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Concentração de fluoreto e quantidade de dentifrício influenciam a desmineralização do esmalte dental in situ

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

Orientador: Prof. Dr. Juliano Pelim Pessan

Coorientador: Prof. Dr. Alberto Carlos Botazzo Delbem

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Dedicatória

Dedico este trabalho

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Epígrafe

"Por mais inteligente que alguém possa ser,
se não for humilde, o seu melhor se perde na arrogância.
A humildade ainda é a parte mais bela da sabedoria."

(Nelson Swel)

Resumo

PAIVA, M.F. **Concentração de fluoreto e quantidade de dentifrício influenciam a desmineralização do esmalte dental *in situ*.** 2017 72 f. Dissertação (Mestrado em Ciência Odontológica, Área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2017.

O presente estudo avaliou o efeito da escovação com dentifrício convencional (DC, 1100 ppm F) e com concentração reduzida de fluoreto (DCRF, 550 ppm F), aplicados em diferentes quantidades sobre a desmineralização do esmalte dental bovino, bem como sobre as concentrações de fluoreto (F) no biofilme dental (biomassa e fluido) formado *in situ*. O estudo compreendeu 5 etapas experimentais de 7 dias cada, nas quais foram testadas 5 combinações de dentifrícios e quantidades: dentifrício placebo (sem F) aplicado sobre todas as cerdas da escova (P); DCRF aplicado pela técnica transversal (0,3 g – T1) ou sobre todas as cerdas da escova (0,6 g – T2); e DC aplicado do tamanho de uma ervilha (0,15 g – T3) ou pela técnica transversal (0,3 g – T4), para se obter intensidades de tratamento comparáveis (concentração de F no dentifrício x quantidade aplicada na escova). Em cada fase, os voluntários (*n*=13, 20-36 anos de idade) utilizaram um dispositivo palatino contendo 4 blocos de esmalte dental bovino, selecionados por meio de dureza de superfície inicial (330 – 370 KHN) e realizaram um desafio cariogênico com solução de sacarose a 30%, 6x/dia. A escovação foi realizada 3x/dia com as combinações previamente descritas, seguindo um protocolo duplo-cego, cruzado e randomizado. Na manhã do 8º dia, o biofilme foi coletado 5 e 60 min após a escovação. Foram realizadas as análises de F no biofilme total e no fluido do biofilme, bem como análise da dureza de superfície final e cálculo da porcentagem de perda de dureza de superfície (%DS) e perda integrada da dureza de subsuperfície (ΔKHN). Os resultados foram analisados por ANOVA de medidas repetidas, teste de Student-Newman-Keuls e coeficiente de correlação de Pearson ($p<0.05$). Para %DS e ΔKHN, a escovação com DC e DCRF promoveu efeitos significativamente superiores aos do P, sendo que a %DS para T3 foi significativamente maior que T4. Para os dados de ΔKHN, os tratamentos de maior intensidade (T2 e T4) promoveram valores significativamente menores em relação aos de menor intensidade (T1 e T3), sem diferenças significativas

entre DC e DCRF dentro de cada intensidade. A concentração de F no biofilme total apresentou uma tendência dose-resposta em relação à concentração de F nos dentifrícios. Entretanto, as concentrações de F no fluido do biofilme atingiram um *plateau* para todos os dentifrícios fluoretados, em ambos os tempos de coleta. Uma forte correlação foi observada entre ΔKHN e a concentração de F no biofilme total (r=-0,71) e no fluido do biofilme (r=-0,72) 5 min após a escovação, enquanto uma correlação moderada foi observada entre ΔKHN e %DS (r=0,60). Concluiu-se que a intensidade do tratamento apresenta influência significativa sobre o desenvolvimento de lesões de cárie, bem como sobre as concentrações de F no biofilme formado *in situ.*

Palavras-chave: Dentifrícios, Esmalte dentário, Fluoretos, Placa dentária.

Abstract

PAIVA, M.F. **Fluoride concentration and amount of dentifrice influence enamel demineralization *in situ.*** 2017 72 f. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2017.

The present study evaluated the effect of brushing with conventional (CD, 1100 ppm F) and low-fluoride (LFD, 550 ppm F) dentifrices, applied in different quantities, on the demineralization of bovine dental enamel, as well as on fluoride (F) concentrations in the dental biofilm (solid and fluid phases) formed *in situ.* The study comprised 5 experimental phases of 7 days each, in which 5 combinations of dentifrices and quantities were tested: placebo dentifrice (F-free) applied on all brush bristles (P); LFD applied by the transversal technique (0.3 g – T1) or on all bristles of the brush (0.6 g – T2); and CD applied in a pea-sized amount (0.15 g - T3) or transversal technique (0.3 g - T4), in order to produce comparable intensities (F concentration in the dentifrice x amount applied to the brush). At each experimental phase, volunteers (*n*=13, 20-36 years old) wore palatal devices containing 4 bovine enamel blocks, selected by initial surface hardness (330 – 370 KHN) and performed a cariogenic challenge using a 30% sucrose solution, 6x/day. Brushing was performed 3x/day, using the above-mentioned combinations, following a double-blind, cross-over and randomized protocol. On the morning of the 8th day, biofilm formed on the enamel blocks was collected at 5 and 60 min after brushing. Biofilm and biofilm fluid samples were analyzed for F concentrations. The analysis of final surface and cross-sectional hardness, and the calculation of the percentage of surface hardness loss (% SH) and integrated loss of subsurface hardness (ΔKHN) were determined for all enamel specimens. The results were analyzed by repeated-measures ANOVA, Student-Newman-Keuls test, and by Pearson's correlation coefficient ($p<0.05$). For %SH and ΔKHN, brushing with CD or LFD promoted significantly superior effects than P; the %SH of T3 was significantly higher than of T4. For the ΔKHN data, the treatments with higher intensity (T2 and T4) promoted values significantly lower than those seen for the lower intensity (T1 and T3). F concentrations in the total biofilm showed a dose-response tendency in relation to F concentrations in the dentifrices. However, F concentrations in the fluid phase reached a plateau for all fluoridated dentifrices at both times of

sample collection. A strong correlation was observed between ΔKHN and F concentrations in the total biofilm (r=-0.71) and biofilm fluid (r=-0.72) 5 min after brushing, while a moderate correlation was observed between ΔKHN and %SH (r=0.60). It was concluded that the intensity of treatment has a significant influence on the development of caries lesions, as well as on F concentrations of dental biofilm formed *in situ.*

Key-words: Dentifrices, Dental enamel, Fluoride, Dental plaque.

Listas

Lista de Tabelas

Lista de Abreviaturas e Símbolos

ANOVA	Análise de Variância
CAAE	Certificado de Apresentação para Apreciação Ética
°C	Graus Celsius
F	Fluoreto
g	Grama
g	Gravidade
h	Hora (s)
HCl	Ácido Clorídrico
KHN	Knoop Hardness Number/*Número de Dureza Knoop*
L	Litro
M	Molar
mL	Mililitro
Mm	Milímetro
Min	Minuto
Mg	Miligrama
Mg	Micrograma
µL	Microlitro
µm	Micro-metro
NaOH	Hidróxido de Sódio
Nm	Nanômetro
p	Probabilidade
pH	Potencial Hidrogeniônico
Ppm	Parte por milhão
Q.S.P	Quantidade Suficiente Para
R	Coeficiente de correlação
rpm	Rotações por minuto
SD	Standard Deviation
SH	Surface hardness
SHi	Initial Surface hardness/Dureza de Superfície Inicial
SHf	Final Surface Hardness/Dureza de Superfície Final
TISAB	Total Ionic Strenght Adjustment Buffer/*Tampão de Ajuste da*

	Força Iônica Total
UNESP	Universidade Estadual Paulista
α	Alfa
B	Beta
ΔKHN	Integrated subsurface hardness/*Dureza integrada de subsuperfície*
%	Porcentagem

Sumário

Sumário

Fluoride concentration and amount of dentifrice influence enamel demineralization *in situ*

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Short tittle: Amount of F dentifrice influences demineralization

Key-words: Dentifrices, Dental enamel, Fluoride, Dental plaque.

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*De acordo com as instruções aos autores do periódico Caries Research (Anexo A).

Abstract

This study evaluated the effect of conventional (CD, 1100 ppm F) and low-fluoride (LFD, 550 ppm F) dentifrices, applied in different quantities, on enamel demineralization, and on fluoride (F) concentrations in the dental biofilm formed *in situ.* Five combinations of dentifrices and quantities were tested: placebo (P – F-free) applied on all brush bristles; LFD applied by the transversal technique (0.3 g – T1) or on all bristles (0.6 g – T2); and CD applied in a pea-sized amount (0.15 g - T3) or by the transversal technique (0.3 g - T4), in order to produce comparable intensities (F concentration in the dentifrice x amount applied to the brush). Volunteers (*n*=13, 20-36 years old) wore palatal devices containing 4 bovine enamel blocks, and performed cariogenic challenges (30% sucrose solution) 6x/day, and brushing 3x/day, following a double-blind, cross-over and randomized protocol. On the 8th day, biofilm was collected 5 and 60 min after brushing. The percentage of surface hardness loss (%SH), integrated loss of subsurface hardness (ΔKHN) and biofilm F concentrations (solid and fluid phases) were determined. Data were analyzed by repeated-measures ANOVA, Student-Newman-Keuls test, and Pearson's correlation coefficient ($p<0.05$). Significantly lower ΔKHN was observed for treatments with higher intensity (T2 and T4) in comparison with the lower intensity (T1 and T3). A strong correlation was observed between ΔKHN and F concentrations in total biofilm ($r=-0.71$) and biofilm fluid ($r=-0.72$) 5 min after brushing. It was concluded that treatment intensity has a significant influence on the development of caries lesions *in situ.*

Introduction

A comprehensive Cochrane review assessing the relative caries-protective effectiveness of dentifrices with different fluoride concentrations concluded that toothbrushing with dentifrices with fluoride concentration of 1000 ppm F or above is an effective measure in preventing caries in children and adolescents [Walsh et al., 2010]. Given that the early use of fluoridate toothpaste has also been shown to be a potential risk factor for the development of dental fluorosis [Wong et al., 2011], professionals and health authorities have advocated the use of dentifrices with fluoride concentrations above 1000 ppm F, but applied in very small quantities for young children, under the assumption that this measure would minimize systemic fluoride exposure from this source without compromising the clinical efficacy of the product [European Academy of Paediatric Dentistry, 2009; American Academy of Pediatric Dentistry, 2014].

