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Campus de Araçatuba

LETÍCIA CABRERA CAPALBO

ANÁLISE *IN VITRO* DA CAPACIDADE DE SOLUÇÕES CONTENDO FLUORETO E/OU HEXAMETAFOSFATO NA REMINERALIZAÇÃO DA DENTINA

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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. Assoc. Juliano Pelim Pessan

Coorientador: Prof. Tit. Alberto Carlos Botazzo Delbem

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LETÍCIA CABRERA CAPALBO

Nascimento	05/08/1994 Araçatuba-SP
Filiação	Marcos Roberto Capalbo Lúcia Sanchez Cabrera Capalbo
2012/2016	Curso de graduação em Odontologia na Universidade Estadual Paulista “Júlio de Mesquita Filho” - UNESP
2016/2019	Especialização em Ortodontia – FACSETE (NEC)
2019/Atual	Curso de Pós-Graduação em Ciência Odontológica – área Saúde Bucal da Criança, nível de Mestrado, na Faculdade de Odontologia de Araçatuba- UNESP
Associações	CROSP- Conselho Regional de Odontologia de São Paulo SBPqO- Sociedade Brasileira de Pesquisa Odontológica IADR – International Association for Dental Research

Dedicatória

Dedicatória

Aos meus pais, Marcos e Lúcia:

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Epígrafe

Epígrafe

"Feliz aquele que transfere o que sabe e aprende o que ensina."

Cora Coralina

"Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota."

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Resumo

Capalbo, LC. **Análise *in vitro* da capacidade de soluções contendo fluoreto e/ou hexametafosfato na remineralização da dentina.** 2020. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

O objetivo do presente estudo foi avaliar a capacidade de soluções contendo HMP e F, sozinhos ou em associação, em induzir a remineralização dentinária em um protocolo *in vitro*. Blocos de dentina radicular bovina (4 × 6 cm, *n* = 100) foram preparados e submetidos à indução de lesões de cárie artificiais em dois terços da superfície; cada bloco foi utilizado como seu próprio controle. Em seguida, os blocos foram divididos em 10 grupos experimentais (*n*=10/grupo), de acordo com as soluções a serem testadas: (1) Placebo (sem F ou HMP); (2) 0,5% HMP; (3) 0,75% HMP; (4) 1% HMP; (5) 250 ppm F; (6) 500 ppm F; (7) 1100 ppm F; (8) 250 ppm F + 0,5% HMP; (9) 500 ppm F + 0,75% HMP e (10) 1100 ppm F + 1% HMP. Os blocos foram tratados por um minuto, duas vezes ao dia com as respectivas soluções, e submetidos a uma ciclagem de pH durante 7 dias. Em seguida, foram determinadas a porcentagem de recuperação da dureza de superfície (%RDS) e a área integrada da lesão de subsuperfície (ΔKHN). Os dados foram submetidos a ANOVA e teste de Fisher LSD ($p<0.05$). Uma relação dose-reposta foi observada entre as concentrações de F nas soluções sem HMP e as variáveis %RDS e ΔKHN; quanto às soluções contendo apenas HMP, uma relação dose-resposta foi observada somente para ΔKHN. Em relação à %RDS, os grupos placebo e 0,5% HMP, e os grupos 0,75% e 1% HMP não apresentaram diferenças significativas entre si. Quando associado ao F, o HMP aumentou a capacidade de remineralização da superfície e subsuperfície dentinária, visto que os grupos contendo F + HMP apresentaram resultados significativamente melhores em relação aos grupos contendo F sozinho. Em acréscimo, a solução contendo 250 ppm F + 0,5% HMP promoveu um efeito remineralizador semelhante à solução contendo 500 ppm F. Já em relação à ∆KHN, diferenças estatísticas foram observadas entre todos os grupos na área tratada, sem diferenças significativas quanto às áreas controle e desmineralizada. Os resultados permitem concluir que a adição de HMP às soluções fluoretadas potencializou o efeito destas sobre a

remineralização das lesões artificiais de cárie em dentina, tanto na superfície quanto em profundidade.

