surface roughness of the metallized

specimen was also evaluated. Dental enamel specimens were prepared for SEM analysis with and without carbon metallization using conventional or environmental modes. Argon−oxygen plasma for carbon layer removal was used and surface roughness was re-evaluated. Roughness obtained by SEM and MeX™ reconstructed images, with or without metallization, did not differ. No significant alteration on surface roughness after carbon layer removal using plasma was found. SEM baseline evaluation using conventional mode without sample preparation and in environmen-tal mode were not comparable. Roughness of enamel 3D images reconstructed with MeX™ software from SEM images, with or without metallization was similar. The 74.98% (%mol/mol) argon−oxygen plasma removed the carbon layer with no effect on enamel roughness.

KEYWORDS
Carbon. Dental Enamel. Plasma. Scanning Electron Microscopy. Software.

INTRODUCTION

Dental enamel surface analyses are necessary to evaluate the effects of preventive and therapeutic agents in different experimental conditions. Longitudinal experimental designs set up repeated measures on the same dental specimen to follow the effect of treatments in different phases of the study.

Contact and laser profilometers, atomic force microscope (AFM) and scanning electron microscopy (SEM) are useful methods for sur- face characterization (Fischer et al., 2019; Talu, Contreras-Bulnes, Morozov, Rodríguez-Vilchis, & Montoya-Ayala, 2016). Dental enamel roughness analysis by AFM and contact profilometry are quantitative methods that demand flat specimen surfaces to avoid reading errors on the peaks and valleys (axis Z) or noises, depending on the topographic characteristics. However, a flattened specimen may lead to biased results as the subsurface enamel layer is less mineralized and more susceptible to the effects of treatments (Sorozini, Perez, & Rocha, 2018) while the whole dental surface properly simulates the clinical reality, increasing the validity and scientific value (Mullan, Mylonas, Parkinson, Bartlett, & Austin, 2018). Furthermore, the loca- tion of the area of interest for the repeated measures depends on the operator care due to the lack of precise tools for delimitation. The uncertainty about the readings being made exactly on the same spot prevents the comparison in different phases of the study. This is a shortcoming of some AFM and profilometry equipment that may compromise reproducibility of the readings.

Quanta 3D FEG scanning electron microscope has high resolution (2–5 nm) and applies an electron beam for image acquisition and specimen surface properties assessment. SEM application for surface morphological analysis could be used for longitudinal studies with repeated measures. SEM analysis in environmental mode maintains a rarefied atmosphere of water (residual pressure of approximately 1 mTorr) in the microscope chamber, allowing the dissipation of the superficial charge of the sample and enabling the evaluation of non- conductive and hydrated materials without any sample preparation (Fitzek, Schroettner, Wagner, Hofer, & Rattenberger, 2015; Mansur, 2012; Tihlafíková & Nedela, 2015). Lacking the environmental mode in the microscope, the deposition of a thin conductive layer on the specimen prevents

charge accumulation on the surface and permits the achievement of images without distortion due to beam repulsion during image acquisition.

SEM environmental mode may be properly applied for surface morphology assessment (Ball, Job, & Walker, 2017; Dall'Oca et al., 2017; Manesh, Giacomin, & Stoll, 2017) presenting a great influence on image resolution (Tihlafíková & Nedela, 2015). In some situations, hydrated biofilms are observed in environmental mode, but the magnification will not be comparable to SEM conventional mode (Holling et al., 2014).

The resolution loss in environmental mode occurs mainly due to the interaction of the beam and the chamber atmosphere before the interaction with the sample. To minimize this effect, it is necessary to spend more time on image acquisition, due to the low signal-to-noise ratio. In conventional mode, more accurate images will be easily obtained with the conductive layer on the sample, as the beam interacts only with the analyzed surface, with no charge accumulation, which increases the resolution (Pereira, Daleprane, Barbosa, Moreira, & Magalhães, 2014) and favors the image quality. That is the main reason for metal coating the sample surface. However, the coating process precludes the specimen reuse in subsequent evaluations, preventing the execution of repeated measures studies.

The most used materials for specimen coating are carbon and gold, usually evaporated or sputtered with the sample at room temperature, which create a residual roughness (in the order of a few nanometers). It is important to consider that in many cases the metallization will not impact on the measure, depending on the expected roughness of the sample. Coating with carbon may be a solution to make the surface conductive, and the application of oxygen plasma appears to be able to remove the carbon (Paredes, Salvagni, Rodriguez, Gil, & Manero, 2014) by volatilization in carbon dioxide, but it is necessary to ensure that there is no alteration to the surface roughness with plasma application.

The resultant micrography of SEM analysis are bidimensional images (2D), and it is advisable to reconstruct the tridimensional (3D) model of the SEM images to effectively measure and visualize the surface characteristics (Gontard, Schierholz, Yu, Cintas, & Dunin-Borkowski, 2016). With the MeX™ software, SEM transforms itself in a 3D metrology device with accurate and reliable results (Pirisinu & Mazzarello, 2016), in which the data of the 3D measure are created from 2D images obtained with SEM (Ball et al., 2017). This software rebuilds the height profile

of the analyzed area with two or three 2D images obtained from different points of visualization (different sam- ple tilts). This technique is known as stereoscopic reconstruction and the measurement precision depends on the desired magnification, quality of image and pixel size (Glon, Flys, Lööf, & Rosén, 2014). The software provides quantitative and visual information and is largely used on several research areas, such as medicine, pharmacology, chemistry and mechanics (Tafti, Kirkpatrick, Alavi, Owen, & Yu, 2015). It would be relevant to bring this technology to the dentistry universe, considering the advantage of using SEM for measurements from millimeters to nanometers (Glon et al., 2014), and to apply it with a meth- odology that needs repeated evaluations of the same specimen at different phases of the study.

This study's objective was to evaluate the influence of sample metallization for dental enamel roughness (Arithmetic average of roughness profile—Ra; root mean square deviation—Rq; surface skewness—Ssk; surface kurtosis—Sku; ten-point height—S10z; maxi- mum valley depth—SUV; maximum peak height—Spa) analysis with 3D images reconstructed with MeX™ software from SEM images. The influence of 74.98% (%mol/mol) argon−oxygen plasma (plasmacleaner) for carbon layer removal on surface roughness of the metal- lized specimen was also evaluated. The first null hypothesis is that there is no alteration in roughness (Ra, Rq, Ssk, Sku, S10z, Sv, Sp) measured from SEM images reconstructed in MeX™ software, with or without metallization. The second null hypothesis is that there is no difference in surface roughness with argon−oxygen plasma for the removal of the carbon metallic layer.

MATERIALS AND METHODS

The dependent variable of this in vitro study is the enamel surface roughness (Ra, Rq, Ssk, Sku, S10z, Sv, Sp), and the independent variables are the sample metallization by carbon and the argon−oxygen plasma application for its removal. Three human third molars were obtained from the Human Teeth Biobank of UFMG Dentistry College (COEP: CAAE-69736817.8.0000.5149, ID 2.129.538), subjected to

prophylaxis and evaluated in 8× optical microscopy. Teeth with cracks and surface

defects were excluded, while sound enamel teeth were sectioned to obtain six specimens of approximately 25 mm^2. These specimens were immersed in thymol for 7 days, washed with water and then maintained in a dry environment for 30 days, for gradual dehydration.

