UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA

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AVALIAÇÃO DO EFEITO DA ASSOCIAÇÃO ENTRE USO DE DENTIFRÍCIO FLUORETADO E ENXAGUATÓRIO FLUORETADO NA INIBIÇÃO DA DESMINERALIZAÇÃO DA DENTINA RADICULAR: ESTUDO *IN VITRO*

Porto Alegre
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Orientador: Prof. Dr. Rodrigo Alex Arthur

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RESUMO

MARTINS, Vanessa Balbé. **Avaliação do efeito da associação entre uso de dentifrício fluoretado e enxaguatório fluoretado na inibição da desmineralização da dentina radicular:** estudo *in vitro.* 2013. 30 f. Trabalho de Conclusão de Curso (Graduação)- Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

O uso de flúor desempenha um papel fundamental no controle e prevenção da doença cárie. Enxaguatório fluoretado (EF) pode ser indicado para tratamento de pacientes cárie ativos. Porém, não existe um consenso se o uso de EF pode trazer um benefício adicional em termos de redução de perda de minerais á indivíduos que fazem uso regular de dentifrício fluoretado (DF).O objetivo do estudo foi avaliar o efeito da associação entre uso de DF 2X/dia e EF 1X/dia na inibição da desmineralização da dentina radicular em comparação com o uso de DF 2 ou 3 x/dia usando um modelo microbiano *in vitro* de desmineralização. Foram utilizados 48 blocos de dentina radicular (4x4x2 mm) obtidos a partir do terço cervical de raízes de incisivos bovinos com dureza de superfície conhecida. A dureza inicial de superfície foi determinada através da média de 5 indentações usando um indentador tipo Knoop, com carga de 10g por 5 segundos. Biofilmes formados por *Streptococcus mutans* e *Lactobacillus casei* foram crescidos na superfície dos blocos de dentina previamente recobertos com película salivar. Esses blocos foram transferidos para uma placa de 24 poços contendo meio de cultura acrescido de inóculo de duas espécies e incubados em microaerofilia por 8 horas. Após essas 8 horas, os blocos de dentina foram transferidos para uma nova placa de 24 poços contendo meio de cultura suplementado com 0,12% de sacarose e incubados a 37ºC sob microaerofilia durante aproximadamente 16 horas. Diariamente, os blocos foram submetidos aos seguintes tratamentos (6 blocos/ grupo): Grupo 1- controle, água destilada e deionisada 3x/dia; Grupo 2 - DF 2x/dia; Grupo 3 - DF 3x/dia; Grupo 4 - associação entre DF 2x/dia + EF 1x/dia. Este regime de tratamento ocorreu durante 3 dias. O experimento foi realizado em duplicata. A porcentagem de perda de dureza de superfície foi determinada para cada condição avaliada. A porcentagem de perda de minerais no grupo exposto a DF 3x/dia foi estatisticamente menor comparada ao grupo exposto a DF 2x/dia, entretanto não foi diferente quando comparada ao grupo tratado com DF 2x/dia+EF 1x/dia. Os resultados do presente estudo sugerem que a associação entre uso de DF+ EF mostrou-se tão eficiente quanto o uso de DF 3X dia na inibição da dentina radicular.

Palavras-chave: Flúor. Dentifrício. Enxaguatório. Biofilme. Cárie radicular. Dentina.

ABSTRACT

MARTINS, Vanessa Balbé. **Effect of the association between fluoridated dentifrice and fluoridated mouthwash use on the inhibition of root dentin demineralization:** in vitro study**.** 2013. 30 f. Final Paper (Graduation in Dentistry)- Faculdade de Odontologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, 2013.