Considering that the clinical effects of fluoride are known to be dose-dependent, the above-mentioned recommendation would be valid except for the fact that the dentifrice is diluted in saliva during toothbrushing, so that reducing the amount of toothpaste during toothbrushing would have a marked impact on the resulting fluoride concentration in the natural dentifrice-saliva slurry that bathes all intraoral surfaces, which, in turn, could affect the clinical efficacy of the toothpaste [Duckworth et al., 1992].

While previous studies have addressed the impact of the amount of dentifrice used during toothbrushing and the resulting fluoride levels in saliva [DenBesten and Ko, 1996; Zero et al., 2010], as well as on the remineralization of enamel caries-like lesions and enamel F uptake [Zero et al., 2010], the effect of the amount of dentifrice as a function of fluoride concentration in the product has only recently been assessed in an *in vivo* study conducted in children. It was shown that brushing with a low-fluoride dentifrice (LFD, 550 ppm F) applied using the transversal technique (perpendicular to the long axis of the brush) led to significantly higher salivary fluoride concentrations than the conventional dentifrice (CD, 1100 ppm F) using a pea-sized amount [Hall et al., 2016], thus confirming the hypothesis that the dilution of the toothpaste in saliva during toothbrushing has indeed a direct impact on intraoral fluoride concentrations.

Based on dose-response considerations, the above-mentioned study could have important clinical implications, raising questions on the

appropriateness of the current recommendation of toothpastes to children, considering the fact that only one intraoral variable was assessed, and given the short-term nature of the above-mentioned study, the results cannot be fully extrapolated to a clinical situation. As dental caries is the result of the slow mineral loss occurring in dental enamel covered by a cariogenic biofilm [Buzalaf et al., 2011], the study of the effects of treatment intensity (i.e., fluoride concentration in the dentifrice versus the amount used during toothbrushing) on enamel mineral loss, as well as on biofilm fluoride concentrations, could provide additional information regarding the relative effect effects LFDs and CDs on caries dynamics.

In view of the above, the present study evaluated the effects of a CD and a LFD applied in different quantities on the demineralization of bovine dental enamel, as well as on the F concentrations in the dental biofilm (solid and fluid phases) formed *in situ* under cariogenic challenge. The null hypothesis was that the treatment intensity would not influence the variables assessed.

Materials and Methods

Ethical aspects and inclusion criteria

This study was approved by the Human Research Ethics Committee of School of Dentistry, Araçatuba - UNESP (CAAE 44712715.5.0000.5420 – ANEXO B). All participants signed an informed consent form and received written and verbal instructions on the research protocol prior to the start of the study (ANEXO C).

Thirteen individuals (20-36 years old) participated in the study, living in the city of Araçatuba-SP, Brazil, which is supplied with artificially fluoridated water at 0.6 to 0.8 mg F/L [Moimaz et al., 2012]. The inclusion criteria involved participants in good general and oral health. Individuals who used drugs that could interfere with the formation of the dental biofilm or salivary flow, smokers, those who received fluoride applications 2 weeks before the experiment, were using orthodontic appliances or had systemic diseases could not participate in the study [Delbem et al., 2010].

Experimental design

The determination of the sample size was based on a previous study [Amaral et al., 2013], in which the F concentrations in the dental biofilm were evaluated after the use of dentifrices containing 500 and 1100 ppm F. A sample of 14 volunteers was calculated considering error-α of 5%, error-β of 20% [DSS, 2010], and a loss of 20%. The protocol used was *in situ*, double-blind and crossover. The volunteers were randomly divided into 5 groups, totaling 4 combinations of dentifrice and quantity, namely: 550 ppm F applied by the transversal technique (0.3 g) or on all bristles of the brush (0.6 g), and 1100 ppm F applied in a pea-size amount (0.15 g) or by the transversal technique (0.3 g). A placebo dentifrice (F-free, negative control) was also included, which was applied on all bristles of the brush (0.6 g). These combinations were based on the study of Hall et al. [2016], which evaluated the F concentrations in saliva after brushing with dentifrices containing 0, 550 and 1100 ppm F, applied in quantities similar to those of the present study. The dentifrice tubes were coded and the quantities used during brushing were determined by an examiner not involved in sample collection and analysis (ACBD) (ANEXO D).

Formulation and determination of F in the experimental dentifrices

The experimental dentifrices were produced in the laboratory of Pediatric Dentistry from School of Dentistry, Araçatuba, using the same basic formulation with the following components: titanium dioxide, carboxymethylcellulose, methyl p-hydroxybenzoate, sodium saccharine, oil peppermint, glycerin, silica abrasive, sodium lauryl sulfate, water and sodium fluoride (NaF, Merck®, Germany 550 e 1100 ppm F). A formulation without F (placebo) was also used. The determination of the ionic F concentration (IF) and total fluoride (TF) [Delbem et al., 2009 – ANEXO E] and pH [Moretto et al., 2010 – ANEXO F] of the toothpastes were performed prior to the beginning of the study.

Enamel blocks and appliance preparation

Two hundred sixty enamel blocks measuring 4 x 4 x 2 mm were obtained from bovine incisors previously stored in 2% neutral formaldehyde solution (pH 7.0) for 1 month. Enamel blocks were sequentially polished (600, 800, 1200 grit), followed by polishing with felt paper and 1μ diamond suspension (ANEXO G). Four enamel blocks were fixed in 4-mm deep spaces of custom-made

acrylic palatal devices, approximately 1 mm below the level of the acrylic surface, in order to allow biofilm accumulation on the enamel specimens. A plastic mesh covered the enamel blocks to prevent biofilm disturbance by mechanical forces [Amaral et al., 2013] (ANEXO H).

Intraoral procedures

For the cariogenic challenge, a 30% sucrose solution was used *ex vivo*. After removal of the appliance from the mouth, 2 drops of the sucrose solution were dispensed on each enamel block, 6x/day, at previously established times (8:00, 11:00, 14:00, 17:00, 19:00, 21:00 h). The appliances were left to rest during 5 min before being returned to the oral cavity, allowing sucrose to diffuse through the biofilm. The treatment of the blocks with the dentifrices was performed 3x/day (7:30, 13:00 and 22:00 h), during 7 days. In each week, volunteers used a combination of dentifrice and amount to be applied on the brush, totaling 5 combinations. The amount of toothpaste to be used was demonstrated individually to all volunteers, in each experimental phase. They also received photographs via cell phone showing the quantity to be applied on the brush, to avoid doubts. The volunteers were instructed to brush their natural teeth with the palatal appliance in the oral cavity for 1 min, and to swish the natural suspension of dentifrice/saliva for 30 seconds. Volunteers then completed the cleaning of their natural teeth without the appliance in their mouths, and rinsed the appliances (20 mL) and their mouths (10 mL) with tap water. They were instructed to remove the appliances only during meals and ingestion of liquids, with no restrictions on their diet (ANEXO I). Prior to each experimental phase (lead in and wash out), the volunteers used a placebo dentifrice (without F) for 7 days.

Sample collection

On the morning of the 8th day of each experimental phase, the volunteers attended the laboratory of Pediatric Dentistry, School of Dentistry, Araçatuba, while fasting, having been instructed not to brush their teeth and not to perform the cariogenic challenge on the specimens. Volunteers then performed the brushing procedures as previously described, but with the dentifrices weighed by the researchers (Shimadzu Balance AUY220, Kyoto - Japan). Biofilm from 2

blocks was randomly collected at 5 and 60 min after brushing. Biofilm was collected with a plastic spatula and immediately transferred to a microcentrifuge tube filled with mineral oil. The tube was weighed (before and after collection) and centrifuged (21000 *g*, 5 min, 4 °C) in order to separate the solid and fluid phases of the biofilm. After centrifugation, a small fraction of the fluid phase was collected, using a micropipette (ANEXO J).

Fluoride analysis in the biofilm fluid

After biofilm fluid separation from its solid phase, it was transferred to the surface of an inverted ion-specific electrode (Orion 9409), immersed in mineral oil [Vogel et al., 1997]. The samples were placed on drops of TISAB III previously placed on the electrode membrane, in a ratio of 10:1 (sample:TISAB III), and were read by the positioning of the reference microelectrode within each sample in order to close the circuit. This electrode was previously calibrated with standard solutions of known F concentrations (ANEXO J).

Fluoride analysis of total biofilm

For extraction of F from the total biofilm, 0.5 M HCl (0.5 mL /10 mg of biofilm, wet weight) was added to the biofilm [Cury et al., 1997]. After vigorous shaking, the samples were placed on a flat orbital shaker (60 rpm, for 3 hours, at room temperature). Then 0.5 M NaOH (0.5 mL /10 mg biofilm, wet weight) was added to the samples. The tubes were vortexed for 1 min and centrifuged (11000 *g*, 2 min, room temperature). The supernatant was analyzed after buffering with TISAB III, using a F ion-specific electrode (Orion 9409), and a reference electrode (Orion 900200), both coupled to an ion analyzer (Orion 720 A^+) (ANEXOS K e L).