A carbon tape was used to stabilize the specimens in the micro- scope and in each specimen an experimental area of 30 μm × 30 μm was delimited with the "*focused ion beam*—FIB" of the double beam microscope "Quanta FEG 3D FEI," operated with 30 kV and a beam current of nA. This delimited experimental area was considered the reference of surface roughness in different moments, on the same specimen.

Images in conventional mode were obtained (high vacuum), with no sample preparation, at 5 kV acceleration, 4.5 spot size and 4 × 10^{-6} Torr residual pressure (R1). Three SEM images were obtained with different sample tilts (angles of −13, 0, and 13°) for each specimen. With the MEV images a 3D model was built in MeX™ software (Alicona Imaging GmbH, Raaba/Graz, Austria) for metrologi- cal analysis and assessment of the surface roughness parameters Ra, Rq, Ssk, Sku, S10z, Sv, and Sp. The software uses the paralaxis effect when tilting the sample around the euccentric position, to determi- nate the differences of height on the area selected for reconstruction (Piazzesi, 1973). The areas of interest were selected on the images, such as those that had less effect of charge accumulation. The lack of contrast is represented on the reconstruction as a plane surface. The procedure is illustrated in Figure 1.

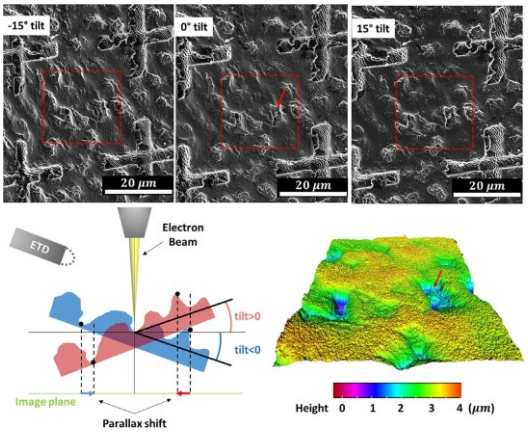
The final value of the z coordinate was obtained by the software with the geometry parameters of the microscope, such as the sample inclination angle, the distance between the sample surface and the lens (working distance) and the calibrated pixel size. Each image pixel was submitted to this procedure to create a digital model of eleva- tions, in a detailed 3D topographic map of the specimen surface. For each one of the presented reconstructions, the best lateral resolution available was selected at around 1800 pixels.

The MeX™ software is suitable for surface metrology and the area measuring parameters can be calibrated based on ISO patterns (ISO 25178-2, 2012; ISO 25178-3, 2012; Pirisinu & Mazzarello, 2016). To calibrate the microscope, the certified sample NIST MRS-6 Magnification Reference Standard was used according to the proce- dure of ASTM E866-98 and ISO 16700. To check the vertical preci- sion

of the reconstruction method, the sample of reference TGXYZ03 AFM/SPM Calibration Grating was used with operating conditions of the microscope similar to those used for the image acquisition of the studied specimens. While reconstructing the 500 nm step height pat-tern in the TGXYZ03 sample, a 3D model with an error of around 40 nm (8% of the determined value) was obtained.

SEM images in environmental mode were obtained, also without any sample preparation, with the same leanings described previously, acceleration of 20 kV, 6.5 spot and residual pressure of 0.08 Torr.

FIGURE 1 - Reconstruction procedure of Specimen 4 from images in conventional mode (high vacuum), with no sample preparation.



However, despite the time of permanence of the beam being around 10 μs, the noise on the images prevented the 3D reconstruction for roughness assessment. As the software makes the calculation on every pixel, a random contrast variation of border pixels (characteristic of the white noise generated by the interaction of the beam with the atmosphere in environmental mode) is recognized as a difference of heights during the reconstruction (Figure 2).

The specimens were metallized with an amorphous carbon layer of 2–4 nm thickness, in a glow discharge process (BalTec Sputter Med20, Baltec Maschinenbau AG, Pfäffikon, Switzerland) and were evaluated in conventional mode on SEM, with the same parameters described previously. The images were exported to MeX™ and reconstructed for surface roughness assessment on the area delimited by FIB (R2).

The specimens were submitted to a 74.98% (%mol/mol) argon−oxygen plasma (Plasma Cleaner Model 1020, E.A. Fischione Instruments Inc, Export) in a

sequence of 10 s exposure until complete removal of the amorphous carbon layer. After 110 s of plasma expo- sure, the specimens were submitted to another metallization with car- bon, SEM evaluation in conventional mode and 3D reconstruction with MeX™ software for roughness assessment (R3). An image of Specimen 4 with the height profile of the same area exemplifies the three steps of the study in Figure 3. The 3D model data was exported to Gwyddion (Nec˘as & Klapetek, 2012) for height profile extraction.

The roughness data were assessed and the measures of variability calculated. Roughness values obtained in the different experimental conditions were compared and the influence of metallization on enamel roughness assessment and the influence of argon−oxygen plasma for carbon removal of metallized specimens were evaluated. The statistical analysis was performed with the software SPSS 17 (Statistical Product and Service Solutions, SPSS, Chicago, IL), with a 5% significance level.

RESULTS

There was no statistically significant difference between roughness (Ra, Rq, Ssk, Sku, S10z, Sv, and Sp) measured with SEM images reconstructed with MeX™, with or without metallization. This result was found with the comparison between R1 and R2 (Ra: p = .917; Rq: p = .917; Ssk: p = .080; Sku: p = .249; S10z: p = .600; Sv: p = .917; Sp: p = .249), and assumes that this null hypothesis was accepted (Table 1).

There was no statistically significant surface roughness alteration (Ra, Rq, Ssk, Sku, S10z, Sv, Sp) with 74.98% (%mol/mol) argon−oxy- gen plasma for carbon removal. This was found with the comparison between R2 and R3 (Ra: p = .463; Rq: p = .500; Ssk: p = .753; Sku: p = .075; S10z: p = .046; Sv: p = .345; Sp: p = .753), which assumes that this null hypothesis was also accepted (Table 2).

Baseline evaluations in conventional mode without sample prepa- ration and in environmental mode were not comparable since environ-
mental mode generated an image of very low quality, less than that necessary for MeX™ reconstruction.

FIGURE 3 - Reconstructions of the same area in the same specimen in the three different steps of the study, from left to right R1, R2, and R3. At the bottom, the height profile along the indicated line is shown for each image.

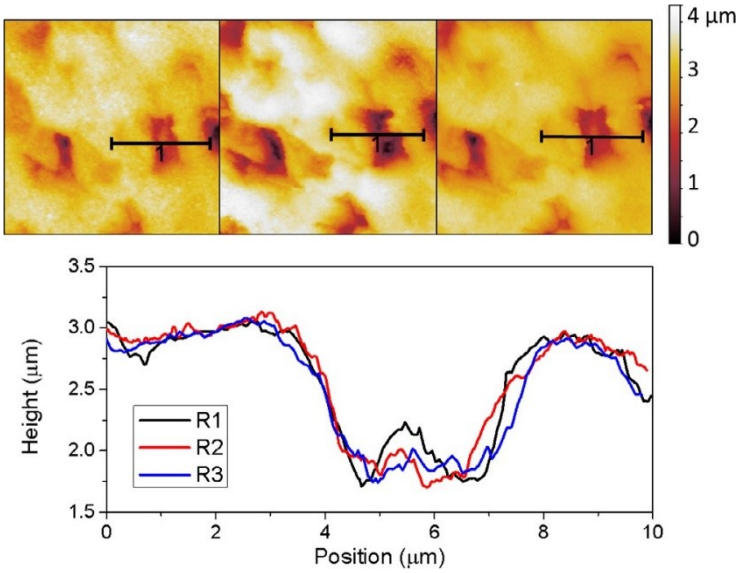


FIGURE 2 - Reconstruction in environmental mode, compared with the conventional mode with no sample preparation.