The use of fluoride plays a key role in the control and prevention of caries. Fluoridated mouthwash (FM) may be prescribed to caries active subjects in an effort to control dental mineral loss. However, there is no consensus whether FM can bring additional benefits to individuals who regularly use fluoridated dentifrice (FD). The aim of this study was to evaluate the association between the use of FD 2X/day and FM 1x/day on the inhibition of root dentin demineralization compared with the use of FD 2 or 3 x / day using an *in vitro* microbial model. A total of 48 root dentin slabs (4x4x2 mm) were obtained from the cervical third of the roots of bovine incisors. Initial hardness of the slabs was determined using a Knoopmicrohardness (KNH) load of 10 g for 5 sec. Biofilms formed by *Lactobacillus casei* and *Streptococcus mutans* were grown on the surface of dentin slabs coated with salivary pellicle. These slabs were transferred to a 24-well plate containing culture medium plus two species inoculum and incubated in microaerophilic conditions for 8 hours. The dentin slabs were then transferred to a new plate 24-well plate containing culture medium supplemented with 0.12% sucrose and incubated at 37°C under microaerophilic conditions for approximately 16 hours. Every day, the slabs were subjected to the following treatments (6 slabs / group): Group 1 - control, distilled and deionized water 3x/day, Group 2 - FD 2x/day, Group 3 - FD 3x/day, Group 4 - association between FD 2x/day + FM 1x/day. This treatment regimen was followed for 3 days. The experiment was done in duplicate. The percentage of surface mineral change was determined for each evaluated condition. There was a statistically greater mineral loss in the control group. Mineral loss found in the presence of FD 3X/day was lower than in the presence of dentifrice 2X/day but it was not statistically different compared with the slabs subjected to FD + FM. The results of this study suggest that the association between the use of FD + FM is as effective as the use of FD 3X/day in the inhibition of root dentin demineralization.

Key-words: Fluoride. Dentifrice. Mouthwash. Biofilm. Root Caries. Dentin.

SUMÁRIO

1 INTRODUÇÃO

A cárie dentária é uma doença multifatorial causada pela interação entre microbiota oral, componentes salivares (tais como proteínas e enzimas, e composição mineral, principalmente em relação as concentrações de cálcio, fosfato e flúor), dieta rica em carboidratos fermentáveis e a superfície dental(SELWITZ et al., 2007; HICKS; GARCIA-GODOY; FLAITZ, 2003). Os carboidratos presentes na dieta são metabolizados pelas bactérias do biofilme que produzem ácidos a partir desses carboidratos (MARSH, 2003).

Nesse contexto, as frequentes quedas de pH produzidas no biofilme devido a fermentação desses carboidratos da dieta induzem uma mudança ecológica no biofilme viabilizando o crescimento dos microorganismos acidúricos (capazes de sobreviver nesses ambientes de reduzido pH) em detrimento dos microrganismos ácido-sensíveis, frequentemente associados a um biofilme compatível com saúde, de acordo com a hipótese da placa ecológica (MARSH, 2003). Adicionalmente, a seleção de acidúricos também contribui para acentuar a acidificação do biofilme, uma vez que os microorganismos acidúricos também são acidogênicos, ou seja, são capazes de produzirem ácidos a partir da metabolização dos carboidratos da dieta.O consumo frequente de carboidratos à longo prazo tem por consequencia um aumento nas proporções de estreptococos do grupo mutans e de lactobacilos na cavidade oral (DE STOPPELAAR et al.,1970; DENNIS et al., 1975; STAAT et al., 1975). Autores sugerem que *Streptococcus* mutans e Lactobacilos são um dos principais microrganismos associados à cárie dental (VAN HOUTE, 1994; BECKER et al., 2002; FEATHERSTONE, 2000).

Essas frequentes quedas de pH na superfície dental alteram o equilíbrio físico-químico entre dente, saliva e fluido do biofilme, o que, à longo prazo, pode resultar em sinais clínicos de desmineralização dental (FEATHERSTONE, 1990; DAWES, 2003; HARA; ZERO, 2010). Dessa forma, os carboidratos da dieta são fatores ambientais necessários para a iniciação e desenvolvimento dessa doença (KIDD; FEJERSKOV, 2004; ZERO et al., 2009).

Dentre os carboidratos fermentáveis, a sacarose é considerada o mais cariogênico da dieta (PAES LEME et al., 2006). Além de ser fermentado por

bactérias orais, o que leva à produção de ácidos no biofilme, esse dissacarídeo é o único substrato para a síntese de polissacarídeos extracelulares insolúveis (PECs) (BOWEN, 2002). PECs constituem-se um importante fator de virulência no biofilme dental, uma vez que promovem a adesão de bactérias à superfície do dente (ROLLA, 1989), e também promovem alterações na estrutura da matriz do biofilme, tornando-a mais porosa (DIBDIN; SHELLIS, 1988). A consequência dessa maior porosidade é uma difusão mais rápida de substratos e ácidos pela matriz do biofilme o que resulta em quedas de pH mais intensas na superfície dental (ZERO et al., 1986). Adicionalmente, estudos *in situ* tem sugerido que o biofilme dental formado na presença de sacarose apresenta menores concentrações de íons cálcio, fosfato e flúor (essenciais para a manutenção do equilíbrio físico-químico entre dente e fluidos orais) o que predispõe a superfície dental à desmineralização (CURY et al., 1997). Muitos estudos sugerem que quanto maior a frequência de ingestão de carboidratos fermentáveis, maior será a perda mineral do dente (GUSTAFSSON et al., 1954; KOING et al., 1968; HEFTI; SCHMID, 1977; et al., 1997).