Palavras–chave: Dentina. Flúor. Fosfato. Remineralização Dentária.

Abstract

Abstract

Capalbo, LC. ***In vitro* analysis of the capacity of solutions containing fluoride and/or hexametaphosphate on the remineralization of dentin.** 2020. Dissertação (Mestrado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2020.

The present study aimed to investigate the ability of solutions containing HMP and F, alone or in association, in promoting dentin remineralization in an *in vitro* protocol. Bovine root dentin blocks (4 × 6 cm, *n* = 100) were prepared, and caries-like lesions were induced in two thirds of the surface; each block served as its own control. Then, blocks were divided into 10 experimental groups (*n* = 10 / group), according to the solutions to be tested: (1) Placebo (without F or HMP); (2) 0.5% HMP; (3) 0.75% HMP; (4) 1% HMP; (5) 250 ppm F; (6) 500 ppm F; (7) 1100 ppm F; (8) 250 ppm F + 0.5% HMP; (9) 500 ppm F + 0.75% HMP and (10) 1100 ppm F + 1% HMP. Specimens were treated for one minute, twice a day with the respective solutions, and subjected to a pH-cycling regime for 7 days. Next, the percentage of the superficial hardness recovery (%SHR) and integrated loss of subsurface hardness (ΔKHN) were determined. Data were submitted to ANOVA and Fisher LSD's test ($p<0.05$). A dose-response relationship was observed between F concentrations in solutions without HMP and the variables %SHR and ΔKHN; as for the solutions containing HMP alone, a dose-response relationship was only observed for ΔKHN. Regarding %SHR, no significant differences were observed Placebo and 0.5% HMP groups, nor between 0.75% and 1% HMP groups. When associated with F, HMP was shown to increase the remineralizing capacity of the solutions both at the surface and the subsurface of dentin specimens, since the groups containing F + HMP showed significantly superior results compared to groups containing F alone. In addition, the solution containing 250 ppm F + 0.5% HMP promoted a remineralizing effect similar to that containing 500 ppm F. Regarding ∆KHN, significant differences were observed among all groups in the treated area, while no significant differences were observed among the groups in the control and demineralized areas. The results allowed the conclusion that the addition of

Abstract

HMP to fluoridated solutions significantly enhanced their remineralizing potential on dentin artificial caries lesions, both at the surface and in depth.

Keywords: Dentin. Fluoride. Phosphate. Tooth Remineralization.

Lista de abreviaturas e símbolos

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LISTA DE ABREVIATURAS E SÍMBOLOS

ANOVA	Analysis of Variance/*Análise de Variância*
Ca^{+2}	Calcium/*Cálcio*
$CaCl_2$	Calcium Chloride/*Cloreto de cálcio*
CaF_2	Calcium Fluoride/*Fluoreto de cálcio*
$Ca(OH)_2$	Calcium Hydroxide/*Hidróxido de cálcio*
$Ca(NO_3)_2$	Calcium Nitrate/*Nitrato de cálcio*
°C	Degrees Celsius/*Graus Celsius*
F	Fluoride/*Fluoreto*
g	Gram/*Grama*
H_2O	Water/*Água*
HMP	Sodium hexametaphosphate/*Hexametafosfato de sódio*
KCL	Potassium Chloride/*Cloreto de potássio*
KHN	Knoop Hardness Number/*Número de Dureza Knoop*
KH_2PO_4	Potassium dihydrogenphosphate/*Fosfato Monopotássico*
L	Liter/*Litro*
Log_{10}	Logarithm, base 10/*Logaritmo na base 10*
mL	Milliliter/*Mililitro*
M	Molar
Mm	Millimolar/*Mili Molar*
mm	Millimeter/*Milímetro*
mg	Milligram/*Miligrama*
mmol	Milimol
MMPs	Matrix Metalloproteinases/*Metaloproteinases da matriz*
NaF	Sodium Fluoride/*Fluoreto de sódio*
NaH_2-PO_4	Sodium dihydrogen phosphate*/Fosfato Monossódico*
NaN_3	Sodium azide/*Azida de sódio*
µm	Micrometer/*Micrometro*
µM	Micromolar/*Micro molar*
p	Probability/*Probabilidade*
pH	Hydrogenionic Potential /*Potencial Hidrogeniônico*
PO_4^{-3}	Phosphate/*Fosfato*
Ppm	Parts per million/*Partes por milhão*

