UNIVERSIDADE ESTADUAL PAULISTA
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Campus de Araçatuba
Departamento de Odontologia Infantil e Social

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# "Efeito *in situ* da combinação de dentifrício fluoretado e gel com trimetafosfato de sódio sobre a desmineralização do esmalte"

Araçatuba-SP

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# "Efeito *in situ* da combinação de dentifrício fluoretado e gel com trimetafosfato de sódio sobre a desmineralização do esmalte"

Trabalho de Conclusão de Curso como parte dos requisitos para a obtenção do título de Bacharel em Odontologia da Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista "Júlio de Mesquita Filho".

Orientador: Prof. Titular. Alberto Carlos Botazzo Delbem

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# Dedicatória

Dedico este trabalho,

Aos meus pais, Cilene Felipe e Wilson Seidy Akabane,

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# Epígrafe

"De tudo ficaram três coisas...
A certeza de que estamos começando,
A certeza de que é preciso continuar,
A certeza de que podemos ser interrompidos
antes de terminar...
Façamos da interrupção um caminho novo,
Da queda, um passo de dança,
Do medo, uma escada,
Do sonho, uma ponte,
Da procura, um encontro!"

Fernando Sabino

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# *Resumo*

Objetivo: O objetivo deste estudo foi avaliar *in situ* o efeito da associação de gel com reduzida concentração de fluoreto suplementado com trimetafosfato de sódio (TMP) e dentifrício fluoretado sobre a desmineralização do esmalte. Materiais e Métodos: Este estudo foi cruzado e duplo-cego consistindo em 5 fases de 7 dias cada. Voluntários (n = 12) utilizaram aparelhos palatinos contendo 4 blocos de esmalte. O desafio cariogênico foi realizado com sacarose a 30% (6x/dia). Os tratamentos foram: dentifrício sem F/TMP - Placebo (PT); dentifrício 1,100 µg F/g (FT); FT + 4,500 µg F/g + 5%TMP gel (FT + TMP gel); FT + 9,000 µg F/g gel (FT + 9000 gel) e FT + 12,300 µg F/g gel (FT + Ácido gel). Após aplicação tópica dos tratamentos durante 1 min, foram removidos 2 blocos para análise de fluoreto fracamente ligado ($CaF_2$), cálcio (Ca), fósforo (P) e fluoreto firmemente ligado (F) formado no esmalte. Após os períodos experimentais de 7 dias, determinou-se a porcentagem de perda de dureza de superfície (%SH), perda integrada de dureza de subsuperfície (ΔKHN), $CaF_2$, Ca, P e F retidos. Além disso, nos biofilmes foram analisados as concentrações de F, Ca, P e polissacarídeos extracelulares insolúveis (EPS). Resultados: O tratamento FT + TMP gel promoveu a menor %SH e ΔKHN ($p < 0,001$). FT + Ácido gel mostrou uma maior concentração de $CaF_2$ formado/retido ($p < 0,001$). Valores semelhantes foram observados para a concentração de Ca/P/F e EPS no esmalte e biofilme para todos os

grupos fluoretados. Conclusão: A associação de FT + TMP gel reduziu significativamente a desmineralização do esmalte *in situ*. Significância clínica: a associação de tratamentos pode ser uma alternativa para pacientes com alto risco de cárie.

Palavras-chave: Cárie; Biofilme; Fluoreto; Desmineralização.

Akabane, S.T.F. *In situ* effect of the combination of fluoridated toothpaste and gel with sodium trimetaphosphate on enamel demineralization. 2017. 46 f. Trabalho de Conclusão de Curso – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2017.

# Abstract

Objective: This study evaluated the *in situ* effect of the association of low-F gel supplemented with sodium trimetaphosphate (TMP) and fluoride toothpaste on enamel demineralization. Methods: This crossover and double-blind study consisted of 5 phases of 7 days each. Volunteers (n = 12) wore palatal appliances containing 4 enamel blocks. The cariogenic challenge was performed with 30% sucrose (6×/day). Treatments were: no fluoride/TMP toothpaste - Placebo (PT); 1,100 µg F/g toothpaste (FT); FT + 4,500 µg F/g + 5%TMP gel (FT + TMP gel); FT + 9,000 µg F/g gel (FT + 9,000 gel) and FT + 12,300 µg F/g (FT + Acid gel). After topical application of treatments for 1 min, 2 blocks were removed for analysis of loosely bound fluoride ($CaF_2$), calcium (Ca), phosphorus (P) and firmly bound fluoride (F) formed in enamel. After the 7-day experimental periods, percentage of surface hardness loss (%SH), integrated subsurface hardness loss (ΔKHN), $CaF_2$, Ca, P, and F retained were determined. Moreover, biofilms were analyzed for F, Ca, P, and insoluble extracellular polysaccharide (EPS) concentrations. Results: FT + TMP gel promoted the lowest %SH and ΔKHN ($p < 0.001$). FT + Acid gel showed a higher concentration of $CaF_2$ formed/retained ($p < 0.001$). Similar values were observed for the Ca/P/F and EPS concentration in enamel and biofilm for all fluoride groups. Conclusion: The association of FT + TMP gel significantly reduced enamel

demineralization in situ. Clinical Significance: The association of treatments may be an alternative for patients with high caries risk.

Keywords: Caries; Biofilm; Fluoride; Demineralization.