Dentro desse contexto de quedas de pH no biofilme dental e de perda de minerais dos tecidos dentais, há um consenso de que o flúor é capaz de reduzir essa perda de minerais sendo assim um dos principais responsáveis pela redução na prevalência de cárie (MARINHO, 2009). Na presença de níveis constantes de flúor na cavidade bucal ocorre redução da desmineralização dental. Segundo Cury e Tenuta (2010) o flúor disponível na forma iônica na cavidade oral é capaz de compensar perdas minerais causadas pela produção de ácido no biofilme, por induzir a precipitação de um mineral menos solúvel na estrutura do dente, a fluorhidroxiapatita. Além disso, o flúor auxilia a saliva na reposição de parte dos minerais perdidos devido ao desafio cariogênico, contribuindo, assim, para reativar a remineralização (GARCIA-GODOY; HICKS, 2008). Ao agir sobre a dinâmica do processo da doença cárie, o flúor é muito eficaz em retardar a progressão das lesões de cárie. No entanto, uma vez que ele não tem um efeito direto sobre os fatores etiológicos responsáveis pela doença (biofilme e açúcar), o flúor não vai evitar o desenvolvimento da doença, e, invariavelmente, a doença vai deixar cicatrizes nos dentes, clinicamente visíveis ou não (CURY; TENUTA 2008). Dessa forma, para o controle da doença cárie, a soma dos fatores de prevenção (controle de dieta, instrução de higiene oral e uso de flúor) devem superar os fatores

patológicos (consumo frequente de carboidratos na dieta, principalmente de sacarose). Se fatores patológicos e protetores estiverem em equilíbrio a cárie não progride. (FEATHERSTONE, 2000). Por isso, devido ao caráter multifatorial da doença, o seu tratamento precisa ser baseado em uma proposta preventiva, no tratamento da doença em si, e não das seqüelas da doença (cavidades) (CURY ; TENUTA, 2010). Dentre os fatores de prevenção, dentifrícios fluoretados têm sido amplamente adotados ao redor do mundo como o principal meio de uso de flúor (ZERO,2006). Esse meio também tem sido considerado como um dos principais fatores relacionados ao declínio na prevalência da carie dental no mundo (HARGREAVES et al., 1981; JENKINS, 1985).

Com o aumento da expectativa de vida tem se observado um envelhecimento gradual da população. Este fato vem sendo seguido por uma maior permanência dos dentes em boca até o final da vida do individuo. Além disso, com um aumento na idade pode ocorrer recessão gengival, havendo exposição da dentina radicular. Além da recessão gengival, outras causas tais como trauma por escovação ou até mesmo doença periodontal também podem provocar exposição da dentina radicular. Alguns autores têm relatado que a prevalência de cárie radicular em adultos na faixa etária de 65 a 83 anos pode variar de 32 a 88% (GUIVANTE-NABET et al., 1998; RIHS et al., 2005). Devido ao seu menor conteúdo mineral e maior conteúdo orgânico, a dentina apresenta-se mais susceptível ao desenvolvimento de cárie quando comparada ao esmalte dental. Alguns autores sugerem que a cárie radicular pode se desenvolver em pacientes cuja cárie em esmalte estavam sob controle. (CURY; TENUTA, 2010). Na presença de reduzido fluxo salivar devido ao uso de medicamentos, condição frequentemente encontrada na população idosa, a dentina radicular quando exposta se torna de alto risco para desenvolvimento de cárie (BANTING et al., 2000).O desenvolvimento da cárie em superfície radicular é semelhante ao da lesão coronária, tendo como fatores etiológicos: exposição da superfície radicular ao ambiente bucal, controle mecânico deficiente de biofilme e dieta cariogênica, que, interagindo em função do tempo, implicam formação e progressão de cárie.