Hardness analysis

Surface hardness was determined prior to the experiments (SH_i) and after the *in situ* phases (SH_f) using the Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan), under 25 grams for 10 seconds (five indentations, spaced 100 µm from each other, made at the center of the enamel surface). Blocks included in the study had SH_i between 330-370 KHN [Takesita et al., 2009], and were randomly distributed among the

study groups. After the experimental phases, SH_f was determined by performing 5 indentations spaced 100 µm apart from each other and from the initial indentations. The percentage of SH loss (%SH) was calculated using the formula: $\%SH=100x(SH_f - SH_i)/SH_i$. For the analysis of the integrated loss of subsurface hardness (ΔKHN), the enamel blocks were then sectioned longitudinally at the center of the exposed area and one of the halves was embedded in acrylic resin with the cut surface exposted and polished. One sequence of 14 indentations was created at the center of the blocks at different distances (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220, and 330 µm) from the outer enamel surface using a Micromet 5114 hardness tester (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s [Danelon et al., 2015]. The integrated area of the hardness (ΔKHN × µm) of the lesion to the sound enamel was calculated using the Trapezoidal rule (GraphPad Prism[k], version 3.02) and subtracted from the integrated area of the hard enamel hardness obtaining the integrated loss of subsurface hardness (ΔKHN) [Spiguel et al., 2009] (ANEXO M).

Statistical analysis

Statistical analysis was performed on the software SigmaPlot version 12.0, at a significance level of 5%. Data of %SH (cubic transformed), ΔKHN, fluoride concentrations in the total biofilm and biofilm fluid (Log_{10} transformed) passed normality (Shapiro-Wilk) and homogeneity tests (Bartlett). Data were then submitted to one-way (hardness data) and two-way (biofilm data), repeated-measures ANOVA, followed by the Student-Newman-Keuls test. The relationship among the variables was assessed by Pearson's correlation coefficient.

Results

Table 1 shows mean total and ionic F concentrations in the experimental dentifrices, which presented a variation lower than 3% when compared with the expected values. Mean pH of the toothpastes ranged from 7.4 (1100 ppm F) to 7.7 (placebo).

The effects of the different treatment intensities on enamel demineralization are displayed on Table 2. Significantly lower %SH and ΔKHN

were observed for all fluoridated toothpastes when compared with the placebo ($p<0.001$). For % SH, the only significant difference among the fluoridated groups was observed for the 1100 ppm F toothpaste applied using a pea-size amount or the transversal technique ($p=0.040$), without significant differences for the other pairwise comparisons. On the other hand, a more marked effect of the treatment intensities was observed for ΔKHN, so that values obtained for the lowest intensity (i.e., 0.30 g of 550 ppm F and 0.15 g of 1100 ppm F) were significantly higher than those for the highest intensity (i.e., 0.60 g of 550 ppm F and 0.30 g of 1100 ppm F), without significant differences between CD and LFD within each intensity.

For total biofilm fluoride concentrations, significant differences were observed among the dentifrices ($F=15.9$, $p<0.001$) and time after brushing ($F=14.2$, $p=0.003$), with a significant interaction between the two variables ($F=0.6$, $p=0.046$), as shown in Table 3. A dose-response relationship was observed between treatment intensity and biofilm F concentrations for both times of sample collection, despite differences among the fluoridated toothpastes were not significant 5 min after brushing, except for the CD applied using the transversal technique . At 60 min, F concentrations remained significantly higher than the placebo for all fluoridated toothpastes, except for the 550 ppm F applied using the transversal technique. For biofilm fluid F concentrations, significant differences were observed among the dentifrices ($F=36.3$, $p<0.001$) and time after brushing ($F=288.6$, $p<0.001$), with a significant interaction between the two variables ($F=12.9$, $p<0.001$). Overall, all fluoridated toothpastes led to significantly higher F concentrations than the placebo group at both times of sample collection, without significant differences among the toothpastes regardless the amount applied on the toothbrush.

A strong correlation was observed between ΔKHN and F concentrations in the total biofilm ($r=-0.71$) and biofilm fluid ($r=-0.72$) 5 min after brushing, while lower coefficients were observed 60 min after brushing. A moderate correlation was seen between F concentrations in the total biofilm 5 and 60 min after brushing ($r=0.67$), whereas a strong correlation was seen for F concentrations in the fluid phase of the biofilm ($r=0.78$). ΔKHN and %SH were moderately correlated ($r=0.60$). Other comparisons are displayed in Table 4.

Discussion

The results of a recent *in vivo* study demonstrated that brushing with a low-fluoride dentifrice (LFD, 550 ppm F) applied using the transversal technique raised salivary F concentrations to levels significantly higher than those attained by the use of a conventional dentifrice (CD, 1100 ppm F) applied in a pea-size amount [Hall et al., 2016]. These results indicated that the treatment intensity (i.e. fluoride concentration in the product x amount applied on the toothbrush) is more relevant than the fluoride concentration in the dentifrice considered alone. Given that salivary fluoride concentration represents only one of the several aspects involved in the clinical performance of toothpastes, the present study was designed to further assess the impact of treatment intensity on the mineral loss of enamel covered by dental biofilm formed *in situ* under cariogenic challenge, as well as on the resulting biofilm fluoride concentrations. The present results showed that the treatment intensity had a direct impact on these variables, leading to the rejection of the study's null hypothesis.

Enamel mineral loss was assessed by the percentage of surface hardness loss (%SH) and integrated loss of subsurface hardness (ΔKHN). For both variables assessed, the use of a placebo dentifrice promoted significantly higher %SH and ΔKHN when compared with the fluoridated dentifrices, thus validating the *in situ* protocol employed (i.e., *in vivo* exposure to the dentifrice slurries and *ex vivo* exposure to sucrose). Interestingly, while brushing with the LFD using larger quantities (transversal technique or full bristles) led to similar %SH values to that observed for the CD applied using the transversal technique, the use of the CD in a pea-size amount promoted the highest %SH among the fluoridated dentifrices, being significantly higher than the CD applied with the transversal technique. Given that a marked reduction in salivary F concentration in children after brushing with a CD applied in a pea-size amount have also been demonstrated [Hall et al., 2016], these data taken together reinforce the concept that the use of very low quantities of a CD might reduce the caries-protective effect of the product under clinical conditions.

A more evident effect of the treatment intensity on enamel mineral loss was observed for ΔKHN data. The highest treatment intensity (i.e., 0.6 g of 550 ppm F or 0.3 g of 1100 ppm F) led to significantly lower ΔKHN when compared with the lowest treatment intensity (i.e., 0.3 g of 550 ppm F or 0.15 g of 1100

ppm F), with no significant differences between CD and LFD within each intensity. These findings are important from a clinical standpoint considering the current recommendations of using very low quantities of a CD [European Academy of Paediatric Dentistry, 2009; American Academy of Pediatric Dentistry, 2014]. Despite reducing the amount of dentifrice during toothbrushing is indeed an effective measure in minimizing F ingestion from the toothpaste, especially by younger children[Kobayashi et al., 2011], the present study clearly demonstrates that this recommendation significantly reduces the protective effect of the dentifrice.

The present *in situ* model was designed to mimic biofilm stagnation sites, what has some implications regarding biofilm fluoride concentrations in the solid and fluid phases. In this sense, while fluoride uptake by total biofilm was shown to be more related to the fluoride concentration in the dentifrices than the treatment intensity, the corresponding values in the biofilm fluid reached a plateau at 5 min after brushing, for all combinations of dentifrices and quantities. Despite the study protocol does not allow extrapolations regarding the interactions between the treatments and the biofilm, it is possible that the salivary fluoride concentrations immediately after the initial brushing strokes would be largely influenced by the fluoride concentration in the product (considering the reduced dilution of the product in the mouth at the beginning of toothbrushing), being, therefore, higher for CD than for LFD. This natural dentifrice-saliva slurry would then bathe all oral surfaces, including the dental biofilm, allowing fluoride to retain in the dental biofilm mainly through calcium bridging [Rose et al., 1996; Vogel, 2011], especially at the outer layers of the biofilm [Pessan et al., 2014], given the reduced penetration of fluoride into deeper layers in thick biofilms [Watson et al., 2005], as in the present study. Unlike for total biofilm, however, biofilm fluid represents a compartment without any mechanism of “retention” [Vogel, 2011], so that fluoride concentrations in this compartment would be related to a complex interplay among the residual salivary fluoride concentration, the release of fluoride from the biofilm biomass retained in more labile forms, as well as salivary clearance. In this sense, it is likely that the plateau observed for biofilm fluid fluoride would result from a saturation state obtained immediately after brushing. Time-course studies

assessing biofilm fluoride concentrations in the solid and fluid phases would be instructive to confirm the above-mentioned hypothesis.

It was noteworthy that enamel mineral loss (ΔKHN) was strongly correlated with fluoride concentrations in the biofilm (solid and fluid phases) collected 5 min after brushing, but lower correlation coefficients were observed for samples collected 60 min after brushing. Given that ΔKHN and biofilm fluoride concentrations are variables related to the long- and short-term effects of fluoride, respectively, the higher correlation coefficients observed for biofilm collected at 5 min indicates that the effect of fluoridated dentifrices on the biofilm immediately after brushing is an utmost aspect when assessing the efficacy of dentifrices on enamel demineralization, as it represents the maximum boosting effect of the treatment in interfering with the de-and re-mineralization processes of tooth enamel. These findings highlight the importance of brushing frequency, given that a higher frequency of exposure to the toothpaste will raise biofilm fluoride levels more often during the day, ultimately having a greater impact on the caries dynamics. This is in line with the results of a Cochrane review, showing that the clinical efficacy of fluoridated dentifrices increases with higher frequency of use [Marinho et al., 2003].