# Figure legends

Figure 1. A Enamel block preparation. B Initial surface hardness analysis. C Acrylic appliance and TAF or brushing with the PT/FT. D *In situ* appliance and Cariogenic challenge. E Biofilm collected for Ca, P, F and EPS analysis. F Final surface hardness analysis. G Integrated loss of subsurface hardness (ΔKHN). H $CaF_2$ formed/retained analysis. I Fluoride, calcium and phosphorus in enamel analysis.

# Table legend

Table 1: Mean (SD) of variables of enamel analyzed according to the toothpastes/gels treatments

Table 2: Mean (SD) of variables of biofilm according to the toothpastes/gels treatments

# List of abbreviations

ºC Degrees Celsius

Ca Calcium

$Ca^{+2}$ Calcium ion

$CaF^{+}$ Calcium fluoride ion

$CaF_2$ Loosely bound fluoride

$CaHPO4^{0}$ Neutral Calcium Phosphate

F Fluoride

Fl Ionic fluoride

FT Total fluoride

g Gram

h Hour

HCl Hydrochloric acid

$HF^{0}$ Neutral hydrogen fluoride

KHN Knoop hardness unit

KOH Potassium hydroxide

l Liter

M Molar

mg Milligram

mg/g Milligram per gram

ml Milliliter

mm Millimeter

mol $L^{-1}$ Mol per liter

mol/kg Mol/kilograms

mV Millivolts

n Volunteers number

$Na^{+}$ Sodium Ion

NaF Sodium Fluoride

NaOH Sodium hydroxide

P Phosphor

pH Hydrogen potential

SD Standard deviation

SHi Initial surface hardness

SHf Final surface hardness

%SH Surface hardness loss

TISAB Total ionic strength adjuster cap

TMP Sodium trimetaphosphate

µg Microgram

µg/mm$^3$ Microgram per cubic millimeter

µL/mg Microliters/milligram

µg F/mL Fluoride microgram per milliliter

µg F/cm$^2$ Fluoride microgram per square centimeter

µm Micrometer

ΔKHN Integrated loss of subsurface hardness

# Summary

# 1. INTRODUCTION

Dental caries (DC) is a disease resulting from colonization of the enamel surface by microorganisms, especially S*treptococcus mutans*, which, by metabolizing fermentable carbohydrates, produce acids, which demineralize the dental surface [1]. Fluoride (F) has been the main agent used in DC prevention worldwide. The use of fluoride toothpaste (FT) is considered the main reason for the reduction in caries prevalence observed in the last decades [2,3].

Topical application of fluoride (TAF) is often used in prevention programs, as well as in patients who are at high risk of developing dental caries as a supporting measure for reduction of lesions [4]. When a product with high F concentration is placed in contact with the dental surface, a precipitate forms on the tooth in the form of calcium fluoride ($CaF_2$), which is covered by calcium, phosphate and saliva proteins, delaying the solubility of the compound, with which it operates as an F releasing agent [5], consequently, interfering with the dynamics of the de-remineralization process [6]. Also, products with high F concentration become toxic when indiscriminately ingested by children who do not exhibit complete development of complex sputum [7].

One of the effective strategies that could be used to reduce the risk of intoxication would be the reduction of F in its composition and supplementation with calcium and/or phosphate, since the remineralization process, although intensified by F, depends primarily on the presence of these ions in saliva [8,9]. Among the phosphate salts with anticariogenic activity, sodium trimetaphosphate (TMP) seems to be the most effective [10]. Danelon et al. [11,12] compared the effectiveness of low-fluoride gels (4,500 µg F/g) supplemented with TMP on the *in vitro* demineralization process and *in situ*

remineralization, demonstrating that the association was able to reduce demineralization and promote remineralization of tooth enamel similar to conventional gels (9,000 µg F/g and 12,300 µg F/g - Acid gel).

In situations of high caries risk, a combination of TAF and toothpaste has been suggested [13]. Ferreira et al. [14], in a double-blind, randomized controlled trial, demonstrated that TAF with acid gel associated with supervised brushing with FT is able to arrest initial carious lesions. However, Paes Leme et al. [15], in an *in situ* study, concluded that a single combination of TAF followed by daily use of FT did not decrease enamel demineralization when compared to the use of isolated FT. However, there is no consensus on the additional effect of association of fluoride methods for DC control.

Thus, the aim of this study was to evaluate the *in situ* effect of the association of low-F gel supplemented with sodium trimetaphosphate (TMP) and FT on enamel demineralization.

The null hypothesis was that the association of treatments (i.e. FT and TMP gel) would promote a similar reduction in demineralization in relation to FT treatment alone.

## 2. MATERIALS AND METHODS

### 2.1 Experimental Design

This study was approved by the Human Ethical Committee of Araçatuba Dental School, São Paulo State University, Brazil (Protocol: 50723315.1.0000.5420), and all participants read and signed an informed consent form prior to study onset. This crossover double-blind study was conducted in 5 phases of 7 days each. The sample size of volunteers was based on a previous study [16], considering the

primary outcome from surface and cross-sectional hardness analysis, the mean difference between groups (30 and 1,300, respectively), standard deviation (20 and 9,000, respectively), an α-error of 5%, and a β-error of 20%. Volunteers (n=12) aged 20-30 years, who were in good general and oral health [17] wore palatal appliances initially containing 4 enamel blocks (APPENDIX A), selected by initial surface hardness (SHi). The cariogenic challenge was performed with 30% sucrose (6×/day). Treatments were: no fluoride/TMP toothpaste - Placebo (PT); 1,100 µg F/g toothpaste (FT); FT + 4,500 µg F/g + 5%TMP gel (FT + TMP gel); FT + 9,000 µg F/g gel (FT + 9,000 gel) and FT + 12,300 µg F/g (FT + Acid gel). After topical application of treatments for 1 min, 2 blocks were removed for analysis of loosely bound fluoride ($CaF_2$), calcium (Ca), phosphorus (P) and firmly bound fluoride (F) formed in enamel. After the 7-day experimental periods, the percentage of surface hardness loss (%SH), integrated subsurface hardness loss (ΔKHN), $CaF_2$, Ca, P, and F retained were determined. Moreover, biofilms were analyzed for F, Ca, P, and insoluble extracellular polysaccharide (EPS) concentrations. Data were analyzed using one-way and two-way ANOVA, followed by Student–Newman–Keuls test ($p < 0.001$).