Listas de abreviaturas e símbolos

SD	Standard Deviation/*Desvio padrão*
SH	Surface hardness
s	Seconds/*segundos*
TISAB	Total Ionic Strenght Adjustment Buffer/*Tampão de Ajuste da Força Iônica Total*
TMP	Sodium Trimetaphosphate/*Trimetafosfato de sódio*
UNESP	São Paulo State University/*Universidade Estadual Paulista*
ΔKHN	Integrated subsurface hardness/*Dureza integrada de subsuperfície*
%SHR	Percentage of surface hardness recovery
%RDS	Porcentagem de recuperação de Dureza de superfície

Sumário

SUMÁRIO

In vitro analysis of the capacity of solutions containing fluoride and/or hexametaphosphate on the remineralization of dentin

LC Capalbo[1], ACB Delbem[1], GP Nunes[1], MAR Buzalaf[2], JP Pessan[1]

[1]Department of Preventive and Restorative Dentistry, School of Dentistry, Araçatuba, São Paulo State University (UNESP), Araçatuba, SP, Brazil
[2]Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Bauru, SP, Brazil

Short title: Solutions containing F and/or HMP on dentin remineralization.

Keywords: Dentin. Fluoride. Phosphate. Tooth Remineralization. Dental caries.

Corresponding author:
Juliano Pelim Pessan
School of Dentistry, Araçatuba, São Paulo State University (UNESP)
Department of Preventive and Restorative Dentistry
Rua José Bonifácio 1193
16015-050 Araçatuba - SP - Brazil
Tel: (+55) 18 3636 3314

Email: juliano.pessan@unesp.br

Declaration of interest

The authors declare no conflict of interest that may affect the manuscript judgment.

Letícia Cabrera Capalbo

In vitro **analysis of the capacity of solutions containing fluoride and/or hexametaphosphate on the remineralization of dentin**

Abstract

Objective*:* to investigate the ability of solutions containing HMP and F, alone or in association, in inducing dentin remineralization *in vitro*. **Methods:** Root dentin blocks (4 × 6 cm, *n*=100) were prepared, and caries-like lesions were induced in 2/3 of the surface; each block served as its own control. Blocks were divided into 10 experimental groups (*n* = 10 / group), according to the solutions to be tested: Placebo (without F or HMP); 0.5% HMP; 0.75% HMP; 1% HMP; 250 ppm F; 500 ppm F; 1100 ppm F; 250 ppm F + 0.5% HMP; 500 ppm F + 0.75% HMP; and 1100 ppm F + 1% HMP. Specimens were treated for one minute, twice a day, with the respective solutions, and submitted to a pH-cycling regimen for 7 days. Next, the percentage of the surface hardness recovery (%SHR) and integrated area of subsurface hardness (ΔKHN) were determined. Data were submitted to ANOVA and Fisher LSD's test ($p<0.05$). **Results:** A dose-response relationship was observed between F concentrations in solutions without HMP and %SHR and ∆KHN. For both response variables, solutions containing F + HMP promoted a significantly higher remineralizing effect compared to groups containing F alone. Also, Groups treated with 500 ppm F or 250 ppm F + 0.5% HMP were not significantly different regarding %SHR. **Conclusion:** the addition of HMP to fluoridated solutions significantly enhanced their remineralizing potential on dentine artificial caries lesions *in vitro*, both at the surface and in depth.

Clinical Significance: The simultaneous use of fluoride and HMP may be a promising alternative for remineralizing dentine caries lesions in individuals of all ages.