Two other aspects related to the correlations deserve comment. First, the coefficients obtained between %SH and total biofilm were shown to be moderate (total biofilm) or weak (biofilm fluid), regardless of the time of biofilm sampling. This seems to be related to the nature of the enamel caries lesion, in which the mineral loss of the superficial layers is much lower than that occurring in the subsurface, what seems to justify the lower correlation coefficients between these variables in comparison to those obtained for ΔKHN and biofilm fluoride concentrations. Second, the correlation between %SH and ΔKHN was shown to be moderate, what raises concerns regarding the suitability of surface hardness as a surrogate method for the assessment of the mineral changes occurring mainly in the subsurface. From the present results, it seems that surface hardness should only be used as an adjunct analysis to other methods such as cross-sectional hardness, transverse micro radiography or micro-computed tomography, so that mineral changes at both the surface and subsurface are analyzed together.

Due to the short-term and *in situ* nature of the protocol used and considering the well-controlled conditions regarding cariogenic challenge, brushing and rinsing procedures, the present results cannot be directly extrapolated to clinical conditions, in which several other variables are known to influence the development of caries lesions *in vivo* [Sheiham and James, 2015; Kumar et al., 2016]. The age-range of the subjects involved might also be considered as a shortcoming, considering that LFDs are usually recommended for children under 6 years of age, but for practical and ethical reasons it would not be possible to conduct an *in situ* study with children. Nonetheless, the present investigation provides unprecedented evidence that the caries-protective effect of dentifrices is largely dependent on both the fluoride concentration in the product and on the amount used during toothbrushing.

To conclude, the results of the present study demonstrated that the amount of dentifrice used during toothbrushing and the fluoride concentration in the product have a direct impact on the development of enamel subsurface lesions and on fluoride concentrations in the biofilm and biofilm fluid, emphasizing the concept that treatment intensity is a more relevant parameter of clinical efficacy than merely observing the fluoride concentration in the dentifrice. Considering the lack of well-controlled studies assessing the real benefits of dentifrices containing 500-550 ppm F on caries control, especially in the deciduous dentition [Walsh et al., 2010], the present data reinforce the need to discuss and re-evaluate the current recommendation of very small quantities of a CD to young children, as this was shown to significantly reduce its effects on enamel demineralization.

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Table captions

Table 1. Mean (SD) fluoride concentrations and pH in the experimental toothpastes (*n* = 3)

Table 2. Mean percentage of surface hardness loss (%SH) and integrated loss of subsurface hardness (ΔKHN) according to the different treatment intensities (fluoride concentration in the dentifrice x amount applied on the toothbrush)

Table 3. Fluoride concentration in the total biofilm and biofilm fluid formed *in situ* according to the different treatment intensities (fluoride concentration in the dentifrice x amount applied on the toothbrush) and time after brushing

Table 4. Correlation coefficients among the variables assessed in the study

Table 1. Mean (SD) fluoride concentrations and pH in the experimental toothpastes (*n* = 3)

Dentifrices	Fluoride concentration (ppm)		pH
	Total fluoride	Ionic fluoride	
Placebo	18.5 (3.3)	13.5 (8.4)	7.69 (0.01)
550 ppm F	545.1 (4.0)	543.5 (16.8)	7.46 (0.13)
1100 ppm F	1075.5 (14.6)	1095.6 (22.7)	7.40 (0.14)

Table 2. Mean percentage of surface hardness loss (%SH) and integrated loss of subsurface hardness (ΔKHN) according to the different treatment intensities (fluoride concentration in the dentifrice x amount applied on the toothbrush)

	Dentifrices	**Placebo (F-free)**	**550 ppm F**		**1100 ppm F**	
	Amount applied on the toothbrush	**0.6 g**	**0.3 g**	**0.6 g**	**0.15 g**	**0.3 g**
%SH	Mean	-51.3[a]	-25.0[bc]	-23.4[bc]	-30.2[b]	-20.2[c]
	(SD)	(26.1)	(13.2)	(8.7)	(13.0)	(8.7)
ΔKHN	Mean	7378.6[a]	3113.4[b]	2604.0[c]	3224.7[b]	2431.6[c]
	(SD)	(1123.0)	(411.7)	(453.4)	(845.5)	(379.4)

Superscript letters indicate significant differences among the means. Data analyzed by one-way, repeated measures ANOVA after cubic and $\log_{10}$ transformation, respectively for %SH and ΔKHN, followed by Student-Newman-Keuls test ($p<0.05$, $n=13$).

Table 3. Fluoride concentration in the total biofilm and biofilm fluid (µg F/g) formed *in situ* according to the different treatment intensities (fluoride concentration in the dentifrice x amount applied on the toothbrush) and time after brushing

		Placebo (F-free)		**550 ppm F**				**1100 ppm F**			
Amount used during brushing		**0.6 g**		**0.3 g**		**0.6 g**		**0.15 g**		**0.3 g**	
Time after brushing (min)		5	60	5	60	5	60	5	60	5	60
Total biofilm	Mean	1.9^{a}	2.1^{A}	5.2^{b}	4.0^{ABC}	8.6^{b}	6.6^{BD}	10.4^{b}	8.9^{CD}	22.9^{c}	9.0^{D}
	(SD)	(1.1)	(1.3)	(2.6)	(3.6)	(6.0)	(6.9)	(6.3)	(8.9)	(27.3)	(6.3)
Biofilm fluid	Mean	0.12^{a}	0.06^{A}	4.37^{b}	0.27^{B}	5.32^{b}	0.67^{B}	5.38^{b}	0.40^{B}	5.38^{b}	0.54^{B}
	(SD)	(0.06)	(0.03)	(5.30)	(0.21)	(4.28)	(0.65)	(5.56)	(0.45)	(5.91)	(0.61)

Lowercase and uppercase superscript letters indicate significant differences among the means, respectively for samples collected at 5 and 60 min after brushing (Two-way, repeated measures ANOVA after $\log_{10}$ transformation, followed by Student-Newman-Keuls test ($p<0.05$, $n=13$).

Table 4. Correlation coefficients among the variables assessed in the study

VARIABLES		ΔKHN	F in the total biofilm 5 min after brushing	F in the total biofilm 60 min after brushing	F in the biofilm fluid 5 min after brushing	F in the biofilm fluid 60 min after brushing
%SH	r	0.60	-0.56	-0.48	-0.35	-0.31
	p	*p<0.001*	*p<0.001*	*p<0.001*	*p=0.004*	*p=0.013*
ΔKHN	r	*	-0.71	-0.44	-0.72	-0.55
	p		*p<0.001*	*p=0.003*	*p<0.001*	*p=0.003*
F in the total biofilm 5 min after brushing	r	-0.71	*	0.67	0.53	0.48
	p	*p<0.001*		*p=0.0013*	*p=0.005*	*p=0.005*
F in the total biofilm 60 min after brushing	r	-0.44	0.67	*	0.17	0.09
	p	*p=0.003*	*p=0.0013*		*p=0.2*	*p=0.5*
F in the biofilm fluid 5 min after brushing	r	-0.72	0.53	0.17	*	0.78
	p	*p<0.001*	*p=0.005*	*p=0.2*		*p<0.001*
F in the biofilm fluid 60 min after brushing	r	-0.55	0.48	0.09	0.78	*
	p	*p=0.003*	*p=0.005*	*p=0.5*	*p<0.001*	

%SH = Percentage of surface hardness loss; ΔKHN = Integrated area of subsurface loss. Pearson's correlation coefficients (r) determined on the Log_{10} transformed outcomes (*n*=65 for each analysis, p<0.05).

Anexos

ANEXO A

Instruções aos Autores

Caries Research

Guidelines for Authors
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Aims and Scope

'Caries Research' is an international journal, the aim of which is to promote research in dental caries and related fields through publication of original research and critical evaluation of research findings. The journal will publish papers on the aetiology, pathogenesis, prevention and clinical control or management of dental caries. Papers on health outcomes related to dental caries are also of interest, as are papers on other disorders of dental hard tissues, such as dental erosion. Aspects of caries beyond the stage where the pulp ceases to be vital are outside the scope of the journal. The journal reviews papers dealing with natural products and other bacterial inhibitors against specific criteria, details of which are available from the Editor.

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Should you experience problems with your submission, please contact:

Prof. David Beighton
(Editor-in-Chef, Caries Research)
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Reviews can have a freer format but should nevertheless commence with a Title page, an Abstract and an Introduction defining the scope.

Current topics are concise articles that present critical discussion of a topic of current interest, or a fresh look at a problem, and should aim to stimulate discussion.

Letters to the Editor, commenting on recent papers in the journal, are published occasionally, together with a response from the authors of the paper concerned.

Preparation of Manuscripts

Text should be one-and-a-half-spaced, with wide margins. All pages and all lines must be numbered, starting from the title page. A conventional font, such as Times New Roman or Arial, should be used, with a font size of 11 or 12. Avoid using italics except for Linnaean names of organisms and names of genes.