## 2.2 Gel/toothpaste formulation and fluoride/pH assessment

An experimental gel with neutral pH was prepared in laboratory using the following ingredients: carboxymethyl cellulose (Synth, Diadema, São Paulo, Brazil), sodium saccharin (Vetec, Duque de Caxias, Rio de Janeiro, Brazil), glycerol (Merck, Darmstadt, Germany), peppermint oil (Synth), and water. Fluoride (NaF; Merck) was added to the gel at concentrations of 4,500, or 9,000 µg F/g. Subsequently, TMP (Sigma-Aldrich Co., St. Louis, MO, USA) was added at 5%

concentration to the gel with F concentrations of 4,500 µg F/g. A commercial acid gel was used as positive control (12,300 µg F/g, Acid gel, pH=4.5, DFL Indústria e Comércio S.A., Rio de Janeiro, RJ, Brazil). In addition, toothpastes without F/TMP (PT), as well as with 1,100 µg F/g (FT: without TMP, NaF: Merck, CAS 7681-49-4, Germany) were prepared. The F concentration in the gels and toothpastes was determined using a F ion-specific electrode (9609 BN; Orion Research Inc., Beverly, MA, USA) attached to an ion analyzer (Orion 720 A+; Orion Research Inc.) and calibrated with standards containing 0.125–2.000 µg F/g. The pH levels of gels and toothpaste were determined using a pH electrode (2A09E, Analyser, São Paulo, Brazil) calibrated with standard pH levels of 7.0 and 4.0 [11,12] (APPENDIX B).

2.3 Clinical phase of experimental groups

The palatal appliance was prepared in acrylic resin (Jet, Articles Classic Odontológico, São Paulo, Brazil), and initially four enamel blocks were fixed, with a different device used in each phase of the experiment. Next, prophylaxis was performed using a non-fluoride paste and rubber cup. An amount of the gel product sufficient to fill stock trays to approximately one-third of their capacity was placed. Teeth were air-dried, the trays were placed over them, and the subject was instructed to close the jaws with the trays in contact for 1 minute [12]. At the same time, treatment of the bovine enamel blocks was performed outside the oral cavity. Immediately after topical application or brushing with the PT/FT, two blocks of each device were removed for determination of $CaF_2$, Ca, P and F formed in enamel. Next, in order to allow biofilm accumulation on the enamel blocks, a piece of plastic mesh was fixed to the acrylic appliance, leaving a 1-mm space from the block surface [da Camara et al., 2015]. To provide a cariogenic challenge, the volunteers were instructed to remove the

device and drip 30% sucrose solution (Sucrose, Synth, Diadema, Brazil) onto each enamel block 6x/day at predetermined times (8:00 am, 11:00 am, 2:00 pm, 5:00 pm, 7:00 pm, and 9:00 pm) [18]. The appliances were used 24 h/day, and the volunteers brushed their natural teeth 3x/day (07:30, 12:30, and 21:30 hrs), during the habitual oral hygiene routine for 2 min. During 7-day pre-experimental period and washout periods, the volunteers brushed their teeth with 1,100 µg F/g toothpaste (APPENDIX G).

2.4 Hardness Analysis

Enamel surface hardness was measured before (SHi)and after (SHf) experiment using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA) and a Buehler OmniMet software (Buehler) [11], followed by calculation of the percentage of surface hardness loss (%SH=[(SHf-SHi)/SHi]*100). For the cross-sectional hardness measurements, the enamel blocks were longitudinally sectioned through their center and embedded in acrylic resin with the cut face exposed and gradually polished. A sequence of 14 indentations was created 100 µm apart 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) and the software Buehler OmniMet (Buehler, Lake Bluff, USA) with a Knoop diamond indenter under a 5 g load for 10 s [Danelon et al., 2013]. Integrated hardness (KHN x µm) of the lesion into sound enamel was calculated by the trapezoidal rule (GraphPad Prism, version 3.02) and subtracted from the integrated hardness for sound enamel to obtain the integrated area of the subsurface regions in enamel, which was named integrated loss of subsurface hardness (ΔKHN; KHN x µm) [12] (APPENDIX C).

## 2.5 Loosely bound fluoride ($CaF_2$) formed and retained analysis in enamel

A digital caliper (Mitutoyo CD-15B, Mitutoyo Corporation, Japan) was used to measure the surface area of enamel blocks (n = 240) [Danelon et al., 2013]. Assessment of loosely bound (alkali-soluble fluoride – $CaF_2$ formed/retained) fluoride uptake by enamel was performed following the methodology of Caslavska et al. [19]. The surface of each specimen, except enamel, was coated with wax. Then, they were immersed in 0.5 mL of KOH 1.0 mol $L^{-1}$ solution for 24 h under constant agitation. The solution was neutralized and buffered with 0.5 mL of TISAB II modified HCl specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an ion analyzer (Orion 720A+, Thermo Scientific, Beverly, MA, USA) previously calibrated with standards 4.00 to 64.00 µg F/mL (100 ppm F, Orion 940907) that were used for the readings. The data obtained in mV were converted to µg F/$cm^2$ using Microsoft Excel (APPENDIX D).