Em adição ao uso de dentifrício fluoretado, enxaguatório fluoretado tem sido prescrito para pacientes cárie-ativos (MARINHO et al., 2003). Nesse contexto, Souza et al. (2010) avaliaram o efeito da associação entre dentifrício fluoretado (1100

ppmF) e uso de enxaguatório fluoretado (250 ppmF) na inibição da desmineralização dental. Esses autores encontraram que o uso diário de enxaguatório fluoretado associado à escovação com dentifrício fluoretado 2x/dia produziu a mesma inibição da desmineralização do esmalte dental em comparação à escovação com dentifrício fluoretado usado 3x/dia. Além disso, não houve diferença na incorporação de fluoreto pelo esmalte dental entre os grupos experimentais. A associação entre métodos de uso de fluoreto apresenta resultados conflitantes em relação à redução da desmineralização do esmalte dental (RINGELBERG et al., 1974; ASHLEY et al., 1977; AXELSSON et al., 1977; KARJALAINEN et al., 1994). Entretanto, ainda pouco se sabe sobre essa associação de métodos na prevenção ou tratamento de cárie radicular. Desta forma, o presente trabalho teve por objetivo avaliar o efeito da associação entre uso de dentifrício fluoretado (DF) 2x/dia e enxaguatório fluoretado (EF) 1x/dia na inibição da desmineralização da dentina radicular em comparação ao uso de DF 2x ou 3x/dia usando um modelo microbiano *in vitro* de desmineralização.

ARTIGO CIENTÍFICO

“Effect of the association between fluoridated dentifrice and fluoridated mouthwash use on the inhibition of dentin root caries”

Running title: Fluoridated dentifrice and mouthwash use and dentin root caries inhibition.

Key-words: Fluoride, Dentifrice, Mouthwash, Biofilm, Root Caries, Dentin.

Abstract

Root dentin caries (RDC) occurs in a location adjacent to gingival margin where dental biofilm accumulates. This disease has become an important dental problem positively related to aging and gingival resection. Elderly groups are especially susceptible to RDC. **Objective:** This study aimed to evaluate the effect of the association between the use of fluoridate dentifrice (FD) 2X/day + fluoridated mouthwash (FM) 1x/day on the inhibition of root dentin demineralization compared with the isolated use of FD 2x or 3x/day. **Methods:** Dual-species biofilms were grown on the surface of salivary-pellicle-coated bovine root dentin slabs with known Knoop surface hardness. Mixed-species suspensions were inoculated in 2.5-ml-wells containing dentin slabs and media supplemented with 0.12% sucrose which was replaced daily. During 3 consecutive days, the slabs were exposed to the following treatments (6 slabs/group): Group 1 – distilled and deionised water 3x/day; Group 2 – FD 2x/day; Group 3 – FD 3x/day; Group 4 – FD 2x/day + FM 1x/day. After 4 days of biofilm formation the counts of viable biofilm microorganisms were performed. Dentin slabs were again assessed regarding surface hardness evaluation and the percentage of surface microhardness change (%SMC) was calculated. The experiment was performed in duplicate and the results were statistically analyzed. **Results:** Statistically greater mineral loss was found in the control group. %SMC of slabs treated with FD 2x/day +FM 1x/day was not statistically different compared to slabs treated with FD 3x/day, which was statistically lower than mineral loss of slabs treated with FD 2x/day ($p<0.05$). **Conclusion:** The use of FD 2x/day + FM 1x/day

provided non additional effect on the inhibition of root dentin demineralization compared with FD used 3x/day. Both fluoridated regimens could be adopted for prevention and treatment of RDC.

Introduction

Dental caries is a multifactorial and biofilm-dependent disease associated with consumption of dietary carbohydrates (Kidd &Fejerskov 2004; Zero et al., 2009). The frequent events of pH fall in dental biofilm due to the fermentation of these carbohydrates by dental biofilm microbiota drives a microbiological imbalance which selects acid-tolerant and lactic-acid producer bacteria, such as mutans streptococci and *Lactobacilli* (Marsh, 2003). In response to the acidic environment, the physicochemical equilibrium between tooth and saliva and fluid of biofilm is disrupted resulting in dental demineralization (Dawes, 2003; Hara & Zero, 2010).