Manuscripts should be prepared as a text file plus separate files for illustrations. The text file should contain the following sequence of sections: Title page; Declaration of interests; Abstract; Introduction; Materials and Methods; Results; Discussion; Acknowledgements; References; Legends; Tables. Each section should start on a new page, except for the body of the paper (Introduction to Acknowledgements), which should be continuous. Lines in the manuscript must be numbered consecutively from the title page until the last page. Submissions which do not conform to these simple guidelines will be returned to the author.

Title page: The first page of each manuscript should show, in order:

- the title, which should be informative but concise;
- the authors' names and initials, without degrees or professional status, followed by their institutes;
- a short title, maximum length 60 characters and spaces, for use as a running head;
- a list of 3-10 key words;
- the name of the corresponding author and full contact details (postal address, telephone and fax numbers, and e-mail address).

Declaration of Interests:Potential conflicts of interest should be identified for each author or, if there are no such conflicts, this should be stated explicitly. Conflict of interest exists where an author has a personal or financial relationship that might introduce bias or affect their judgement. Examples of situations where conflicts of interest might arise are restrictive conditions in the funding of the research, or if an author or their employer holds patent(s) on a product used in the study, or payment to an investigator from organisations with an interest in the study (including employment, consultancies, honoraria, ownership of shares, travel grant). Investigators should disclose potential conflicts to study participants and should state whether they have done so.

The possible existence of a conflict of interest does not preclude consideration of a manuscript for publication, but the Editor might consider it appropriate to publish the disclosed information along with the paper.

Abstract: The abstract should summarise the contents of the paper in a single paragraph of no more than 250 words (to ensure that the abstract is published in full by on-line services such as PubMed). No attempt should be made to give numerical results in detail. References are not allowed in the abstract.

Introduction: This section should provide a concise summary of the background to the relevant field of research, introduce the specific problem addressed by the study and state the hypotheses to be tested.

Materials and Methods (or Subjects and Methods): All relevant attributes of the material (e.g. tissue, patients or population sample) forming the subject of the research should be provided. Experimental, analytical and statistical methods should be described concisely but in enough detail that others can repeat the work. The name and brief address of the manufacturer or supplier of major equipment should be given.

Statistical methods should be described with enough detail to enable a knowledgeable reader with access to the original data to verify the reported results. When possible, findings should be quantified and appropriate measures of error or uncertainty (such as confidence intervals) given. Sole reliance on statistical hypothesis testing, such as the use of P values, should be avoided. Details about eligibility criteria for subjects, randomization and the number of observations should be included. The computer software and the statistical methods used should be specified. See Altman et al.: Statistical guidelines for contributors to medical journals [Br Med J 1983;286:1489–93] for further information.

Manuscripts reporting studies on human subjects should include evidence that the research was ethically conducted in accordance with the Declaration of Helsinki (World Medical Association).In particular, there must be a statement in Materials and Methods that the consent of an appropriate ethical committee was obtained prior to the start of the study, and that subjects were volunteers who had given informed, written consent.

Information detailing the power and sample size calculations must be included in the manuscript.

Randomized clinical trials should be reported according to the standardised protocol of the CONSORT Statement. The CONSORT checklist must be submitted together with papers reporting clinical trials.

Randomized clinical trials must be registered at clinicaltrials.gov or similar national authority and the trial number included in the manuscript.

Trials beginning after 1 July 2012 must be registered before recruitment of the first patient. Caries Research will accept 'retrospective registration' of trials that began before 1 July 2012 (retrospective meaning registration occurs after patient enrolment begins). When submitting a paper on a clinical trial, the trial registration number should be stated at the end of the abstract in the following format: Trial registration: [name of the trial registry, the registry URL and the trial registration number].

In studies on laboratory animals, the experimental procedures should conform to the principles laid down in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and/or the National Research Council Guide for the Care and Use of Laboratory Animals.

Unless the purpose of a paper is to compare specific systems or products, commercial names of clinical and scientific equipment or techniques should only be cited, as appropriate, in the 'Materials and Methods' or 'Acknowledgements' sections. Elsewhere in the manuscript generic terms should be used. In any manuscript involving microradiography, the following information must be included: the radiation source and filters used and the kV used (this determines the wavelength of radiation and hence the validity of using Angmar's equation).

Manuscripts on experimental enamel caries should show that the lesions retain a relatively well-preserved surface layer, i.e. are not surfacesoftened lesions. Proof of surface integrity can be provided either as illustrations in the paper or as supplementary material for the reviewers. Transverse microradiography, polarized light microscopy of a section immersed in water or backscattered scanning electron microscopy of a polished cross-section can be used to provide the necessary proof. To allow the nature of experimental changes to be assessed, microradiographs or micrographs should be provided to show part of the experimental lesion and the adjacent control (e.g. figure 2 of Zaura et aI.: Caries Res 2007;41:489–492). Again, these images can be provided as part of the paper or as supplementary material for review purposes.

Results: Results should be presented without interpretation. The same data should not be presented in both tables and figures. The text should not repeat numerical data provided in tables or figures but should indicate the most important results and describe relevant trends and patterns.

Discussion: This section has the functions of describing any limitations of material or methods, of interpreting the data and of drawing inferences about the contribution of the study to the wider field of research. There should be no repetition of preceding sections, e.g. reiteration of results or the aim of the research. The discussion should end with a few sentences summarising the conclusions of the study. However, there should not be a separate 'Conclusions' section.

Acknowledgements: Acknowledge the contribution of colleagues (for technical assistance, statistical advice, critical comment etc.) and provide the position(s) of author(s) employed by commercial firms. This section should describe the source(s) of funding that have supported the work inlcuding relevant grant numbers. Please also include this sentence: "The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript." If this statement is not correct, you must describe the role of any sponsors or funders, and amend the sentence as needed. Additionally, the roles of all authors must be described (For example: Conceived and designed the experiments: AA, BB. Performed the clinical examination: AA, CC. Performed the experiments: DD, FF. Analyzed the data: BB, FF. Wrote the paper: AA, CC, FF, EE).

Legends: The table headings should be listed first, followed by the legends for the illustrations.

Tables: Tables should be numbered in Arabic numerals. Each table should be placed on a separate page. Tables should not be constructed using tabs but by utilising the table facilities of the word-processing software.

Illustrations:

- Illustrations should be numbered in Arabic numerals in the sequence of citation. Figure numbers must be clearly indicated on the figures themselves, outside the image area.

- Black and white half-tone illustrations must have a final resolution of 300 dpi after scaling, line drawings one of 800-1200 dpi.
- Figures with a screen background should not be submitted.
- When possible, group several illustrations in one block for reproduction (max. size 180 x 223 mm).

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References

Reference to other publications should give due acknowledgement to previous work; provide the reader with accurate and up-to-date guidance on the field of research under discussion; and provide evidence to support lines of argument. Authors should select references carefully to fulfil these aims without attempting to be comprehensive.

Cited work should already be published or officially accepted for publication. Material submitted for publication but not yet accepted should be cited as 'unpublished results', while unpublished observations communicated to the authors by another should be cited as 'personal communication', with credit in both cases being given to the source of the information. Neither unpublished nor personally communicated material should be included in the list of references. Abstracts more than 2 years old and theses should not be cited without a good reason, which should be explained in the covering letter accompanying the paper.

References should be cited by naming the author(s) and year. Where references are cited in parenthesis, both names and date are enclosed in square brackets. Where the author is the subject or object of the sentence, only the year is enclosed in brackets.
One author: [Frostell, 1984] or Frostell [1984].
Two authors: [Dawes and ten Cate, 1990] or Dawes and ten Cate [1990].
More than two authors: [Trahan et al., 1985] or Trahan et al. [1985].
Several references cited in parenthesis should be in date order and separated by semi-colons: [Frostell, 1984; Trahan et al., 1985; Dawes and ten Cate, 1990].

Material published on the World Wide Web should be cited like a reference to a print publication, and the URL included in the reference list (not in the text), together with the year when it was accessed.
The reference list should include all the publications cited in the text, and only those publications. References, formatted as in the examples below, should be arranged in strict alphabetical order. All authors should be listed. For papers by

the same authors, references should be listed according to year. Papers published by the same authors in the same year should be distinguished by the letters a, b, c, ... immediately following the year, in both the text citation and the reference list. For abbreviation of journal names, use the Index Medicus system. For journals, provide only the year, volume number and inclusive page numbers.

Examples

(a) Papers published in periodicals: Lussi A, Longbottom C, Gygax M, Braig F: Influence of professional cleaning and drying of occlusal surfaces on laser fluorescence in vivo. Caries Res 2005;39:284-286.

(b) Papers published only with DOI numbers: Theoharides TC, Boucher W, Spear K: Serum interleukin-6 reflects disease severity and osteoporosis in mastocytosis patients. Int Arch Allergy Immunol DOI: 10.1159/000063858.

(c) Monographs: Matthews DE, Farewell VT: Using and Understanding Medical Statistics. Basel, Karger, 1985.

(d) Edited books: DuBois RN: Cyclooxygenase-2 and colorectal cancer; in Dannenberg AJ, DuBois RN (eds): COX-2. Prog Exp Tum Res. Basel, Karger, 2003, vol 37, pp 124-137.

(e) Patents: Diggens AA, Ross JW: Determining ionic species electrochemically. UK Patent Application GB 2 064 131 A, 1980.

(f) World Wide Web: Chaplin M: Water structure and behavior. www.lsbu.ac.uk/water, 2004.