## 2.6 Firmly bound fluoride, calcium and phosphorus formed and retained analysis in enamel

Firmly bound fluoride (F) formed/retained present in enamel was determined as described by Weatherell et al. [20], as modified by Alves et al. [21]. Self-adhesive polishing discs (diameter, 13 mm) and 400-grit silicon carbide (Buehler) were fixed to the bottom of polystyrene crystal tubes (J-10; Injeplast, Sao Paulo, SP, Brazil). One layer of enamel (50.4 ± 0.4 µm) was removed from each block, after the addition of 0.5 ml HCl 1.0 mol $L^{-1}$, and these were kept under constant stirring for 1 hour. For analysis of F formed/retained, specific electrode 9409BN (Thermo Scientific, Beverly, MA, USA) and microelectrode reference (Analyser, São Paulo, Brazil) coupled to an

ion analyzer (Orion 720A+, Thermo Scientific, Beverly, MA, USA) were used. The results were expressed in µg/mm³. Ca formed/retained analysis was performed using the Arsenazo III colorimetric method [Fiske and Subbarow, 1925]. The absorbance readings were recorded at 650 nm, using a plate reader (PowerWave 340, Biotek, Winooski, VT, USA). P formed/retained was measured according to Fiske and Subbarow [22], and the absorbance readings were recorded at 660 nm. The results were expressed as µg/mm³ (APPENDIX E).

## 2.7 Analysis of dental biofilm composition

The biofilm formed on enamel was collected and stored in microcentrifuge tubes. The biofilm samples were dried in vacuum over P pentoxide for 12h at room temperature. F was analyzed using an ion specific electrode (Orion 9409 BN) and a potentiometer (Orion 720 Aplus). The Ca concentration was analyzed by a colorimetric test [23]. The P concentration was measured using a colorimetric method [22]. EPS was extracted by adding 1.0 mol $L^{-1}$ NaOH (10 µL/mg dry weight) to the biofilm [24,25]. The amount of EPS was determined using the phenol-sulfuric acid method [26]. The results were expressed as moles/kilograms (F, Ca, and P) and milligrams/grams (EPS) dry weight (APPENDIX F).

## 2.8 Statistical analysis

SigmaPlot 12.0 software was used for statistical analysis, and the significance level was set at 5%. The statistical power calculated was 85%, considering all the differences between groups of each outcome. Data from the dental biofilm analysis (Ca, P, F and EPS content) and enamel analysis (%SH, ΔKHN, $CaF_2$, Ca, P and F formed/retained) exhibited a normal (Shapiro–Wilk) and homogeneous (Bartlet) distribution, and were therefore subjected to

one-way and two-way repeated measures ANOVA, followed by the Student–Newman– Keuls test.

## 3. RESULTS

Mean (SD) of the total (TF) and ionic fluoride (IF) concentrations (µg/g) in the PT, FT were respectively: 10.5 (0.9); 10.0 (1.2); 1189.0 (33.1); 1102.4 (28.5). Ionic fluoride (IF) concentrations (µg/g) in the TMP gel, 9000 gel, Acid gel were respectively: 4,514.6 (29.7); 9,308.2 (22.5); 12,497.1 (12.3). The mean pH value from the toothpastes was 7.3 (0.1) ranging from 7.2 to 7.4. The pH of neutral gels was 7.2 [0.2], ranging from 7.0 to 7.4. All gels had neutral pH, except for the Acid gel.

The use of FT + TMP gel resulted in 58% and 67% decrease in %SH in comparison with FT + Acid gel and FT ($p < 0.001$) (Table 1) (APPENDIX H). No significant difference was observed between groups FT + 9000 gel and FT + acid gel ($p=0.678$). In addition, the capacity to reduce the lesion body (ΔKHN) was ~28% higher with FT + TMP gel ($p < 0.001$) when compared to FT; FT + 9,000 gel and FT + Acid gel showed similar values ($p > 0.001$) (Table 1) (APPENDIX H).

A higher concentration ($p < 0.001$) of $CaF_2$ formed after topical application of gels/toothpaste was observed in the FT + Acid gel when compared to other groups ($p < 0.001$). For the other groups, the concentration of $CaF_2$ formed was FT + 9000 gel > FT + TMP gel > FT > PT ($p < 0.001$). After 7 days of demineralization, the $CaF_2$ retained was reduced in all groups ($p < 0.001$).

Similar values of F and Ca formed/retained in enamel was observed for all groups, except for the PT group, which showed a lower concentration ($p < 0.001$). No significant difference was observed between groups regarding enamel P formed/retained concentrations ($p > 0.001$).

As for the biofilm composition, all treatments showed the highest retention of Ca and F when compared with PT ($p < 0.001$) (Table 2) (APPENDIX H), while the P values were similar for the treatments ($p =$

0.052). All treatments showed lowest values for alkali-soluble EPS concentration when compared with PT ($p < 0.001$). Similar concentrations were observed for the FT, FT + TMP gel, FT + 9000 gel and FT + Acid gel treatments ($p = 0.709$), which were significantly lower than that of the PT group ($p < 0.001$).