In this scenario, fluoride use is commonly associated to the control and prevention of this disease (Marinho, 2009). Fluoridated dentifrice is well known as the most effective method for dental caries prevention since it helps to maintain low and constant concentrations of fluoride in oral cavity (Mellberg & Chomicki, 1985; Cury & Tenuta, 2010). In addition to fluoridated dentifrice (FD) use, fluoridated mouthwash (FM) has been prescribed to caries-active subjects (Marinho et al., 2003). In this context, Souza et al. (2010) evaluated the effect of the association between FD and FM use on the inhibition of enamel demineralization. The authors found that daily use of FM associated to twice daily toothbrushing with FD did not increase the inhibition of enamel demineralization compared to three-times daily toothbrushing with FD. Besides that, there was not any difference in fluoride uptake by dental enamel among the tested groups. The literature is also inconclusive in respect to the beneficial effect of the association between different fluoride delivery methods in decreasing enamel demineralization (Ringelberg et al,, 1974; Ashley et al., 1977; Axelsson et al., 1977; Karjalainen et al., 1994). It is also unclear whether this association would be beneficial on the treatment of root dentin caries (RDC).

RDC may occur in gingival resection areas where root dentin is exposed to oral environment. In addition to it, mechanical toothbrushing trauma and chronic periodontitis may lead also to root dentin exposure. Considering the increase of life expectancy, these above mentioned conditions are frequently found in elderly

groups. Some authors have shown that the prevalence of RDC in elderly groups (65 to 83 year-old) could be as high as 88% (Guivante-Nabet et al., 1998; Rihs et al., 2005). Considering that dentin has lower mineral content than enamel, it is more prone to caries progression. Under a low salivary flow, which is frequently found in elderly as a side-effect of daily use of medicines, root dentin has a higher risk to RDC (Banting et al., 2000).

Therefore, since there are not evidences about the beneficial effect of association between fluoridated methods use on the inhibition of RDC demineralization, the present study aimed to evaluate the effect of the association between FD 2x/day + FM 1x/day use on the inhibition of RDC compared with the isolated use of FD 2x or 3x/day using an *in vitro* biofilm model to produce dentin carious lesions. The null-hypothesis is the association between FD + FM use does not decrease the demineralization of root dentin compared to the use of FD twice- or three-times daily.

Materials and Methods

Experimental design

A dual-species biofilms were grown on the surface of sound bovine root dentin slabs with known baseline surface hardness. The slabs were covered with salivary pellicle previous to biofilm formation. Daily (during 3 consecutive days), the slabs were exposed to the following treatments (6 slabs/group): Group 1 – distilled and deionised water 3x/day; Group 2 – FD 2x/day; Group 3 – FD 3x/day; Group 4 – FD 2x/day + FM 1x/day. After 4 days of biofilm formation the counts of viable biofilm microorganisms were performed in all studies. Dentin slabs were evaluated regarding mineral loss, via determination of surface microhardness change.

Specimen preparation

Dentin slabs (4x4 mm) were prepared from bovine root incisors. Using two parallel diamond disks separated by a 4-mm spacer a root slice was cut from upper third of the root. The root slices were sectioned mesio-distally and dentin slabs were obtained from the buccal and lingual root surfaces. They were flattened and polished by using 400, 600, 1,200 grades (Aires et al., 2002). Slabs with any cracks, scratches or exposed dentin were discarded. Initial hardness of the sound dentin slabs was

determined using a Knoopmicrohardness (KNH) load of 10 g for 5 sec. Five indentations distant 100 µm of each other were placed 2 mm far from the edge of the slabs. Baseline hardness of selected slabs was 31.24 ± 5.07KNH. Slabs were then covered by a 1 mm nail varnish strip on the right side of the slab leaving a 3 x 4 mm area of exposed enamel. Slabs were then randomized and balanced into experimental groups by mean KNH values. All groups had the same baseline hardness. The slabs were mounted on acrylic cubes attached to the lid of 24-well plate (Arthur et al., 2013) and sterilized by hydrogen peroxide prior to inoculation with an *in vitro* dual-species biofilm model. Forty-eight dentin slabs were obtained.

In vitro biofilm model

Inoculum preparation

Lactobacillus casei (ATCC 4646) and *Streptococcus mutans* (UA 159) were cultivated on Columbia Agar supplemented with 5% of sheep blood (CBA). Colony-forming units (CFU) were individually transferred from CBA plates to tubes containing Tryptic Soy Broth (TSB) supplemented with 0.5% sucrose and incubated at 37^{o}C for 24 hours. Then, aliquots of 1 mL of each suspension were individually transferred to 9 mL of fresh TSB supplemented with 0.5% sucrose and incubated for more 18 hours until optical density of 1.0 ± 0.1 at 550 nm (Arthur et al., 2013). Aliquots of each strain were then combined to form a dual-species microbial consortia.