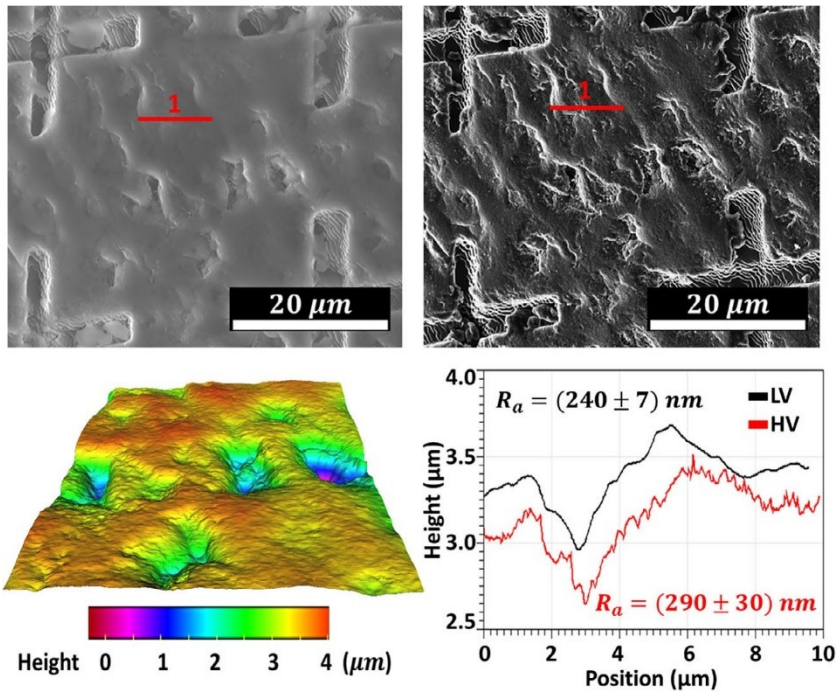


TABLE 1 - Means and standard deviations of surface roughness in R1 and R2

R1 conventional mode baseline		R2 conventional mode after metallization with carbon
Ra (nm)	177.6 ± 48.7 A	174.6 ± 66.7 A
Rq (nm)	230.3 ± 65.9 A	268 ± 92.8 A
Ssk	−0.5 ± 0.6 A	−0.8 ± 0.7 A
Sku	4.3 ± 1.8 A	5.4 ± 2.7 A
S10z (nm)	2,300 ± 800 A	2,400 ± 800 A
Sv (nm)	1,600 ± 800 A	1,600 ± 900 A
Sp (nm)	1,100 ± 400 A	800 ± 300 A

Notes: Wilcoxon Test: Letter A for comparisons between R1 and R2. Equal letters are not statistically different ($p > .05$).

TABLE 2 - Means and standard deviations of surface roughness in R2 and R3.

R2 conventional mode after metallization with carbon		R3 conventional mode after plasma and new metallization
Ra (nm)	174.6 ± 66.7 B	170 ± 61.1 B
Rq (nm)	268 ± 92.8 B	216.3 ± 77.2 B
Ssk	−0.8 ± 0.7 B	−0.7 ± 0.9 B
Sku	5.4 ± 2.7 B	5.0 ± 2.8 B
S10z (nm)	2,400 ± 800 B	2000 ± 700 B
Sv (nm)	1,600 ± 900 B	2000 ± 1,000 B
Sp (nm)	800 ± 300 B	800 ± 300 B

Notes: Wilcoxon test: Letter B for comparisons between R2 and R3. Equal letters are not statistically different ($p > .05$).

DISCUSSION

The main objective of this study was to evaluate the influence of sam- ple metallization for enamel surface roughness assessment with SEM and 3D images reconstructed with MeX™ software. The effect of a 74.98% (%mol/mol) argon−oxygen plasma for carbon layer removal on the surface was also evaluated. The first null hypothesis that there is no difference between roughness (Ra, Rq, Ssk, Sku, S10z, Sv, and Sp) assessed with the SEM images reconstructed in MeX™ software, with or without metallization, was accepted. The second null hypothe- sis that there is no surface roughness alteration with argon−oxygen plasma application for the removal of the carbon layer was also
accepted.

This study was first designed with the purpose of evaluating den- tal enamel specimens' roughness submitted to surface treatments without flattening (Sorozini et al., 2018), in different moments (repeated measures), without the necessity of metallization. Although the most appropriate methods have been discussed (Fischer et al., 2019; Talu et al., 2016), there was still a gap in this topic. The first attempt was to obtain images in environmental mode, without speci- men metallization. However, this approach generated noisy images and the 3D reconstructions in MeX and the roughness values were not reliable. The surface metallization with carbon application was then considered for conventional mode assessment and argon−oxy- gen plasma application for carbon layer removal. Initially, it was sup- posed that the 74.98% (%mol/mol) argon−oxygen plasma would be able to remove all the carbon. In a previous study, it was observed that the oxygen plasma decreases the carbon content and surface contaminants, and induces alterations in the atomic composition of the oxide layer (Paredes et al., 2014). In chromium−cobalt alloys, differences in the OH^- percentages were observed with the oxygen plasma application, which showed that the method is effective for cleaning and surface activation (Paredes et al., 2014). The argon plasma application with 1.0 vol% oxygen admixture also has the effect of enamel chemical cleaning, reducing the organic compounds and increasing mineral compound concentration (Jablonowski et al., 2017).

The 74.98% (%mol/mol) argon−oxygen plasma was chosen for this study

instead of only argon plasma or only oxygen plasma, as the argon plasma on its own could induce some surface alteration, and then the observed roughness would not be real. Also, whether the oxygen plasma on its own would be able to remove all the carbon layer is speculated. In a previous study, with equal exposure times, the argon plasma induced better material removal and higher surface roughness alteration, when compared to oxygen plasma. The protec- tive oxide layer that the oxygen plasma produces may remove surface contaminants without creating defects on it (Berman & Krim, 2012). With the oxygen plasma application, the molecules were pinned on the original untreated surface at low coverages, which is consistent with the presence of contamination and pinning sites (Berman & Krim, 2012). In the present study, there was no surface roughness alteration (Ra, Rq, Ssk, Sku, S10z, Sv, and Sp) with the application of 74.98% (%mol/mol) argon−oxygen plasma (Table 2), and, as shown in Figure 3,
there was no significant modification in the surface features after the removal of carbon by the plasma process. The complete removal of the coating layer was assured by visual inspection of the surface shade using a low magnification bench stereomicroscope.

The oxygen plasma application generates roughness values that show a random distribution of peaks and valleys, which is consistent with the surface contaminants' removal without roughness alteration (Paredes et al., 2014). The application of argon−oxygen plasma is able to remove the organic material of the dental enamel, while the min- erals are left on the surface. An increase of PO_4 may take place, which confirms that the hydroxyapatite is left on the surface after plasma exposure (Jablonowski et al., 2017).