## 4. DISCUSSION

The application of high concentration fluoride products in association with fluoride toothpaste is recommended for patients at high risk of developing dental caries. Thus, it is important to perform *in vitro* and *in situ* studies to evaluate the anticariogenic potential of new gel formulations prior to clinical study. The results of this study indicate that the association of FT + TMP gel treatments significantly decreased enamel demineralization, presenting better results when compared to conventional gels (9000 gel and Acid gel). Thus, the null hypothesis was rejected.

The choice of 5% TMP concentration was based on the *in situ* study of Danelon et al. [12], which demonstrated that supplementation of 4,500 µg F/g gel with 5%TMP promoted similar effect in enamel remineralization when compared to Acid and 9000 gels. In the present study, analysis of the surface hardness loss (%SH) and integrated subsurface hardness loss (ΔKHN) was superior for the supplemented group when compared to conventional gels. Lagerweij and Ten Cate [27] evaluated the remineralization of *in situ* enamel lesions after daily applications of high concentration gel (12,300 µg F/g, Acid gel) associated with FT (1,450 µg F/g). There was an increase in mineral volume in 27% for FT associated with acid gel compared to 11% for FT, concluding that the association increased the F incorporation in lesions for the FT + Acid gel group, compared

only to FT. The good results of this study can also be related to the frequency of gel application and consequently to the greater incorporation of F in enamel. In addition, Paes Leme et al. [15] evaluated *in situ* the association of FT + Acid gel on enamel demineralization, showing no reduction in %SH and ΔKHN when compared to the FT regimen. Differently from the findings of Paes Leme et al. [15], our results showed (%SH) statistical difference between the FT, FT + 9000 gel and FT + Acid gel groups; however, the use of FT daily did not increase the effect on demineralization between the FT + 9000 gel and FT + Acid gel groups ($p > 0.001$). A possible explanation for the lack of differences between these groups (i.e. FT, FT + 9000 gel and FT + Acid gel) for the ΔKHN data is due to the fact that the toothpaste was used daily, with the gel applied only once. However, for the results of TMP, the characteristics and properties of this salt can explain the best results obtained with its use.

Based on previous studies [11,12] and confirmed by the present results, it is known that the association of TMP and F, in ideal proportions, reduces the mineral loss, and its effect can be explained by the adsorption of TMP in enamel, even with the presence of biofilm. When TMP is adsorbed on enamel, this interaction may alter the selective permeability and diffusion of ions to the interior [11,12,28]. Thus, TMP possibly retains positively charged ions of $CaF^{+}$ and $Ca^{2+}$, replacing the $Na^{+}$ of the cyclic structure and promoting reduction in acid diffusion [28]. In the case of de-remineralization process, there is formation and flow of neutral species ($CaHPO_4^0$ and $HF^0$) in enamel, whose diffusion coefficient is a thousand times higher than their charged counterparts [29]. The treatment F + TMP gel promoted reduction in the lesion (ΔKHN) by ~ 28% when compared to the FT + 9000 gel and by 25% in relation to the FT + Acid gel (Table 1). The more

pronounced effect of FT + TMP gel on ΔKHN suggests that, under clinical conditions, a subsurface lesion would take longer to develop compared to conventional gels (9000 gel and Acid gel). This is extremely significant from a clinical perspective, considering that a cavity could take longer to occur, or even would not occur when using the formulation tested in the present study. The use of TMP in this study and the good results are also in agreement with the study of Danelon et al. [11], demonstrating that the gel supplemented with TMP presents great potential in reducing enamel demineralization.

The effect on reducing enamel demineralization by topical products with high F concentration is related to the deposition of $CaF_2$ to enamel and its dissolution during the *in situ* experiment [30], which is concentration- and pH-dependent [5]. The concentration of $CaF_2$ measured immediately after gel application or brushing with toothpaste ($CaF_2$ formed) increases with the F concentration present in the products, the highest value being observed for the FT + Acid gel group (Table 1), followed by the FT + 9000 gel group. Still, the remaining concentration of $CaF_2$ (Retained) for all groups except for PT was higher (Table 1), suggesting that the effect of high F concentration gels on enamel demineralization is prolonged, since a significant amount is observed even under a severe cariogenic challenge. Studies have shown that the presence of F can reduce enamel demineralization if sucrose consumption is not greater than 6x/day but is not able to reduce demineralization in major challenges [31]. Thus, the presence of F in the oral environment may interfere with the caries development process by inhibiting demineralization. As TMP does not benefit the precipitation of $CaF_2$ on enamel [11], this effect should reduce the obstruction of pores on the enamel surface, facilitating the diffusion of ions into enamel. Nevertheless, TMP should

be able to retain F and Ca during treatment to produce a direct impact on the incorporation of F and Ca in enamel (Table 1). The hypothesis rests on the possibility of TMP binding to ions $Ca^{2+}$ and $CaF^{+}$.

With regard to the effects of treatments on the biofilms formed, the statistically significant effect of the FT (Table 2) and the higher F concentration found after fluoridated toothpaste treatment in comparison with PT may be attributed to the daily use of F toothpaste. In addition, F is probably firmly bound to the dental plaque structure. The statistically significant effect of fluoride gels application (Table 2) and the higher F concentration in the biofilm from the groups receiving fluoride gels application compared with PT may be attributed to the release of $CaF_2$ formed on enamel by the pre-treatment [32,33]. Nevertheless, when pre-treatment with fluoride gels was combined with daily use of FT, the F concentration in dental plaque was not statistically higher than treatment with FT alone (Table 2).