Salivary pellicle coating

Saliva was obtained from healthy donors (18 to 24 year old) who had mean stimulated saliva flow rate ≥ 0.7 ml/min and no report of antibiotics use for the last two months or use of any medication that modifies salivary secretion. Potential subjects with periodontal disease or general systemic illness were excluded. The study protocol was approved by the Ethics in Research Committee of Federal University of Rio Grande do Sul (CAAE 092897.12.6.0000.5347). Subjects signed a written consent prior to saliva collection. The saliva samples were pooled, centrifuged and the supernatant was mixed with 1:1 dilution in distilled water with 25% of physiological saline and filter-sterilized (0.22 µm general purpose filter system, Corning, NY, USA) (Guggenheim et al., 2001). Prior to incubation with the microbial

consortia, the slabs were immersed in 2.5 ml of sterile saliva filtrate and kept under agitation for 45 min at room temperature.

In vitro microbial demineralization

Slabs were then transferred to a new 24-well plate (Arthur et al., 2013) and immersed in 1.4 mL of TSB supplemented with 0.12% sucrose. An aliquot of 500ul of the dual-species inoculum (containing approximately 10^7 CFU/ml of each species) was added to each well. Plates were incubated at 37^{o}C for 8 hours. The slabs were then transferred to another 24-wells plate containing fresh TSB 0.12% sucrose and incubated at 37^{o}C.

After overnight incubation with microbial inoculum (at 8am next day morning) the slabs were immersed in the following suspensions (6 slabs/group; 2mL/well):fluoridated dentifrice slurry (FD, 1,100 ppmF, sodium fluoride; Colgate Total – Lot n^o 3196BR123D; Exp – 07/2016) or fluoridated mouthwash (FM; 0.05% sodium fluoride; Colgate Plax – Lot n^o BR121CVAL; Exp – 04/2016). Dentifrice slurry was prepared by diluting dentifrice in sterile distilled and deionized water in a proportion of 1:3. The treatment groups were: Group 1 – distilled and deionised water 3x/day; Group 2 – FD 2x/day; Group 3 – FD 3x/day; Group 4 – FD 2x/day + FM 1x/day. In all groups, the first slurry treatment was done at 8 am. The second slurry treatment in Group 3 was done at noon and the last slurry treatment for Groups 2, 3 and 4 was done at 5 pm. All treatments were done during 3 minutes under slow agitation. At 5 pm, immediately after dentifrice slurry treatment, slabs of Group 4 were exposed to FM for additional 3 minutes. After each treatment, the specimens were dip-washed in sterile saline and transferred back to the same plate containing the TSB provided early in the morning. This protocol was repeated for the following 2 days. The experiment was done in duplicate.

Harvesting of microbial biofilms and determination of counts of viable cells

At the end of the experimental period, dentin slabs were aseptically removed from the lids and individually transferred to tubes containing 1 ml of sterile 0.9% NaCl. The

biofilm suspension was vigorously mixed. Aliquots of the suspension were serially diluted and inoculated on CBA plates using the drop-technique for counts of *S. mutans* and *L. casei*. Plates were incubated at 37°C for 48 h. CFU were then examined under stereomicroscope and the results expressed as CFU/mL.

Post-biofilm formation enamel surface hardness

Surface KNH on each slab was determined again at the end of each experimental period. The average of post-biofilm KNH were determined from one parallel row of five adjacent indentations placed 100 µm to the left of the baseline row. The mean values of baseline (B) hardness and post-biofilm (P) were averaged and the percentage of surface microhardness change (%SMC) was calculated as %SMC= (P-B)/B)*100.

Statistical Analysis

The mean and standard deviation of each outcome (bacteria counts and %SMC) were calculated for each tested condition. Analysis of variance (ANOVA) was used to determine the effect of each tested condition on response variables. Variables that did not satisfy assumptions for ANOVA (counts of *S. mutans* and *L. casei*) were log transformed. Tukey test was used when ANOVA indicated statistical differences. The level of significance was set as 5%. Analyzes were performed on software BioEstat 5.3.

Results

According to Table 1, counts of *S. mutans* in biofilms of control group and in the presence of FD+FM were statistically higher than the counts in the presence of FD 3x/day which was not statistically different compared with FD 2x/day. The counts of *L. casei* found in the presence of FD 3x/day ($p<0.05$) were statistically lower than counts found in the control group which was not different compared with counts in the presence of FD 2x/day or FD 2x/day + FM 1x/day. There were not differences in counts of *L. casei* among biofilms exposed to any of the fluoridated treatments.