Supplementary Material

Supplementary material is restricted to additional information which is directly pertinent to the contentand conclusion of the paper. Please note that all supplementary files will undergo editorial review and should be submitted together with the original manuscript. The editors reserve the right to reject or limit the scope and length of supplementary material. Supplementary material must meet production quality standards for web publication without the need for any modification or editing. In general, supplementary files should not exceed 10 MB in size. Acceptable file formats are word or pdf, excel spreadsheets (only if the data cannot be converted properly to a pdf file), video files (.mov, .avi, .mpeg), and audio files (.wav), either free standing or incorporated into html or ppt files in each case to illustrate the sound.
Accepted supplementary material will be published as submitted and no proofs will be provided to the authors.

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ANEXO B

Certificado de aprovação do Comitê de Ética em Pesquisa

FACULDADE DE ODONTOLOGIA - CÂMPUS DE ARAÇATUBA - JÚLIO DE

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Influência da quantidade de dentifrício e concentração de fluoreto na retenção intrabucal de fluoreto: estudo in situ.

Pesquisador: Mayra Frasson Paiva

Área Temática:

Versão: 5

CAAE: 44712715.5.0000.5420

Instituição Proponente: Universidade Estadual Paulista Júlio de Mesquita Filho

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.323.893

Apresentação do Projeto:

O estudo será realizado em 5 fases sucessivas, cada fase com duração de duas semanas. Os voluntários serão aleatoriamente divididos em 5
grupos e farão uso de dentifrício convencional (1100 ppm F, NaF) e com concentração reduzida de F (550 ppm F, NaF), totalizando 4 combinações
de dentifrício e quantidade, a saber:a) 550 ppm F aplicado pela técnica transversal (0,3 g);b) 550 ppm F aplicado sobre todas as cerdas da escova
(0,6);c) 1100 ppm F aplicado com quantidade do tamanho de uma ervilha 0,15 g)d) 1100 ppm F aplicado pela técnica transversal (0,3 g).Além
destas, será incluída uma etapa com um dentifrício placebo (controle negativo), aplicado sobre todas as cerdas da escova (0,6 g). Estas
combinações foram baseadas em um estudo concluído recentemente pelo nosso grupo de

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pesquisa que
avaliou as concentrações de fluoreto (F) na
saliva após a escovação com dentifrícios contendo diferentes concentrações de F, em diferentes quantidades (0,1, 0,3 e 0,5 g) [Hall, 2015].
Entretanto, para que os tratamentos tenham intensidades comparáveis (quantidade x concentração de F no produto), optamos por utilizar quantidades que guardassem proporção entre si (dobro ou metade), de forma a permitir uma comparação direta entre os resultados após a conclusão do experimento. Os tubos dos dentifrícios serão codificados as quantidades a serem utilizadas
durante a escovação serão determinadas
por um examinador não envolvido na coleta e na análise das amostras, para que o protocolo duplo-cego seja utilizado.

Objetivo da Pesquisa:
O objetivo do presente estudo será avaliar as concentrações de fluoreto no biofilme dental e no fluido do biofilme após a escovação com dentifrícios
contendo diferentes concentrações de fluoreto (0, 550 e 1100 ppm F, pH neutro) em diferentes quantidades. Também será avaliada a influência
destes fatores sobre a desmineralização do esmalte dental bovino sob desafio cariogênico.

Avaliação dos Riscos e Benefícios:
Na utilização do aparelho removível poderá ocorrer desconforto mínimo quanto a fala, especialmente no primeiro dia de uso. No entanto, é comum
em estudos desta natureza que o voluntário se adapte rapidamente ao uso do mesmo.
Ao participar desta pesquisa o (a) voluntário (a) não terá nenhum benefício direto resultante da mesma. Entretanto, os participantes receberão uma
limpeza dentária e instruções de higiene bucal previamente ao início do estudo. Caso haja

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Continuação do Parecer: 1.323.893

necessidade de
restauração dos dentes, será oferecido
tratamento na Faculdade de Odontologia de Araçatuba-UNESP.

Comentários e Considerações sobre a Pesquisa:

O projeto está bem elaborado, descrito, embasado em literatura atualizada e os resultados trarão importante contribuição à área.

Considerações sobre os Termos de apresentação obrigatória:

Todos termos forma devidamente apresentados.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

Não havendo pendências recomenda-se a aprovação do protocolo de pesquisa.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 16/05/2016.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_504180.pdf	20/10/2015 09:24:31		Aceito
Projeto Detalhado / Brochura Investigador	Projeto.docx	20/10/2015 09:23:12	Mayra Frasson Paiva	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE Mayra 2.doc	02/06/2015 10:51:00		Aceito
Folha de Rosto	Folha de Rosto assinada.pdf	06/05/2015 10:21:10		Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

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Continuação do Parecer: 1.323.893

ARACATUBA, 16 de Novembro de 2015

Assinado por:
André Pinheiro de Magalhães Bertoz
(Coordenador)

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Página 04 de 04

ANEXO C

Instruções aos Voluntários

- Antes do início de cada fase experimental, você deverá fazer uso do dentifrício placebo (sem F) por 7 dias;
- Para cada fase experimental, você receberá uma escova dental específica. A primeira escova (somente utilizada com o dentifrício placebo) deverá ser guardada para posterior uso entre uma fase e outra (washout 7 dias);
- Durante cada fase experimental e entre elas, não será permitido o uso de produtos fluoretados;
- Durante o período de descanso (washout – 7 dias) entre uma fase e outra, utilizar somente o dentifrício placebo fornecido pelos pesquisadores.

Protocolo do estudo:

- Na tarde que antecede cada fase, você receberá o kit a ser utilizado na pesquisa, contendo uma escova nova, o dentifrício correspondente a cada fase, o dispositivo palatino, solução de sacarose, copo medidor para enxágue da boca e gaze;
- Neste mesmo dia, você deverá instalar o dispositivo na cavidade bucal antes de dormir, e não realizar a escovação com o mesmo no interior da cavidade bucal;
- A partir do dia seguinte (1º dia da fase experimental), você deverá iniciar o desafio cariogênico dos blocos de esmalte com sacarose a 30%, e continuar durante 6 dias, como descrito a seguir:

  Com o dispositivo fora da boca, dispensar duas gotas da solução de sacarose 30% em cada bloco, 6x/dia nos horários pré-determinados (8:00, 11:00, 14:00, 17:00, 19:00, 21:00). Após o gotejamento, o dispositivo palatino será deixado em descanso por 5 minutos, e após esse tempo, deverá ser reinserido na cavidade bucal.
  OBS: Armazenar a solução de sacarose a 30% na geladeira, e retirar 5 minutos antes do desafio.

- A escovação deverá ser realizada 3x/dia, com o aparelho na cavidade bucal, entretanto a escova não deverá tocar a região protegida pela tela plástica, de acordo com os passos a seguir:

  Após escovar durante 1 minuto com a quantidade de dentifrício determinada no frasco, a suspensão formada de dentifrício + saliva deverá ser bochechada por 30 segundos, com o aparelho ainda na boca. Em seguida, expelir a suspensão, remover o dispositivo da cavidade bucal e finalizar a escovação. A cavidade bucal deverá ser enxaguada com 10 ml de água, e o dispositivo com 10 ml para a região do palato e 10 ml para a região dos blocos.

- O dispositivo deverá ser removido da cavidade bucal apenas durante as refeições ou ingestão de líquidos (inclusive água);
- Quando estiver fora da boca, em nenhum momento o dispositivo deve ser deixado a seco. Guarde-o no porta-aparelho, com gaze umedecida sobre a área dos blocos de esmalte. Troque a gaze diariamente ou sempre que julgar necessário.
- No 8º dia da pesquisa, você deverá comparecer ao laboratório de Odontopediatria em jejum, sem realizar a escovação dos dentes e do dispositivo e sem realizar o desafio cariogênico. Assim que chegar ao laboratório, será realizada escovação sob supervisão do pesquisador. Neste dia, o dentifrício será aplicado na escova e pesado pelo pesquisador, utilizando uma balança de precisão. Em seguida serão iniciadas as coletas de biofilme.
- Em seguida, você terá um período de 7 dias sem utilizar o dispositivo (washout), utilizando somente o dentifrício placebo sem restrições quanto a quantidade a ser aplicada na escova, número de escovações por dia e enxágue da boca;
- É de suma importância que você avise ao responsável pela pesquisa se o dentifrício estiver acabando, caso contrário prejudicará a pesquisa;
- Quando realizar atividades aquáticas, o dispositivo deverá ser mantido no recipiente fornecido;
- Caso haja necessidade da utilização de algum tipo de medicamento, ou em caso de qualquer dúvida, entrar em contato com a pesquisadora responsável: Mayra Frasson Paiva - Contato: (44) 99800-3041.