The use of FT + fluoride gels or FT alone promoted significant changes in biofilm composition. It is noteworthy that increase in F concentrations in the biofilm is often correlated with Ca concentration [34,35,36] as observed for all treatment groups when compared to the PT group. In the fluoride treatment groups, the biofilm served as a reservoir of F and Ca ions during the high cariogenic challenge, which could contribute to minimizing the mineral loss from the enamel surface, being greater for the FT + TMP gel group.

Regarding alkali-soluble EPS concentration, our results showed an expressive reduction for fluoride treatments. The correlation between the increase of F/Ca and the reduction of EPS is a point that must be considered. TMP could act on the biofilm indirectly, since high

F and Ca concentrations in biofilm alter the bacterial metabolism and biological activity as well as EPS formation [37]. These data are based on an *in situ* demineralization protocol, and we suggest the accomplishment of other similar studies with biofilm accumulation and different cariogenic challenges (frequency x exposure to sucrose), to simulate patients with different caries activities. The greater inorganic composition of saliva and plaque fluid, *in situ* remineralization protocol, and *in vivo* studies would be of great importance to confirm our findings.

## 5. CONCLUSION

We conclude that the association of FT + TMP gel treatments was more efficient in reducing enamel demineralization *in situ* when compared to conventional treatments (i.e. daily use of fluoride toothpaste and/or association with conventional fluoride gels). Thus, it can be an alternative for patients with high levels of carious lesions.

## 6. ACKNOWLEDGMENTS

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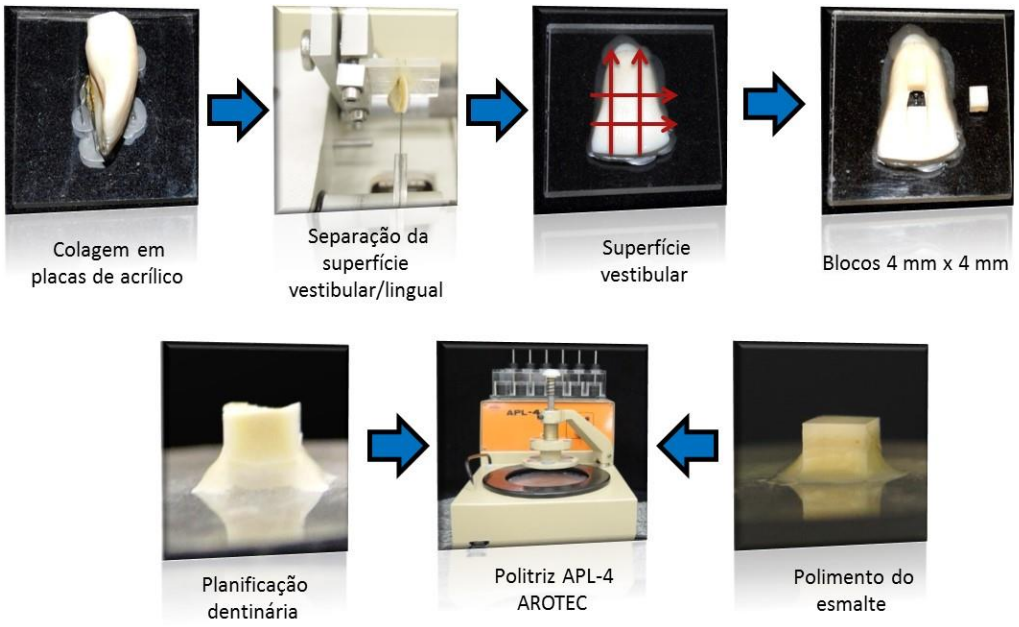
# 8. APPENDIX

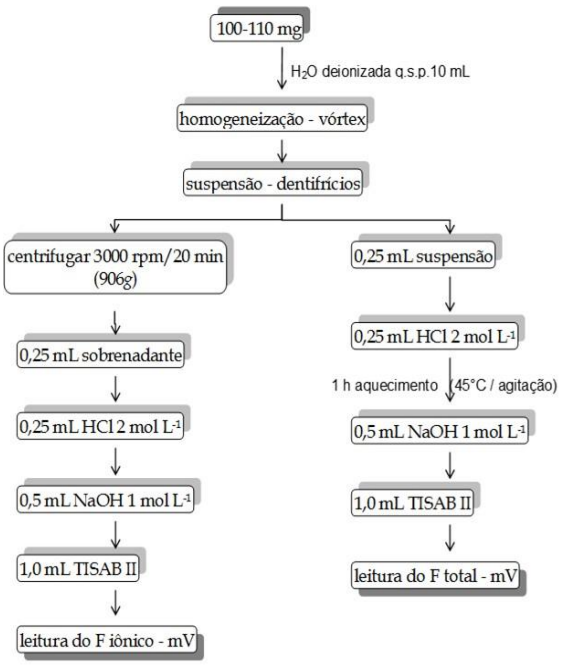
## APPENDIX A

### PREPARATION OF ENAMEL BLOCKS



# APPENDIX B

## TOOTHPASTE/GEL FORMULATION AND FLUORIDE AND pH ASSESSMENT




- Eletrodo específico para F; Orion 9409-BN
- Microeletrodo de referência
- Analisador de íons