Regarding mineral loss, the highest %SMC was found on control group which was statistically different from all the other groups. The mineral loss found on slabs treated with FD 3x/day was statistically lower than on slabs treated with FD 2x/day but it was not statistically different compared to slabs treated with FD 2x/day + FM 1x/day. Additionally, no statistically differences were found in %SMC between slabs treated with FD 2x/day or FD 2x/day + FM 1x/day ($p<0.05$).

Table 1. Counts of viable cells on biofilms (Log_{10}(CFU); n=10) and percentage of surface microhardness change (%SMC; n=12) on dentin specimens according to treatments (mean ± sd):

Groups	Counts of *S. mutans*	Counts of *L. casei*	% SMC
1 – Control	8.01 ± 0.13^{a}	6.70 ± 0.38^{a}	-60.85 ± 10.11^{a}
2 - FD 2x/day	6.71 ± 0.40^{bc}	6.36 ± 0.32^{ac}	-37.30 ± 14.22^{b}
3 - FD 3x/day	6.45 ± 0.32^{b}	6.04 ± 0.52^{bc}	-26.23 ± 11.20^{c}
4 - FD 2x/day + FM 1x/day	7.04 ± 0.65^{ac}	6.62 ± 0.80^{ac}	-34.59 ± 11.07^{bc}

Means followed by distinct letters are statistically different among groups by Tukey test ($p<0.05$).
FD: fluoridated dentifrice; FM: fluoridated mouthwash. (n =48)

Discussion

In this study, bovine root dentin slabs were submitted to a demineralization process by an *in vitro* microbial model. Self-use topical fluoridated products were used in order to replace part of lost minerals due to the cariogenic challenge and to test the effect of the association between FD + FM in the inhibition of demineralization. The choice for the use of bovine teeth was due to the fact they are easier to obtain and to manipulate. Evidence suggests that both bovine root dentin and human dentin have the same behavior in relation to the development of carious lesions (Hara et al., 2003). Therefore, it has been suggested that bovine dentin can be safely used instead of human dentin to study development and inhibition of dental caries (Hara et al., 2003).

Recently, Souza et al. (2010) evaluated the effect of the association between FD + FM use in the inhibition of dental enamel demineralization. Those authors found that daily use of FM (1x/day) associated with use of FD (2x/day) produced the same inhibition of enamel demineralization compared to FD used 3x/day. The data of the present study agree with those of Souza et al. (2010). It seems that the association between FD and FM is as advantageous as the use of FD 3x/day in terms of

inhibition of dentin demineralization (Table 1). However, although FD 3x/day and FD+FM were not statistically different in terms of %SMC, it is interesting to observe that the use of FD 3x/day induced a mineral loss about 25% lower than those found in the presence of FD+FM. That might be related to the reduced counts of *S. mutans* in the presence of FD 3x/day compared with those found in the presence of FD+FM. Considering that our study was a short-term one, we hypothesize that under longer periods of biofilm growth and cariogenic challenge the effect of the association between FD+FM would not be as strong as the use of FD 3x/day in the decrease of mineral loss. Accordingly, it is also suggested that root dentin seems to require a significantly higher fluoride uptake than enamel in order to replace some of the minerals lost during cariogenic challenge (Petersson, LG MKambara2004). That might explain the slightly better results when using FD 3x/day.

All fluoride regimens tested in this study were able to reduce significantly the mineral loss of root dentin slabs in comparison with the control group. Actually, these results were expected mainly if we take in to account the physicochemical effect of fluoride in reducing demineralization and enhancing remineralization (Cury&Tenuta, 2010). Our results suggest that the fluoride provided by these treatments may have been able to diffuse through the biofilm saturating it (Cury&Tenuta 2010).This high level of saturation might have contributed to balance the mineral equilibrium between the slab surface and the aqueous phase surrounding it leading to lower mineral loss.