ANEXO D

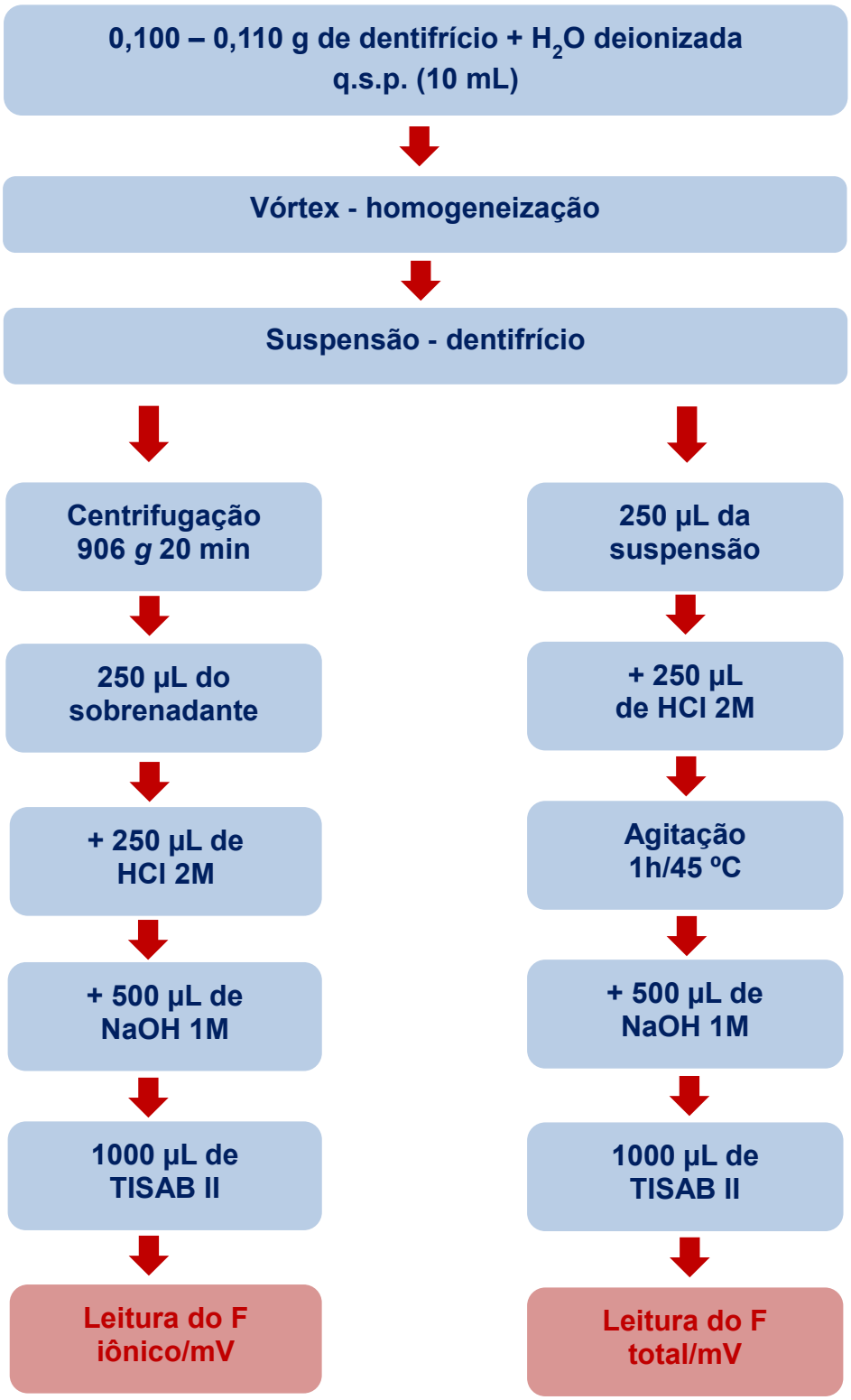
Dentifrícios Experimentais e Quantidades Utilizadas

Dentifrícios experimentais codificados por pesquisador não envolvido na etapa experimental: Placebo (sem F), 550 µg F/g, 1100 µg F/g

Quantidades de dentifrício utilizadas na pesquisa: a) grão de ervilha, b) transversal, c) cerda cheia

ANEXO E

Esquema representativo da Dosagem de F nos Dentifrícios



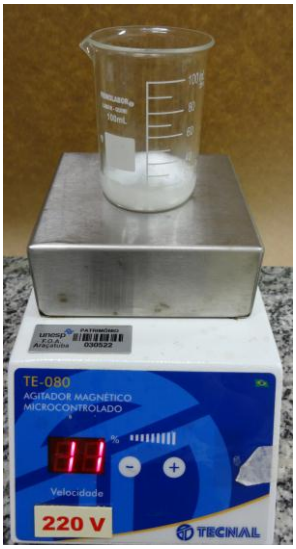
Delbem *et al.*, 2009
Ref. complete: Delbem *et al.* Caries Res. 2009;43(5):359-365

ANEXO F

Dosagem de pH nos Dentifrícios Experimentais

Pesagem de 1 g de dentifrício com auxílio de um Bécker de vidro

Adição de 3 mL de água deionizada ao Bécker de vidro



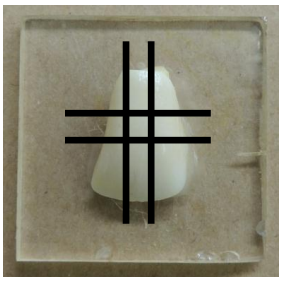
Agitação em agitador magnético até completa homogeneização

Dosagem do pH com eletrodo de pH 2A14-OC (Analyser) e analisador de pH Mettler Toledo

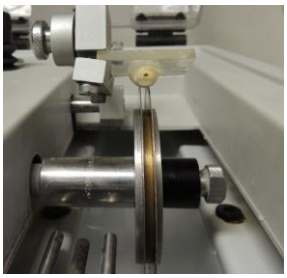
ANEXO G

Preparo dos Blocos de Esmalte

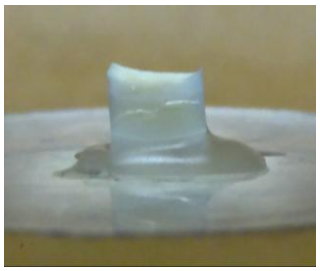
Separação coroa/raiz



Região da superfície vestibular (V) a ser seccionada



Secção da superfície V em cortadeira



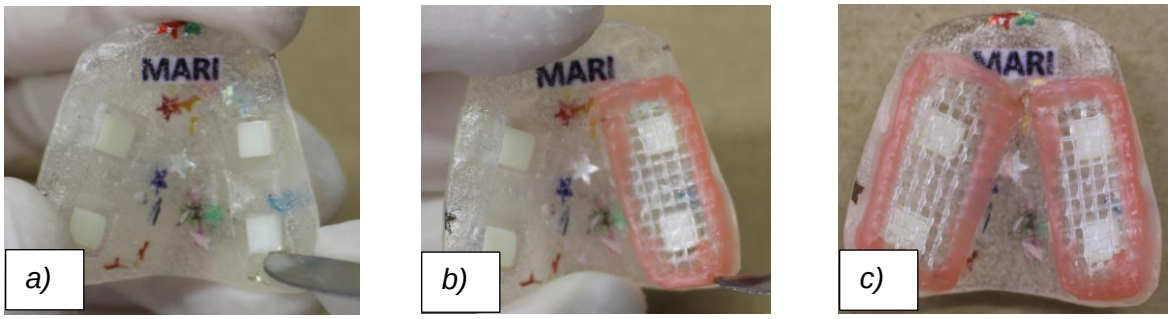
Superfície dentinária antes do polimento

Planificação da dentina e polimento do esmalte em politriz

Bloco de esmalte após polimento

ANEXO H

Preparo dos Dispositivos Palatinos e Desafio Cariogênico



a) b) c)

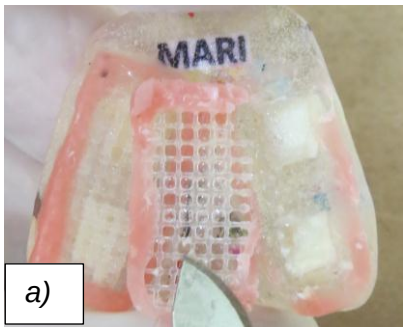
Preparo dos dispositivos palatinos: a) colagem dos blocos de esmalte; b) colagem da tela plástica; c) dispositivo finalizado

Kit de tratamento: escova dental, dentifrício experimental, solução de sacarose, copo para enxágue, gaze e estojo contendo o dispositivo palatino

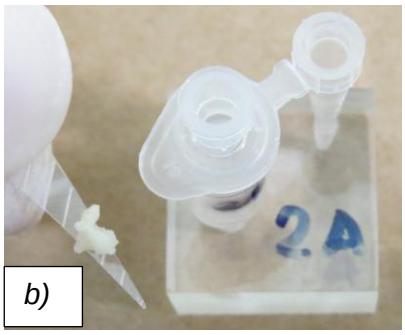
Desafio cariogênico: 2 gotas de sacarose a 30% aplicadas sobre cada bloco de esmalte

ANEXO I

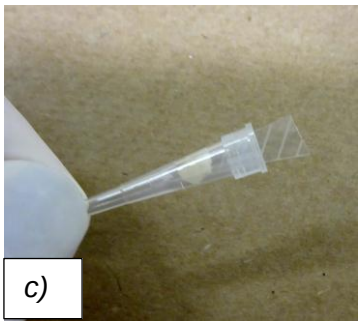
Coleta e Pesagem do Biofilme



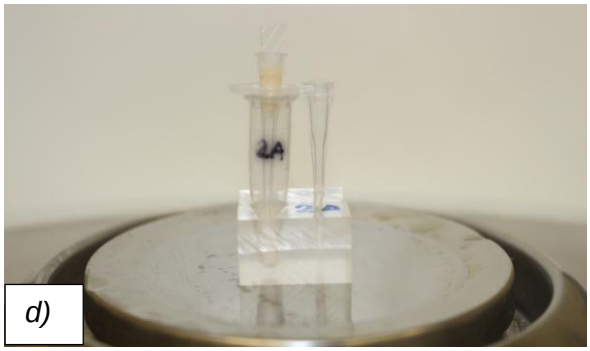
a)



b)



c)



d)

a) Remoção da tela plástica para a coleta do biofilme; b) espátula plástica para a transferência do biofilme ao conjunto de pesagem; c) inserção da espátula com biofilme em óleo mineral; d) pesagem do conjunto após coleta do biofilme