The treatment with a nonthermal argon plasma does not integrate the argon to the substrate, and may induce a decrease in the concen- tration of carbon and nitrogen, and an increase of oxygen, calcium and phosphorus on the enamel (Chen et al., 2013), in the same way as the argon−oxygen plasma (Jablonowski et al., 2017). Regarding surface morphology, no significant difference was observed in the differ- ent times of exposure to plasma, although a slightly rougher surface has been observed with the only argon plasma application for a longer time (30 s) (Chen et al., 2013). The prolonged exposure to argon−oxygen plasma induces an increase of calcium, phosphorus and oxygen concentration due to the increase of the superficial hydroxyapatite on the enamel and exposure of its interprismatic structure

(Jablonowski et al., 2017). The application of an argon plasma with 1 vol% oxygen for 300 s is able to remove tooth biofilm effectively and seems to remove the smear layer and associated proteins (Jablonowski et al., 2017). In the present study the surface covered with amorphous car- bon was exposed to the plasma cleaner for 110 s, when a visual inspection determined the total removal of the metallizing layer. Plasma exposure for 110 s did not induce any surface roughness alteration (Table 2).

The initial evaluations in conventional mode with no sample prep- aration and in environmental mode were not comparable since the evaluation in environmental mode generated a low-quality image, preventing MeX™ reconstruction. Since there are water molecules between the electron beam and the sample, the beam interacts with the atmosphere before reaching the sample, generating high noise in each pixel and a loss of resolution and contrast. For the stereoscopic reconstruction there is a dependence relation of the image resolution to generate a suitable MeX™ reconstruction that allows roughness evaluation (Bariani, De Chiffre, Hansen, & Horsewell, 2005). SEM analysis in conventional mode demands a dehydrated specimen, since the microscope should operate in a high vacuum. For this reason, this methodology is more suitable for dental specimens constituted only of enamel, since dentin substrate has considerable humidity.

In the present study there was no difference between roughness (Ra, Rq, Ssk, Sku, S10z, Sv, and Sp) measured with SEM images reconstructed on MeX™ with or without metallization, which was observed in the comparison between R1 and R2 (Table 1). However, on the specimens with no metallization there was more noise on the images and consequently the visualization of the fine details of the structure was compromised.

The use of MeX™ software presents particularities regarding the method. The most important parameters that may affect the precision of the SEM analysis and surface 3D reconstruction are the leaning angle precision and the calibration of image ampliation, as well as the image quality and spacial resolution (Tafti et al., 2015). For the present analysis, low magnifications were used—around 5,000 and 10,000×— which are significantly lower than the ampliation limit of the SEM (350,000×).

The great advantage of using SEM to measure topography is the ability to evaluate samples with a good range of magnifications. It is possible to locate in seconds the area of interest, visualizing surface characteristics in low or high magnifications (Glon et al., 2014). How- ever, in the present study the experimental

areas delimited by FIB were not promptly found, and it was necessary to sweep the total area of the specimen and assess increasing magnification images to facilitate the localization.

Tridimensional reconstruction is suitable as a nondestructive method for roughness evaluation (Ball et al., 2017; Gontard et al., 2016; Manesh et al., 2017). The MeX™ software is useful for 3D data collection and surface modification evaluation. The MeX™ precision is assured examining surfaces of different sizes and characteristics
(Pirisinu & Mazzarello, 2016). With other software, when a sample is nearly uniform considering its roughness and therefore has low- contrast zones, the reconstruction error is considerable (Tondare, Villarrubia, & Vlada, 2017). The MeX™ software generates precise 3D images, allowing surface measurings, such as roughness, area, volume, height and depth (Pirisinu & Mazzarello, 2016). The results of this study establish that this noninvasive analytical approach of applying SEM + MeX constitutes a valid and precise tool for enamel roughness evaluation.

SEM evaluation and MeX™ software reconstruction was a valid method especially when metallized specimens with carbon coverage and conventional mode SEM evaluation were used. The sample prepa- ration and conventional mode analysis resulted in higher quality images and reliable reconstructions in MeX™ to properly achieve roughness values.

CONCLUSIONS

Roughness measures (Ra, Rq, Ssk, Sku, S10z, Sv, and Sp) performed on 3D images reconstructed with MeX™ software from SEM images with or without metallization were similar. The 74.98% (%mol/mol) argon−oxygen plasma was able to remove the carbon layer with no effect on enamel roughness.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1 Ball, A. D., Job, P. A., & Walker, A. E. L. (2017). SEM-micro- photogrammetry, a new take on an old method for generating high- resolution 3D models from SEM images. *Journal of Microscopy*, *267*, 214–226. https://doi.org/10.1111/jmi.12560

1 Bariani, P., De Chiffre, L., Hansen, H., & Horsewell, A. (2005). Investigation on the traceability of three dimensional scanning electron microscope measurements based on the stereo-pair technique. *Precision Engineer- ing*, *29*, 219–228. https://doi.org/10.1016/j.precisioneng.2004.08.002 Berman, D., & Krim, J. (2012). Impact of oxygen and argon plasma expo- sure on the roughness of gold film surfaces. *Thin Solid Films*, *520*,

2 6201–6206. https://doi.org/10.1016/j.tsf.2012.06.033

3 Chen, M., Zhang, Y., Driver, M. S., Caruso, A. N., Yu, Q., & Wang, Y. (2013). Surface modification of several dental substrates by non-thermal, atmospheric plasma brush. *Dental Materials*, *29*, 871–880. https://doi. org/10.1016/j.dental.2013.05.002

4 Dall'Oca, C., Maluta, T., Micheloni, G. M., Cengarle, M., Morbioli, G., Bernardi, P., … Magnan, B. (2017). The biocompatibility of bone cements: Progress in methodological approach. *European Journal of Histochemistry*, *61*(2), (2673), 1–11. https://doi.org/10.4081/ejh.2017.

5 2673

6 Fischer, N. G., Dang, J., Takamizawa, T., Tsujimoto, A., Barkmeier, W. W., & Baruth, A. G. (2019). The role of spatial frequency analysis in correlating atomic force microscopy and optical profilometry with self-etch adhesive enamel bond fatigue durability. *Microscopy Research and Technique*, *82*, 1419–1429. https://doi.org/ 10.1002/jemt.23294

7 Fitzek, H., Schroettner, H., Wagner, J., Hofer, F., & Rattenberger, J. (2015).

Experimental evaluation of environmental scanning electron micro- scopes at high chamber pressure. *Journal of Microscopy*, *260*, 133–139. https://doi.org/10.1111/jmi.12275

8 Glon, F., Flys, O., Lööf, P. J., & Rosén, B. G. (2014). 3D SEM for surface topography quantification — A case study on dental surfaces. 14th international conference on metrology and properties of engineering surfaces. *Journal of Physics: Conference Series*, *483*, 012026, 1–8. https://doi.org/10.1088/1742-6596/483/1/012026

9 Gontard, L. C., Schierholz, R., Yu, S., Cintas, J., & Dunin-Borkowski, R. E. (2016). Photogrammetry of the three-dimensional shape and texture of a nanoscale particle using scanning electron microscopy and free software. *Ultramicroscopy*, *169*, 80–88. https://doi.org/10.1016/j. ultramic.2016.07.006

10 Holling, N., Dedi, C., Jones, C. E., Hawthorne, J. A., Hanlon, G. W., Salvage, J. P., … Jones, B. V. (2014). Evaluation of environmental scan- ning electron microscopy for analysis of *Proteus mirabilis* crystalline biofilms *in situ* on urinary catheters. *FEMS Microbiology Letters*, *355*,

11 20–27. https://doi.org/10.1111/1574-6968.12451

12 ISO 25178-2:2012 (2012). Geometrical product specification (GPS) – Sur- face texture: Areal — Part 2: Terms, definitions and surface texture parameters. *International Organization for Standardization*.