# APPENDIX **C**

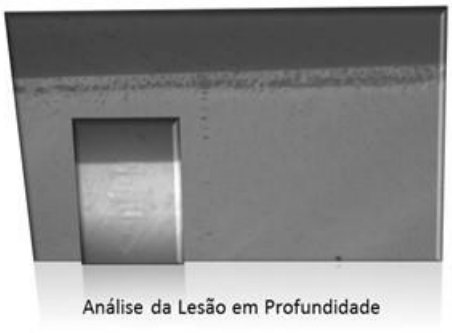
## HARDNESS ANALYSIS

Secção dos blocos de esmalte no sentido longitudinal

Embutimento dos blocos de esmalte com resina acrílica

Aspecto final dos blocos de esmalte

Microdurômetro Buehler
Carga 5g; Tempo 10 segundos



Análise da Lesão em Profundidade

# APPENDIX **D**

## LOOSELY BOUND FLUORIDE ($CAF_2$) FORMED AND RETAINED ANALYSIS IN ENAMEL

- **Agitadora TECNAL- TE - 420**

- Eletrodo específico para F; Orion 9409-BN
- Microeletrodo de referência
- Analisador de íons

# APPENDIX **E**

## FIRMLY BOUND FLUORIDE, CALCIUM AND PHOSPHORUS FORMED AND RETAINED ANALYSIS IN ENAMEL

Micrômetro eletrônico digital com saída acoplado a uma base de microscópio e blocos fixados

Desgaste 50 µm Lixa 400 (CARBIMET – BUEHLER)

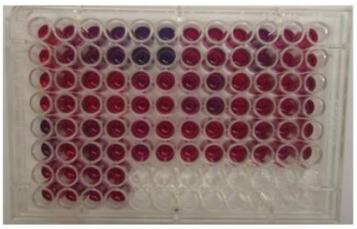
0,5 mL de HCl 1,0 mol/L

Agitação por 1 hora

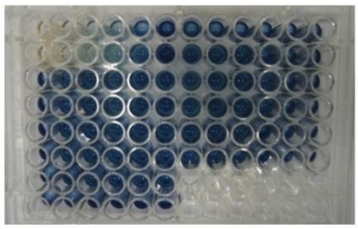
**0,3 mL da amostra + 0,3 mL de Tisab II**

**Modificado com NaOH**


Espectrofotômetro de microplaca EONC, Biotek, USA



**Cálcio** - Método colorimétrico Arsenazo III, Fiske e Subbarow, 1925.



**Fósforo -** Método colorimétrico Fiske e Subbarow, 1925.

# APPENDIX **F**

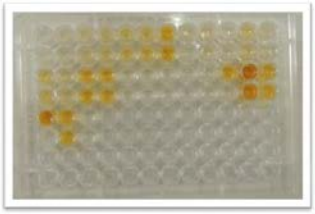
## ANALYSIS OF DENTAL BIOFILM COMPOSITION

**Biofilme Coletado**

**Fluoreto**- Eletrodo específico (Orion 9409) - Eletrodo de referência (Orion 900200) - Analisador de íons (Orion 720A+).

**Cálcio** - Método colorimétrico Arsenazo III, Vogel et al., 1983.

**Fósforo** - Método colorimétrico Fiske e Subbarow, 1925.



**EPS** - Método Fenol-Sulfúrico Dubois, 1956.

APPENDIX **G**

**FIGURE**

Figure1

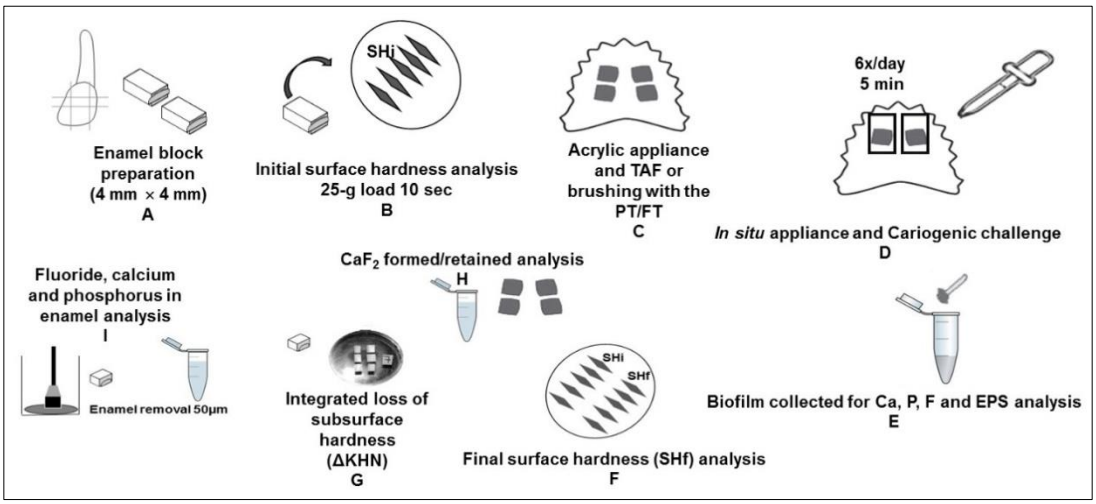



Figure 1. A Enamel block preparation. B Initial surface hardness analysis. C Acrylic appliance and TAF or brushing with the PT/FT. D In situ appliance and Cariogenic challenge. E Biofilm collected for Ca, P, F and EPS analysis. F Final surface hardness analysis. G Integrated loss of subsurface hardness (ΔKHN). H $CaF_2$ formed/retained analysis. I Fluoride, calcium and phosphorus in enamel analysis.