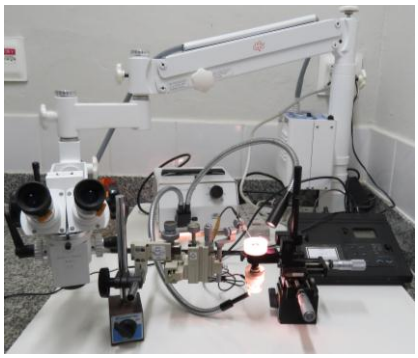
ANEXO J

Processamento e Leitura de F do Fluido do Biofilme

a)

b)

a) Centrifugação do biofilme para obtenção da fase fluida (21.000 g*, 4 ºC, 5 min)*
b) aspecto após separação do fluido do biofilme de sua fase sólida (círculo amarelo indica a localização da fase fluida)



Equipamento utilizado para análise de F no fluido do biofilme

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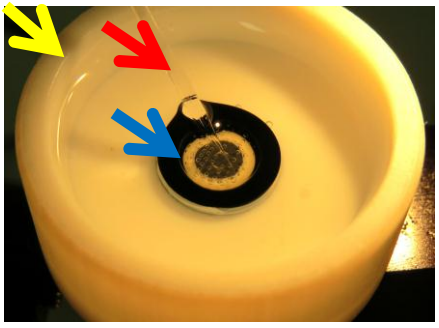
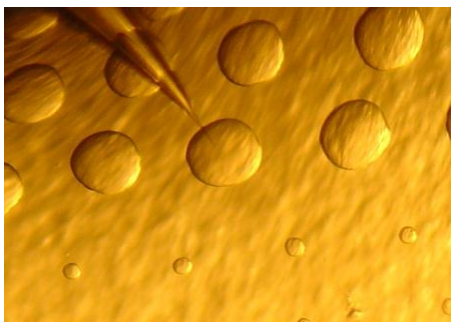


Cubeta de teflon (seta amarela); eletrodo de referência (seta vermelha); membrana do eletrodo íon-específico (seta azul)

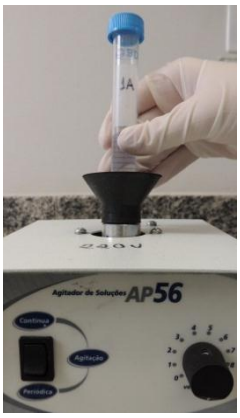


Eletrodo de referência posicionado no interior da amostra de fluido tamponada com TISAB III, sobre a superfície do eletrodo Orion 9409

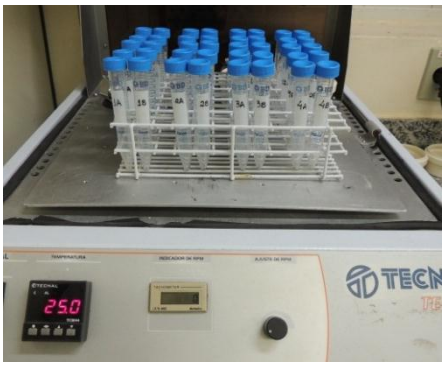
ANEXO K

Extração de F do Biofilme Total

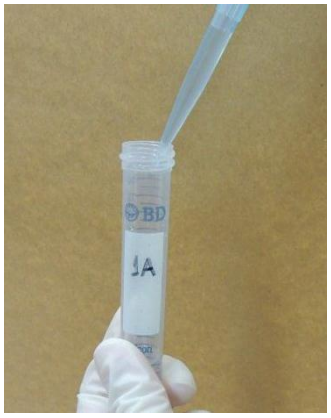
Adição de HCl 0,5 M ao biofilme



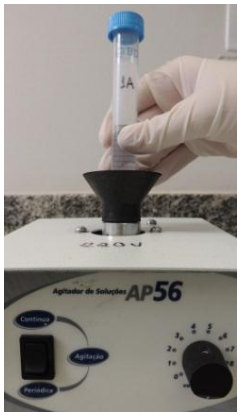
Agitação em vórtex (1 min)



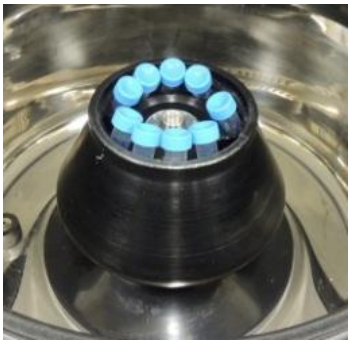
Agitação em mesa agitadora orbital plana (60 rpm, 3 h, 25 ºC)



Adição de NaOH 0,5 M ao biofilme



Agitação em vórtex (1 min)

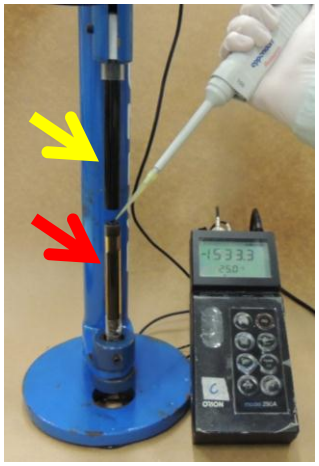


Centrifugação (11.000 g, 2 min, temperatura ambiente)

ANEXO L

Análise de F do Biofilme Total

Adição de 6 µL de TISAB III a 60 µL do sobrenadante obtido após extração ácida e neutralização com NaOH

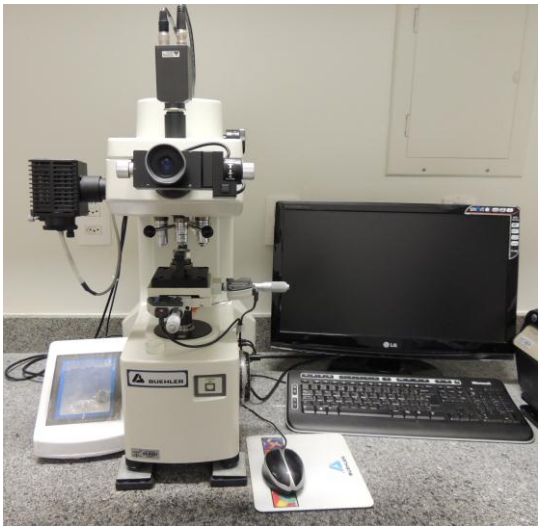


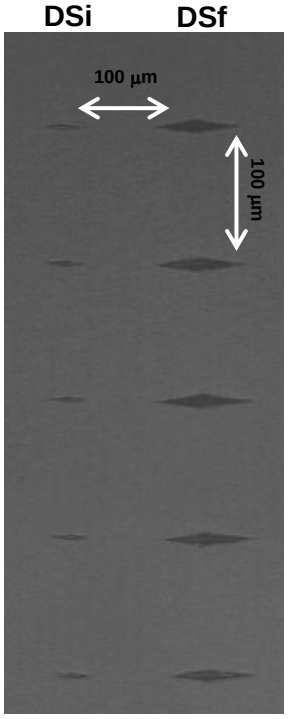
Amostra tamponada dispensada sobre a membrana do eletrodo de referência ORION 900200 em posição invertida (seta vermelha), com eletrodo íon-específico para F ORION 9409 acima (seta amarela)

Vista aproximada da amostra na superfície do eletrodo de referência ORION 900200

ANEXO M

Análise de Dureza de Superfície e em Secção Longitudinal





Dureza de Superfície: 25 g/10 s
5 impressões equidistantes e a 100 µm em relação às impressões iniciais

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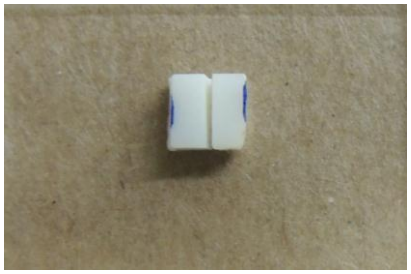
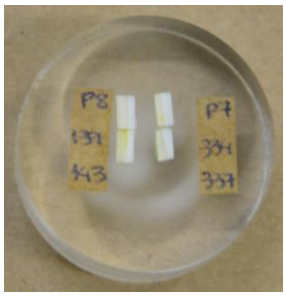
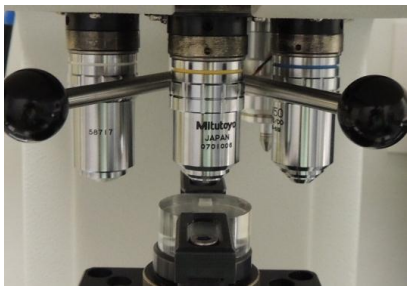


Secção dos blocos no sentido longitudinal

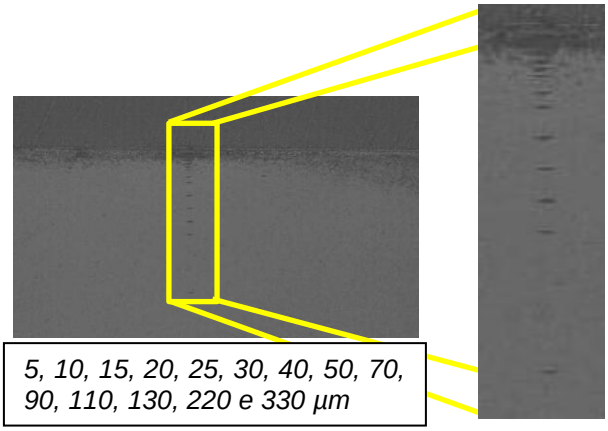
Inclusão dos blocos em resina acrílica



Blocos embutidos, com a região interna voltada para cima



Dureza em secção longitudinal: 5 g/10 s
14 impressões a partir da superfície externa do esmalte



5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 e 330 µm