13 ISO 25178-3:2012 (2012). Geometrical product specification (GPS) – Sur- face texture: Areal — Part 3: Specification operators. *International Organization for Standardization*.

14 Jablonowski, L., Fricke, K., Matthes, R., Holtfreter, B., Schlüter, R., Woedtke, T. V., … Kocher, T. (2017). Removal of naturally grown human biofilm with an atmospheric pressure plasma jet: An *in-vitro*

15 study. *Journal of Biophotonics*, *10*, 718–726. https://doi.org/10.1002/ jbio.201600166

16 Manesh, V. S., Giacomin, P., & Stoll, R. (2017). Quantitative evaluation of root canal surface roughness after filling with adaptive reciprocating and continuous rotary instruments. *Microscopy Research and Tech- nique.*, *80*, 657–661. https://doi.org/10.1002/jemt.22845

17 Mansur, H. S. (2012). Materials characterization techniques. In *Biomateriais*

18 *Fundamentos & Aplicações (Chapter 7)*. Guanabara: Koogan.

19 Mullan, F., Mylonas, P., Parkinson, C., Bartlett, D., & Austin, R. S. (2018). Precision of 655nm confocal laser profilometry for 3D surface texture characterisation of natural human enamel undergoing dietary acid mediated erosive wear. *Dental Materials*, *34*, 531–537. https://doi. org/10.1016/j.dental.2017.12.012

20 Nečas, D., & Klapetek, P. (2012). Gwyddion: An open-source software for SPM data analysis. *Central European Journal of Physics*, *10*, 181–188. https://doi.org/10.2478/s11534-011-0096-2

21 Paredes, V., Salvagni, E., Rodriguez, E., Gil, F. J., & Manero, J. M. (2014). Assessment and comparison of surface chemical composition and oxide layer modification upon two different activation methods on a CoCrMo alloy. *Journal of Materials Science, Materials in Medicine*, *25*, 311–320. https://doi.org/10.1007/s10856-013-5083-2

22 Pereira, C. N.d. B., Daleprane, B., Barbosa, P. F., Moreira, A. N., & Magalh~aes, C. S. (2014). Qualitative evaluation of scanning electron microscopy methods in a study of the resin cement/dentine adhesive interface. *Microscopy and Microanalysis*, *20*, 268–275.

23 Piazzesi, G. (1973). Photogrammetry with the scanning electron micro- scope. *Journal of Physics E: Applied Physics*, *6*, 392–396.

24 Pirisinu, M., & Mazzarello, V. (2016). 3D profilometric characterization of the aged skin surface using a skin replica and alicona MeX software. *Scanning*, *38*, 213–220. https://doi.org/10.1002/sca.21256

25 Sorozini, M., Perez, C. R., & Rocha, G. M. (2018). Enamel sample prepara- tion for AFM: Influence on roughness and morphology. *Microscopy Research and Technique*, *81*, 1071–1076. https://doi.org/10.1002/ jemt.23073

26 Tafti, A. P., Kirkpatrick, A. B., Alavi, Z., Owen, H. A., & Yu, Z. (2015). Recent advances in 3D SEM surface reconstruction. *Micron*, *78*, 54–66. https://doi.org/10.1016/j.micron.2015.07.005

27 Talu, S., Contreras-Bulnes, R., Morozov, I. A., Rodríguez-Vilchis, L. E., & Montoya-Ayala, G. (2016). Surface nanomorphology of human dental enamel irradiated with an Er:YAG laser. *Laser Physics*, *26*, 1–9. https:// doi.org/10.1088/1054-660X/26/2/025601

28 Tihlafíková, E., & Nedela, V. (2015). Repetitive observation of coniferous samples in ESEM and SEM. *Microscopy and Microanalysis*, *21*, 1695–1696. https://doi.org/10.1017/S1431927615009253

29 Tondare, V. N., Villarrubia, J. S., & Vlada, R. A. E. (2017). Three-dimensional (3D) nanometrology based on scanning electron microscope (SEM) stereophotogrammetry. *Microscopy and Microanalysis*, *23*, 967–977. https://doi.org/10.1017/S1431927617012521

7 CONSIDERAÇÕES FINAIS

Esta pesquisa foi idealizada a partir de uma questão clínica na qual uma paciente desejava realizar o clareamento dental em consultório, mas fazia uso de antidepressivos que acarretaram baixo fluxo salivar. Em minha dissertação de mestrado, entre outros aprendizados, foram retiradas conclusões acerca das consequências deletérias que o peróxido de hidrogênio em alta concentração pode induzir ao esmalte dentário. Embora tenha sido usada apenas saliva artifical nesse primeiro estudo, ressaltou-se a importância do papel da saliva no controle das alterações no esmalte. O acompanhamento profissional durante o clareamento dentário se faz importante para minimizar os efeitos indesejáveis e aumentar a longevidade do efeito alcançado, reduzindo o manchamento por corantes e a recidiva da cor. Nesse contexto, o presente estudo é um desdobramento do conhecimento adquirido no desenvolvimento da dissertação de mestrado, e foi motivado pela suposição de que o baixo fluxo salivar não promove o adequado reparo dos efeitos deletérios induzidos pelo peróxido em alta concentração usado na técnica de clareamento em consultório. Isso justificaria a inclusão de medidas adicionais, como o uso de agentes remineralizantes, no protocolo de realização do procedimento.

A presente pesquisa foi cuidadosamente delineada para avaliar o efeito da saliva humana *in vitro* e do fluxo salivar normal e reduzido, *in situ*, na rugosidade eno conteúdo mineral do esmalte clareado com peróxido de hidrogênio a 35%. Avaliou-se também o pH, as concentrações de cálcio e fósforo e a capacidade tampão da saliva em participantes com fluxo salivar normal e reduzido, e seus efeitos na recuperação da rugosidade do esmalte. A inclusão de um grupo experimental no qual os espécimes foram mantidos em saliva humana *in vitro*, teve oobjetivo de validar a metodologia usada em nossos estudos laboratoriais prévios. Além disso, foram incluídos dois grupos controle: o esmalte não clareado, e não exposto à saliva, para registrar os parâmetros da superfície original, sem nenhum tipo de tratamento; e o esmalte clareado e não exposto à saliva, para comparação dos efeitos deletérios induzidos pelo peróxido de hidrogênio em alta concentração. Os demais grupos experimentais foram submetidos à condição *in situ*, na qual os

espécimes foram fixados na cavidade bucal de voluntários com fluxo salivar normale com fluxo reduzido. Isso permitiu investigar não só o efeito da presença da saliva humana, mas também a influência do movimento salivar no fluxo intrabucal, considerando as diferentes fases de salivação do ciclo circadiano. Outras variáveis explicativas incluídas foram o pH, a capacidade tampão e as concentrações de cálcio e fósforo da saliva.