# APPENDIX **H**

## TABLES

Table 1: Mean (SD) of variables of enamel analyzed according to the toothpastes/gels treatments

| Analysis | Treatments | | | | |
|---|---|---|---|---|---|
| | PT | FT | FT + TMP gel | FT + 9000 gel | FT + Acid gel |
| [1] %SH | -58.3$^{a}$ | -27.4$^{b}$ | -9.15$^{c}$ | -20.7$^{d}$ | -21.7$^{d}$ |
| (KHN) | (6.3) | (5.0) | (6.7) | (8.0) | (7.3) |
| [2] ΔKHN | 6413.8$^{a}$ | 2753.9$^{b}$ | 1992.1$^{c}$ | 2768.6$^{b,d}$ | 2648.5$^{b,d}$ |
| (ΔKHN) | (1265.7) | (568.7) | (618.6) | (604.9) | (646.1) |
| [3] F Formed | 0.1$^{a,A}$ | 0.4$^{b,A}$ | 0.3$^{b,A}$ | 0.3$^{b,A}$ | 0.3$^{b,A}$ |
| (µg/mm$^3$) | (0.1) | (0.1) | (0.1) | (0.1) | (0.1) |
| [3] F Retained | 0.2$^{a,A}$ | 0.3$^{b,A}$ | 0.4$^{b,A}$ | 0.5$^{b,B}$ | 0.4$^{b,B}$ |
| (µg/mm$^3$) | (0.1) | (0.1) | (0.3) | (0.2) | (0.2) |
| [4] Ca Formed | 379.2$^{a,A}$ | 457.9$^{a,A}$ | 441.3$^{a,A}$ | 479.5$^{a,A}$ | 433.9$^{a,A}$ |
| (µg/mm$^3$) | (78.2) | (105.1) | (86.1) | (104.5) | (157.5) |
| [4] Ca Retained | 198.7$^{a,B}$ | 274.0$^{b,B}$ | 326.6$^{b,B}$ | 278.5$^{b,B}$ | 286.8$^{b,B}$ |
| (µg/mm$^3$) | (36.7) | (53.8) | (98.6) | (87.1) | (102.1) |
| [5] P Formed | 215.9$^{a,A}$ | 223.1$^{a,A}$ | 228.5$^{a,A}$ | 223.9$^{a,A}$ | 218.9$^{a,A}$ |
| (µg/mm$^3$) | (52.4) | (73.3) | (44.7) | (62.4) | (64.3) |
| [5] P Retained | 147.6$^{a,B}$ | 179.7$^{b,A}$ | 218.9$^{b,A}$ | 158.8$^{a,B}$ | 186.8$^{a,A}$ |
| (µg/mm$^3$) | (33.1) | (54.4) | (48.2) | (54.1) | (55.8) |
| [6] $CaF_2$ Formed | 0.5$^{a,A}$ | 1.3$^{b,A}$ | 3.9$^{c,A}$ | 14.1$^{d,A}$ | 29.2$^{e,A}$ |
| (µg/cm$^2$) | (0.1) | (0.1) | (1.9) | (5.2) | (5.9) |
| [6] $CaF_2$ Retained | 0.3$^{a,B}$ | 0.8$^{b,B}$ | 1.8$^{c,B}$ | 2.6$^{d,B}$ | 6,3$^{e,B}$ |
| (µg/cm$^2$) | (0.1) | (0.3) | (0.9) | (1.4) | (3.9) |

Lowercase letters indicate differences between groups in each analysis and capital letters indicate the differences between formed and retained (Student-Newman Keuls's test; $p < 0.001$).

[1]%SH: percentage of surface hardness loss - KHN
[2]ΔKHN: integrated loss of subsurface hardness - KHN x µm
[3]F: firmly bound fluoride in enamel formed/retained - µg/mm$^3$
[4]Ca: calcium in enamel - µg/mm$^3$
[5]P: phosphorus in enamel - µg/mm$^3$
[6]$CaF_2$: loosely bound fluoride in enamel formed/retained - µg F/cm$^2$

Table 2: Mean (SD) of variables of biofilm according to the toothpastes/gels treatments

| Analysis | Treatments | | | | |
|---|---|---|---|---|---|
| | PT | FT | FT + TMP gel | FT + 9000 gel | FT + Acid gel |
| F (mol/kg) | 2.49E-04[a] (3.54E-05) | 2.93E-03[b] (3.85E-03) | 1.17E-03[b] (1.26E-03) | 1.22E-03[b] (1.10E-03) | 1.39E-03[b] (1.74E-03)[b] |
| Ca (mol/kg) | 8.47E-02[a] (1.57E-02) | 1.33E-01[b] (4.89E-02) | 1.39E-01[b] (3.23E-02) | 1.29E-01[b] (3.94E-02) | 1.39E-01[b] (3.23E-02) |
| P (mol/kg) | 3.90E-02[a] (1.75E-02) | 7.12E-02[a] (4.09E-02) | 6.00E-02[a] (3.00E-02) | 5.49E-02[a] (3.27E-02) | 6.00E-02[a] (3.36E-02) |
| EPS (mg/g) | 2.23[a] (0.79) | 1.62[b] (0.57) | 1.33[b] (0.63) | 1.29[b] (0.75) | 1.25[b] (0.81) |

Distinct superscript letters indicate statistical significance among the toothpastes/gels for each analysis (Student Newman-Keuls's test; $p < 0.001$). Values between parentheses indicate the standard deviation of the mean.

# APPENDIX I

## SUBMISSION OF SCIENTIFIC ARTICLE

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Manuscript Draft

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Title: In situ effect of the combination of fluoridated toothpaste and gel with sodium trimetaphosphate on enamel demineralization

Article Type: Full Length Article

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