Letícia Cabrera Capalbo

1. Introduction

Dental caries is a complex, multifactorial disease, being biofilm-sucrose-dependent [1,2]. It is caused by bacterial acid production through the metabolisms of fermentable carbohydrates in the dental biofilm [3], and results from successive demineralization and remineralization cycles at the dental hard tissues [4]. When demineralization prevails, mineral loss leads to the formation of a subsurface lesion. As this process evolves, the lesion may progress, compromising the structural integrity of the enamel (chemical process) until it reaches the underlying dentin (chemical and biological process), which requires detailed knowledge of this tissue's capacity to resist to mineral losses. In addition to carious lesion at coronal dentin (a more advanced process involving tooth cavitation), root dentin may also be afflicted by dental caries starting from a subsurface lesion, similar to enamel. Dentin has smaller hydroxyapatite crystals compared to enamel, is formed in a collagen matrix, and has a higher carbonate content, which explains its greater solubility. Thus, during the formation of a dentin caries lesion, both processes of mineral dissolution and protein degradation take place [5].

The prevention and treatment of initial dental caries lesions, especially in high-risk patients, is a constant challenge in the clinical practice. Efforts have been directed towards the search for technological advances that promote the remineralization of carious lesions, as well as reverse the caries process at the earliest possible stage [6]. The administration of fluoride (F) in community-based strategies, as well as in vehicles for self- and professional application, is a well-established strategy for the control of dental caries, since it acts both in reducing demineralization, and promoting tissue remineralization [7,8].

In the dentin, this process becomes even more complex ought to tissue heterogeneity. Thereby, two main strategies (focused on the de-/re-mineralization and the enzymatic inhibition) have been extensively investigated. The increase in the therapeutic effects of fluoridated products on mineral dynamics can potentially improve its effects on the control of dental caries, and the addition of organic or inorganic phosphate salts to fluoridated products is an effective alternative to increase their effectiveness on the progression of enamel

caries lesions, according to *in vitro* and *in situ* studies [9–11]. Simultaneously, new strategies aimed at the preservation of collagen fibrils and the promotion of dentin remineralization have been proposed, in a process known as biomimetics [12]. This approach is mediated by specific bioactive agents that improve and reinforce dentin through localized changes in biochemical and biomechanical properties [13]. The viability of an agent that could reconcile these two properties could optimize both the desired results, enabling its use in clinical practice.

Sodium trimetaphosphate (TMP) and sodium hexametaphosphate (HMP) are cyclic inorganic phosphates that have been added to fluoridated products such as toothpastes, gels, varnishes, and mouthwash solutions [10,11,14–17] to increase their ability in reducing enamel demineralization and enhancing its remineralization. It has recently been shown that treatment of dentin artificial caries lesions with 1,5% TMP in association with $Ca(OH)_2$ was successful in promoting remineralization [18]. However, contradictorily to the evidence of the role of TMP and F on enamel caries (when co-administered), this association has been shown to be ineffective in the remineralization of dentin lesions [19], which may be associated with use of an unfavorable F:TMP molar ratio.

As for HMP, it presents a high capacity to adsorb to enamel and to reduce its solubility [10], in addition to having a strong tendency to form complexes with cations [20,21]. This leads to its precipitation on rich electron-donor sites on the enamel surface, which promotes greater adsorption of Ca^{2+} and PO_4^{3-} ions [22]. Considering the promising results of the association of HMP with F on enamel de- and re-mineralization, it would be interesting to evaluate the effect of this association on dentine caries lesions. Based on the similarity of enamel and dentin structures (*i.e.*, mineralized dental tissues), it is possible that the enhanced remineralizing potential of F and HMP observed for enamel might also be verified for dentin. Such effects and their extent, however, remain unknown.

Thus, this study aimed to investigate the capacity of solutions containing HMP and F, alone or in association, to induce dentin remineralization in an *in vitro* protocol. The study's null hypothesis was that the use of the test solutions

(containing F and HMP) would not lead to different remineralization rates compared with their respective controls (containing F alone).