O estudo da rugosidade superficial do esmalte por meio da perfilometria tridimensional de não-contato a laser foi desenvolvido em nosso grupo de pesquisa, desde a minha dissertação de Mestrado, em parceria com o Departamento de Física do Instituto de Ciências Exatas da UFMG. O uso desse método permitiu a análise da superfície original do esmalte, sem a necessidade de planificação e polimento, que removeriam a sua camada externa, limitando a validade externa do estudo. Para responder à questão da pesquisa, além da rugosidade superficial, outro desfecho relevante observado foi o conteúdo mineral do esmalte, representado pelas porcentagens de cálcio e fósforo e a proporção cálcio/fósforo, determinadas por espectroscopia de energia dispersiva por Raios X.

Os resultados encontrados permitiram concluir que:

- o contato dos espécimes com a saliva humana *in vitro* restabeleceu os níveis de rugosidade do esmalte encontrados antes do clareamento com peróxido de hidrogênio a 35%;

- o fluxo salivar reduzido não foi capaz de restabelecer a rugosidade de superfície do esmalte clareado aos valores iniciais, mas o fluxo salivar normal foi capaz;

- o clareamento dentário com peróxido de hidrogênio a 35% não promoveu diferença na proporção cálcio/fósforo e nas porcentagens isoladas de cálcio e fósforo do esmalte dentário humano;

- o contato com a saliva humana não promoveu alterações na proporção cálcio/fósforo e nas porcentagens isoladas de cálcio e fósforo do esmalte dentário humano clareado;

- a recuperação da rugosidade do esmalte clareado foi maior em fluxo salivar normal que em fluxo salivar reduzido, independentemente do pH, da concentração de Ca eP e da capacidade tampão da saliva.

Para viabilizar esse estudo, envolvendo áreas diversas do conhecimento, foram estabelecidas parcerias com o Departamento de Física, para a realização da perfilometria, Departamento de Química, para a espectrofotometria de absorbância, Centro de Desenvolvimento da Tecnologia Nuclear, para a esterilização dos espécimes por raios gama e para a análise por espectroscopia de energia dispersiva por Raios X. Outra parceria fundamental foi firmada com o Centro de Microscopia da UFMG, com o qual desenvolvemos um estudo preliminar, com o objetivo de avaliar um método alternativo para a análise da rugosidade dentária. Foi utilizada a microscopia eletrônica de varredura (MEV) associada ao software MeX® para a obtenção de imagens tridimensionais a partir das quais foram gerados parâmetros de rugosidade da superfície dos espécimes de esmalte dentário. Diante da necessidade de metalização das amostras com filme de carbono para análise em MEV, testou-se o uso do plasma de oxigênio para remoção da camada de carbono, pois o estudo envolvia medidas repetidas em um mesmo corpo de prova. O método mostrou-se viável, e esse estudo preliminar gerou outra produção científica, publicada na revista Microscopy Research and Technique.

Ao final dessa trajetória, posso apontar como as maiores dificuldades encontradas no desenvolvimento desse estudo, aquelas relacionadas à seleção e ao controle dos participantes. Para equilibrar gênero e idade entre participantes de fluxo salivar normal (G4) e baixo fluxo salivar (G5) foi preciso realizar um número de coletas de saliva aproximadamente 5 vezes maior que o tamanho amostral estimado. A coleta de saliva foi realizada pela manhã para minimizar a influência do círculo circadiano. O protocolo envolvia a exigência de não ter se alimentado ou realizado a higienização bucal antes da coleta, representando certo desconforto para os participantes, que procurei minimizar realizando os procedimentos de forma ágil.

A riqueza do aprendizado vivenciado ao longo do curso consiste não só na elucidação da pergunta central que motivou o estudo de tese, mas estende-se à busca de diferentes métodos adequados para responder aos objetivos, ao

desenvolvimento de raciocínio científico para explicar os mecanismos envolvidos nas respostas aos testes aplicados, além de levantar novos questionamentos, aprofundando o conhecimento. O produto final desse estudo é fruto do trabalho em equipe que permitiu o meu aperfeiçoamento no uso de novas técnicas metodológicas e a geração de um conhecimento novo acerca do tema abordado.

Como perspectiva de estudos futuros, seria importante determinar o nível limite de fluxo salivar em que o clareamento em consultório com peróxido de hidrogênio em altas concentrações seria contra-indicado, e se algum agente remineralizante poderia minimizar os efeitos negativos do clareador. Em caso afirmativo, qual agente poderia ser indicado para atuar na remineralização do esmalte dentário de pacientes com baixo fluxo salivar, para diminuição da rugosidade de superfície e aumento da durabilidade da cor conseguida com o clareamento.

REFERÊNCIAS

Affoo RH, Foley N, Garrick R, Siqueira WL, Martin RE. Meta-analysis of salivary flow rates in young and older adults. **Journal of American Geriatrics Society**, v.63,n.10, p.2142–2151, 2015.

Alkattan R, Lippert F, Tang Q, Eckert GJ, Ando M. The influence of hardness and chemical composition on enamel demineralization and subsequent remineralization. **Journal of Dentistry**, v.75, p.34-40, 2018.

Andrade MRTC, Salazar SLA, Sá LFR, Portela M, Ferreira-Pereira A, Soares RMA, Leão ATT, Primo LG. Role of saliva in the caries experience and calculus formation of young patients undergoing hemodialysis. **Clinical Oral Investigations**, v.19, p.1973-1980, 2015.

Aps JKM, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. **Forensic Science International**, v.150, p.119-131, 2005.

Azer SS, Hague AL, Johnston WM. Effect of bleaching on tooth discolouration from food colourant *in vitro*. **Journal of Dentistry**, v.39, Suppl 3, p.e52-e56, 2011.

Bardow A, Lykkeaa J, Qvist V, Ekstrand K, Twetman S, Fiehn N. Saliva composition in three selected groups with normal stimulated salivary flow rates, but yet major differences in caries experience and dental erosion. **Acta Odontologica Scandinavica**, v.72, p.466-473, 2014.

Bardow A, Moe D, Nyvad B, Nauntofte B. The buffer capacity and buffer systems of human whole saliva measured without loss of CO_2. **Archives of Oral Biology**, v.45, p.1-12, 2000.

Bardow A, Vissink A. Dental caries: the disease and its clinical management. In: FEJERSKOV O, NYVAD B, KIDD E. **Dental caries – Pathophysiology and treatment**. 3.ed. Rio de Janeiro: Guanabara Koogan, 2017. pp 73-93.

Batista GR, Torres CRG, Sener B, Attin T, Wiegand A. Artificial saliva formulations versus human saliva pretreatment in dental erosion experiments. **Caries Research**, v.50, n.1, p.78-86, 2016.

Berman N, Vivino F, Baker J, Dunham J, Pinto A. Risk factors for caries development in primary Sjogren syndrome. **Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology**, v.128, n.2, p.117-122, 2019.

Bersezio C, Martín J, Prieto MV, Meneses P, Angel P. One-year bleaching efficacy using two HP products with different pH: a double-blind randomized clinical trial. **Journal of Esthetic and Restorative Dentistry**, v.31, n.5, p.493-499, 2019.

Carpenter GH. The secretion, components, and properties of saliva. **Annual Review Food Science Technology**, v.4, p.267-276, 2013.