Under a clinical and mechanistic perspective, it is believed that FM can lead to higher levels of fluoride retention in the oral cavity compared with FD, depending on individual's behavior after brushing. Zero et al. (1992) reported that salivary fluoride retention after FM rinse (226 ppm F) was significantly greater than after brushing with a FD (1100 ppm F) two hours later. Those authors discussed that the common practice to rinse the mouth with tap water after toothbrushing reduces the retention of fluoride in the oral cavity. In this context, the combination of FD followed by FM rinse could be beneficial in terms of increasing the availability of fluoride in oral cavity and a greater potential to inhibit demineralization (Zero DT 2006). That might explain the reason the association between FD+ FM was not statistically different compared with FD 3x/day. However, Marinho et al. (2003) argued that the combination of FD and FM does not necessarily increase the frequency of exposure to fluoride once the rinses are usually applied immediately after brushing representing only a single

fluoride application. It is noteworthy, however, that the consumer is instructed to use FM immediately after toothbrushing with FD according to the instructions provided on the label of the products. To better simulate a clinical condition, we chose to use FM 1x/day (after the last episode of brushing) in order to represent a bed-time use. However, we are aware that our experimental conditions are not able to simulate the natural clearance present in the oral cavity due to the salivary flow. Immediately after use of FD and FM, the slabs were dip-washed in distilled and deionized water. The fluoride provided by the treatments was cleared from the surrounding aqueous phase faster than the clearance present in the oral cavity what could underestimate the potential of the treatments in the inhibition of demineralization. Adaptions to the present microbial model, such as the growth of biofilms in a flow-chamber, should be adopted for further studies. Nonetheless, all fluoridated treatments were able to significantly reduce mineral loss compared with non-fluoridated treated slabs (control group).

There is not consensus on the use of FM in the prevention of dental caries. Some authors have suggested that FM should be used by high caries risk individuals or individuals with active carious lesions (Stamm et al. 1984, Disney et al., 1990). However, whether the use of FM can bring additional benefits to individuals who regularly use FD is questioned by others (Twetman et al. 2004). Our results, suggest tough that the association between FD and FM is as effective as the use of FD 3x/day in decreasing mineral loss. As previously discussed by Souza et al. (2010), it would be more cost-effective to increase the frequency of FD use instead of adopting another fluoridated treatment (such as mouthwash) in daily oral hygiene procedures mainly due to the fact that FM use would require patient's compliance in order to be effective. Additionally, it is important to discuss that adding another fluoridated method in daily oral hygiene procedures might be more expensive considering a long-term use. However, Twetman et al. (2004) recommend that further research is needed to determine whether FM is effective in caries active individuals and at high risk of developing dental caries.

In respect to the microbiology of dentin root caries, although studies have suggested that the biofilm formed on root dentin carious lesion is predominantly formed by *Actinomyces, Propionibacterium, Bifidobacterium*, *Streptococcus* and *Lactobacillus* (Hashimoto et al., 2011), other studies show that dentin root surfaces

that have high counts of *S. mutans* and *Lactobacillus* present higher risk of caries development (Ellen et al., 1985, Bowden et al., 1990; Beighton et al., 1993). Other studies showed that active root dentin lesions have a high proportion of *S. mutans* (Keltjen et al. 1987). These findings led us to adopt an *in vitro* model of root caries considering *S. mutans* and *L. casei* as inoculum. We understand, though, the limitations related with such condition, especially considering the microbial diversity and the interactions between microorganisms in the oral cavity. These conditions are not possible to be fully reproduced *in vitro*. However, the biofilm model used in this study allowed the development of carious lesions in a biological fashion way as a result of the metabolism of these microorganisms and in response to the carbohydrates present during the biofilm growth.

Root caries seems to be a challenge in terms of public health due to the presence of a greater number of teeth with exposed root surfaces in the oral cavity of elderly population for a longer period of time (Beck JD. 1990)(LeakeJL.2001). The results of this study suggest that no additional effect was found in reducing the demineralization of dentin between the association of FD+FM and FD used 3x/day. Despite the inherent limitations of *in vitro* models for development of caries lesions in relation to the physiological conditions of the oral cavity, these results can bring a new perspective to prevention and treatment of root caries.

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3 CONCLUSÃO

Os resultados do presente estudo sugerem que a associação entre uso de dentifrício fluoretado e enxaguatório fluoretado mostrou-se tão eficiente quanto o uso de dentifrício fluoretado três vezes ao dia na inibição da desmineralização da dentina radicular. Apesar das limitações inerentes aos modelos *in vitro* de desenvolvimento de lesões de cárie em relação ás condições fisiológicas dinâmicas da cavidade bucal, esses resultados podem trazer uma nova perspectiva para prevenção e tratamento de cárie radicular.

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