2. Material and Methods

2.1. *Experimental Design*

Sample size was based on a previous pilot study, considering the primary outcome from surface and cross-sectional hardness analysis, in terms of the mean difference between groups (10 and 163.5, respectively), standard deviation (5.2 and 85.3, respectively), an α-error of 5% and a β-error of 20%. Root dentine blocks (6 × 4 × 2 mm, *n* = 100) were obtained from bovine incisors and kept in thymol solution 0.1% for 30 days prior to experimental procedures. The surface of the blocks was serially polished, followed by induction of subsurface lesions, and the blocks were randomly assigned into 10 groups (*n* = 10/group): placebo (without F/HMP); 250 ppm F (250F); 500 ppm F (500F); 1100 ppm F (1100F); 0.5% HMP; 0.75% HMP; 1% HMP; 250F+0.5% HMP; 500F+0.75% HMP; 1100F+1% HMP. Blocks were subjected to pH cycling and treatment with the solutions. Following, surface and cross-sectional hardness were assessed. Blocks remained under moist conditions (deionized water) at 4ºC prior to hardness analyzes.

2.2. Blocks preparation and caries-like lesion

The teeth were cut using an IsoMet Low Speed Saw (Buehler Ltd., Lake Bluff, Ill., USA) under water cooling and then serially polished with a grinder polisher (Buehler, Lake Bluff, Illinois, USA). The blocks were divided into three areas:

1 – Sound area (positive control area): One-third of the surface was covered with acid-resistant varnish to prevent contact with the demineralizing solution (caries-like lesions induction) and with the treatment solutions;

2 – Demineralized area (negative control area): The specimens were immersed in demineralizing solution to form a subsurface dentin lesion (in the two thirds not covered by acid-resistance varnish). Thereafter, one third of this area was also covered with acid-resistant varnish to prevent contact with the treatment solutions;

3 – Treated area (experimental area): After demineralization, the dentin surface (one third) was submitted to one of the experimental treatment solutions and pH-cycling.

To produce dentin subsurface lesions, the blocks were immersed in a demineralizing solution (50 mM acetic acid, 2.2 mM $CaCl_2$, 2.2 mM KH_2PO_4, 47.6 µM NaF; pH = 5,0 [23]) for 30 minutes, followed by a remineralizing solution (1.5 mmol/l $Ca(NO_3)_2 \times H_2O$; 0.9 mmol/l NaH_2-$PO_4 \times H_2O$; 150 mmol/l KCl in 0.02 mol/l cacodylic buffer, pH 7.0; 0.05 mg F/ml as NaF [23]) for two hours, three times-day, during two days. The blocks were kept in remineralizing solution overnight. After this period, the specimens were immersed in the demineralizing solution for five additional days, totaling seven days of the experimental procedure.

2.3. *Treatments and pH-cycling*

One hundred dentin blocks were randomly divided into 10 experimental groups (*n* = 10/group) according to the treatment solutions, and submitted to a 7-day pH-cycling regimen at 37°C. The specimens were treated for one minute and then immersed in demineralizing solution (1.5 mM $CaCl_2$, 0.9 mM KH_2PO_4, and 50 mM lactic buffer; pH 5.0) for 8 hours. Next, the blocks were treated again, followed by immersion in remineralizing solution (5 mM $CaCl_2$, 0.9 mM KH_2PO_4, 130 mM KCl, 20 mM HEPES, and 5 mM NaN_3; pH 7.0) for 16 hours [18,19,24]. Between all steps, the dentin specimens were rinsed with deionized water.

2.4. Dentin hardness analysis

After the 7-day pH cycling, surface hardness (SH) was measured using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL, USA) and a Buehler OmniMet software (Buehler). A Knoop diamond indenter was used under a 10 g load, for 10 seconds [19]. Five indentations were produced in each of the three areas with a distance of 100 μm among them, and positioned in the center of each area.

For cross-sectional hardness (CSH), the dentin blocks were longitudinally sectioned, included in acrylic resin with the cut face on the top and the serially polished. 14 indentations were performed in each area of the sample at different depths (5, 10, 15, 20, 25, 30, 40, 50, 70, 90, 110, 130, 220 and 330 μm) under a 2 g load applied for 10 s.

Following, the integrated area (cross-sectional profiles of hardness into dentine) was calculated using the trapezoidal rule (GraphPad Prism, version 3.02) in each depth, from the outer surface up to 300 μm in depth, for the three areas (sound, demineralized and treated dentine). These values were used for the calculation of the integrated recovery of subsurface hardness (∆KHN; KHN × μm) [10,25].