Cavalli V, Rosa DA, Silva DP, Kury M, Liporoni PCS, Soares LES, Martins AA. Effects of experimental bleaching agents on the mineral content of sound and demineralized enamels. **Journal of Applied Oral Sciences**, v.26, n.e20170589, p.1-11, 2018.

Cockburn N, Pradhan A, Taing MW, Kisely S, Ford PJ. Oral health impacts of medications used to treat mental illness. **Journal of Affective Disorders**, v.223, p.184-193, 2017.

Cunha AG, De Vasconcelos AA, Borges BC, Vitoriano JeO, Alves-Junior C, Machado CT *et al*. Efficacy of in-office bleaching techniques combined with the application of a casein phosphopeptide-amorphous calcium phosphate paste at different moments and its influence on enamel surface properties. **Microscopy Research and Technique**, v.75, n.8, p.1019-1025, 2012.

Daniel FI, Lima LD, Grando LJ, Castro R, Cordeiro EAK, Santos CR. Salivary evaluation in radioactive I^{131} treated patients with thyroid carcinoma. **Acta Odontologica Scandinavica**, v.76, n.2, p.148-152, 2018.

Daniel FI, Lima L, Santos CR. Salivary calcium and phosphate stability in different time and temperature storage. **Brazilian Journal of Pharmaceutical Sciences**, v.52, n.4, p.679-684, 2016.

Dawes C, Pedersen AML, Villa A, Ekström J, Proctor GB, Vissink A, Aframian D, McGowan R, Aliko A, Narayana N *et al*. The functions of human saliva: a review sponsored by the World Workshop on Oral Medicine VI. **Archives of Oral Biology**, v.60, p.863-874, 2015.

Dawes C, Wong DTW. Role of saliva and salivary diagnostics in the advancement of oral health. **Journal of Dental Research**, v.98, n.2, p.133-141, 2019.

de Abreu DR, Sasaki R, Amaral FLB, Florio FM, Basting RT. Effect of home-use and in-office bleaching agents containing hydrogen peroxide associated with amorphous calcium phosphate on enamel microhardness and surface roughness. **Journal of Esthetics and Restorative Dentistry**, v.23, n.3, p.158-168, 2011.

Dominguez JA, Bittencourt B, Michel M, Sabino N, Gomes JC, Gomes OM. Ultrastructural evaluation of enamel after dental bleaching associated with fluoride. **Microscopy Research and Technique**, v.75, n.8, p.1093-1098, 2012.

Dourado Pinto AV, Carlos NR, Amaral FLBD, França FMG, Turssi CP, Basting RT. At-home, in-office and combined dental bleaching techniques using hydrogen peroxide: Randomized clinical trial evaluation of effectiveness, clinical parameters and enamel mineral content. **American Journal of Dentistry**, v.32, n.3, p.124-132, 2019.

Ferreira SD, Araujo JLN, Morhy ON, Tapety CMC, Youssef MN, Sobral MAP. The effect of fluoride therapies on the morphology of bleached human dental enamel. **Microscopy Research and Technique**, v.74, n.6, p.512-516, 2011.

Flink H, Tegelberg A, Lagerlöf F. Influence of the time of measurement of unstimulated human whole saliva on the diagnosis of hyposalivation. **Archives of Oral Biology**, v.50, p.553-559, 2005.

Gopinath S, James V, Vidhya S, Karthikeyan K, Kavitha S, Mahalaxmi S. Effect of bleaching with two different concentrations of hydrogen peroxide containing sweet potato extract as an additive on human enamel: An *in vitro* spectrophotometric and scanning electron microscopy analysis. **Journal of Conservative Dentistry**, v.16, n.1, p.45-49, 2013.

Hauss Monteiro DD, Valentim PT, Elias DC, Moreira AN, Machado Cornacchia TP, Magalhães CS. Effect of surface treatments on staining and roughness of bleached enamel. **Indian Journal of Dental Research**, v.30, p.393-398, 2019.

Hayashida JN, Minami S, Moriyama M, Toyoshima T, Shinozaki S, Tanaka A, Maehara T, Nakamura S. Differences of stimulated and unstimulated salivary flow rates in patients with dry mouth. **Journal of Oral and Maxillofacial Surgery, Medicine and Pathology**, v.27, p.96-101, 2015.

Henn-Donassollo S, Fabris C, Gagliola M, Kerber I, Caetano V, Carboni V, Salas MMS, Donassollo TA, Demarco FF. *In situ* and *in vitro* effects of two bleaching treatments on human enamel hardness. **Brazilian Dental Journal**, v.27, n.1, p.56-59, 2016.

Heshmat H, Ganjkar MH, Miri Y, Fard MJK. The effect of two remineralizing agents and natural saliva on bleached enamel hardness. **Dental Research Journal**, v.13, p.52-57, 2016.

Inoue H, Ono K, Masuda W, Morimoto Y, Tanaka T, Yokota M, Inenaga K. Gender difference in unstimulated whole saliva flow rate and salivary gland sizes. **Archives of Oral Biology**, v.51, p.1055-1060, 2006.

Jager DHJ, Vieira AM, Ligtenberg AJM, Bronkhorst E, Huysmans MCDNJM, Vissink
A. Effect of salivary factors on the susceptibility of hydroxyapatite to early erosion.
Caries Research, v.45, p.532–537, 2011.

Johnsson M, Winder M, Zawia H, Lödöen I, Tobin G, Götrick B. *In vivo* studies of effects of antidepressants on parotid salivary secretion in the rat. **Archives of Oral Biology**, v.67, p.54-60, 2016.

Justino LM, Tames DR, Demarco FF. *In situ* e *in vitro* effects of bleaching with carbamide peroxide on human enamel. **Operative Dentistry**, v.29, n.2, p.219-225, 2004.

Karadas M, Tahan E, Demirbuga S, Seven N. Influence of tea and cola on tooth colorafter two in-office bleaching applications. **Journal of Research Dentistry**, v.2, n.2, p.83-87, 2014b.

Karlinsey RL, Mackey AC, Blanken DD, Schwandt CS. Remineralization of eroded enamel lesions by simulated saliva *in vitro*. **Open Dentistry Journal**, v.6, p.170-176, 2012.

Kim YS, Kwon HK, Kim BI. Effect of nano-carbonate apatite to prevent re-stain after dental bleaching *in vitro*. **Journal of Dentistry**, v.39, n.9, p.636-642, 2011.

Kuchler EC, Pecharki GD, Castro ML, Ramos J, Barbosa Jr F, Brancher JA, Vieira AR, Gerlach RF, Trevilatto PC. Genes involved in the enamel development are associated with calcium and phosphorous level in saliva. **Caries Research**, v.51,n.3, p.225-230, 2017.

Li Q, Xu BT, Li R, Yu H, Wang YN. Quantitative evaluation of colour regression and mineral content change of bleached teeth. **Journal of Dentistry**, v.38, p.253-260, 2010.

Liu J, Duan Y. Saliva: a potential media for disease diagnostics and monitoring. **Oral Oncology**, v.48, n.7, p.569–577, 2012.

Llena C, Esteve I, Forner L. Effects of in-office blaching on human enamel and dentin. Morphological and mineral changes. **Annals of Anatomy**, v.217, p.97-102, 2018.

Loke C, Lee J, Sander S, Mei L, Farella M. Factors affecting intra-oral pH – a review.
Journal of Oral Rehabilitation, v.43, p.778-785, 2016.

Mori AA, Lima FF, Benetti AR, Terada RSS, Fujimaki M, Pascotto RC. Susceptibility to coffee staining during enamel remineralization following the in-office bleaching technique: an *in situ* assessment. **Journal of Esthetic and Restorative Dentistry**, v.28, n.1, p.S23-31, 2016.

Mullan F, Austin RS, Parkinson CR, Bartlett DW. An *in-situ* pilot study to investigate the native clinical resistance of enamel to erosion. **Journal of Dentistry**, v.70, p.124-128, 2018a.

Mullan F, Mylonas P, Parkinson C, Bartlett D, Austin RS. Precision of 655nm confocal laser profilometry for 3D surface texture characterisation of natural human enamel undergoing dietary acid mediated erosive wear. **Dental Materials**, v.34, p.531-537, 2018b.

Muralidharan D, Fareed N, Pradeep PV, Margabandhu S, Ramalingam K, Ajith Kumar BV. Qualitative and quantitative changes in saliva among patients with thyroid dysfunction prior to and following the treatment of the dysfunction. **Oral Surgery OralMedicine Oral Pathology Oral Radiology**, v.115, p.617-623, 2013.

Pini NIP, Lima DANL, Sundfeld RH, Ambrosano GMB, Aguiar FHB, Lovadino JR. Tooth enamel properties and morphology after microabrasion: an *in situ* study. **Journal of Investigative Clinical Dentistry**, p.1–8, 2016.

Públio JC, D'Arce MB, Brunharo NM, Ambrosano GM, Aguiar FH, Lovadino JR, *et al*. Influence of surface treatments on enamel susceptibility to staining by cigarette smoke. **Journal of Clinical and Experimental Dentistry**, v.5, n.4, p.e163-e168, 2013.

Pyati AS, Naveen Kumar R, Kumar V, Praveen Kumar NH, Parveen Reddy KM. Salivary flow rate, pH, buffering capacity, total protein, oxidative stress and antioxidant capacity in children with and without dental caries. **Journal of Clinical Pediatric Dentistry**, v.42, n.6, p.445-449, 2018.

Rabiei M, Asli HN, Mohamadi MH. Comparison of salivary calcium level in dentulous and edentulous patients. **European Journal of Dentistry**, v.13, n.1, p.36-41, 2019.

Sa Y, Chen D, Liu Y, Wen W, Xu M, Jiang T *et al*. Effects of two in-office bleaching agents with different pH values on enamel surface structure and color: an *in situ* vs.*in vitro* study. **Journal of Dentistry**, v.40, Suppl 1, p.e26-e34, 2012.

Santos DR, Souza RO, Dias LB, Ribas TB, Oliveira LCF, Sumida DH, Dornelles RCM, Nakamune ACMS, Chaves-Neto AH. The effects of storage time and temperature on the stability of salivary phosphatases, transaminases and dehydrogenase. **Archives of Oral Biology**, v.85, p.160-165, 2018.

Uspenskaya AO, Trefilova OV. Effect of bleaching on the biochemical composition of oral fluid and dental hard tissues structure. **Stomatologiia**, v.97, n.5, p.27-30, 2018.

Varoni EM, Federighi V, Decani S, Carrassi A, Lodi G, Sardella A. The effect of clinical setting on the unstimulated salivary flow rate. **Archives of Oral Biology**, v.69, p.7-12, 2016.

Vogel AI, Mendham J, Denney RC, Barnes JD, Thomas MJK. Cap 17. In: VOGELI AI. **Análise Química Quantitativa**. 6a ed. Rio de Janeiro: Editora LTC, 2002. Cap. 17, p.351-385.

Wang J, Yang X, Sun K, Sun P, Pan J, Zhu W, *et al*. Tooth enamel evaluation after tooth bleaching with hydrogen peroxide assisted by a DC nonthermal atmospheric-pressure plasma jet. **IEEE Transactions on Plasma Science**, v.40, n.9, p.2157-2162, 2012.

Yamamoto K, Kurihara M, Matsusue Y, Imanishi M, Tsuyuki M, Kirita T. Whole saliva flow rate and body profile in healthy young adults. **Archives of Oral Biology**, v.54, p.464-469, 2009.

Zeczkowski M, Tenuta LMA, Ambrosano GMB, Aguiar FHB, Lima DANL. Effect of different storage conditions on the physical properties of bleached enamel: an *in vitro*vs. *in situ* study. **Journal of Dentistry**, v.43, p.1154-1161, 2015.

ANEXO A – Aprovação do estudo pelo comitê de ética

UNIVERSIDADE FEDERAL DE MINAS GERAISCOMITÊ DE ÉTICA EM PESQUISA - COEP

Projeto: CAAE – 69736817.8.0000.5149

Interessado (a): Profa. Cláudia Silami de Magalhães
Departamento de Odontologia restauradoraFaculdade de Odontologia- UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 21 de junho de 2017, o projeto de pesquisa intitulado: "**Efeito da saliva humanae do fluxo salivar normal e reduzido na rugosidade e proporção de cálcio e fósforo do esmalte clareado: estudo *in situ* e *in vitro***" bem comoo Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Dra. Vivian Res  a do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

ANEXO B - Questionário aos participantes da pesquisa:

"Fluxo salivar e clareamento dentário"

Nome:_____

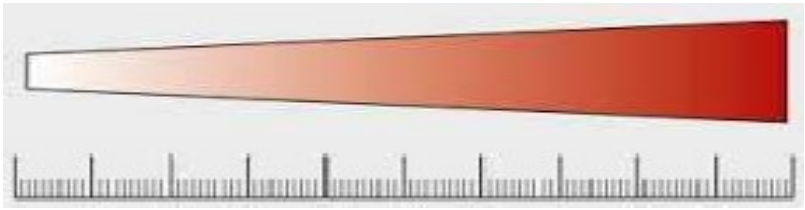
Data de nascimento:__/__/____ ____anos Sexo: ( ) Masculino ( ) Feminino

Endereço:_____

_____

Telefones:_____

1. Qual é o nível de fluxo salivar que você julga ter em boca?



0%_____100%

2. Quantos copos de água (250ml) você bebe por dia?
   ( ) 1 a 3 ( ) 4 a 6 ( ) 7 a 9 ( ) 10 ou mais

3. É fumante?
   ( ) Não ( ) Sim

   Quantidade:_____

4. Tem o hábito de usar diariamente alguma solução de bochecho como listerine, plax, etc.?
   ( ) Não ( ) Sim

5. Tem alguma doença sistêmica?
   ( ) Não ( ) Sim
   Qual? _____

6. Faz uso de algum medicamento?
   ( ) Não ( ) Sim
   Qual? _____

7. Tem alguma desordem gástrica como gastrite ou refluxo? ( ) Não ( ) Sim

8. Já foi submetido a radioterapia para câncer de cabeça e pescoço? ( ) Não ( ) Sim

9. Qual a freqüência de consumo:

	Nunca	1x por semana ou menos	2 a 4x por semana	1x por dia	2x ou mais por dia
Leite e seus derivados Quantidade:					
Iogurte Quantidade:					
Refrigerante Quantidade:					
Bebidas isotônicas (Gatorade) Quantidade:					
Frutas e suco de fruta Quantidade:					
Café Quantidade:					
Bebida alcoólica Quantidade:					

10. Na sua opinião, qual o seu estado emocional mais freqüente? ( ) Calmo(a) ( ) Estressado(a)