2.5. Statistical Analysis

SigmaPlot 12.0 software was used for statistical analysis, and a 5% level of significance was set. Data analysis considered the values of %SHR and ∆KHN. %SHR (raw) and ∆KHN (log_{10}-transformed) data passed normality (Shapiro-Wilk) and homogeneity (Barlett) tests, and were submitted to one-way ANOVA and Fisher LSD's test. Regarding the sound (control) area, ∆KHN data were submitted to one-way ANOVA; for the demineralized area, ∆KHN data were analyzed by Kruskal-Wallis test.

3. Results

The present *in vitro* protocol induced subsurface caries-like lesions in dentin with an average depth of 130 μm. At greater depths, hardness values approached those of the control area. At the control area (sound dentine), mean (SD) hardness values were 72.7 (4.5) and 30.9 (0.5), respectively for SH and ΔKHN. At the demineralized, the corresponding values were 27.7 (2.3) and 16.8 (0.4). No significant differences were observed among the groups regarding %SHR and ΔKHN, both at the sound (control) and demineralized areas.

Table 1 shows %SHR and ΔKHN according to the groups. A dose-response relationship was observed between F concentrations in the experimental solutions without HMP and %SHR and ΔKHN (Placebo < 250 ppm F < 500 ppm F < 1100 ppm F). The lowest %SHR values were observed for Placebo and all groups treated with HMP alone, which were significantly different from the other groups. On the other hand, 500F+0.75% HMP, 1100F, and 1100F+1% HMP groups had the highest %SHR values. No significant differences were observed between Placebo and 0.5% HMP, 0.75% HMP and 1% HMP, or 250F+0.5% HMP and 500F.

Regarding ΔKHN, significant differences were observed among all groups. Placebo and groups containing HMP alone presented the lowest ΔKHN, following a dose-response trend between HMP concentrations and the remineralizing effects. As for the other groups, a pattern similar to %SHR was observed.

Table 1. Mean (SD) percentage of surface hardness recovery (%SHR) and integrated recovery of subsurface hardness (ΔKNH) according to the groups

Groups	%SHR	ΔKHN
Placebo	9.5 (2.4)[a]	56.5 (4.1)[a]
0.5% HMP	11.9 (3.3)[a]	87.6 (6.8)[b]
0.75% HMP	18.9 (3.8)[b]	101.2 (7.4)[c]
1% HMP	17.8 (3.5)[b]	133.2 (9.8)[d]
250 ppm F	26.2 (2.4)[c]	370.0 (27.5)[e]
500 ppm F	41.2 (2.2)[d]	533.5 (85.3)[f]
1100 ppm F	56.2 (5.2)[e]	782.1 (58.6)[g]
250 ppm F + 0.5% HMP	38.7 (4.7)[d]	493.4 (38.1)[h]
500 ppm F + 0.75% HMP	48.8 (5.0)[f]	655.4 (42.8)[i]
1100 ppm F + 1% HMP	66.6 (5.0)[g]	859.0 (36.4)[j]

Different superscript letters indicate significant differences among the groups within each column. One-way ANOVA on the natural outcomes (%SHR) or $\log_{10}$-transformed data (ΔKNH), and Fisher LSD's test ($p<0.05$, n=10/group).

4. Discussion

Approaches to increase the benefits of F, while minimizing its side-effects, have been intensively investigated, yet with much to be explored. Those strategies include the use of polyphosphate salts, nano-hydroxyapatite particles, calcium glycerophosphate, amorphous calcium phosphate, and functionalized β-tricalcium phosphate [26]. This is the first study evaluating the remineralizing potential of F solutions supplemented with HMP in dentin lesions. Given that the addition of HMP to F solutions resulted in a higher remineralizing potential than their counterparts without HMP, both in surface and in depth, the study's null hypothesis was rejected.

Fluoride is the most widely used agent for controlling root caries, especially when applied at high concentrations [27]. It is considered as the gold standard for dental caries prevention regarding its ability to prevent dental demineralization as well as to promote remineralizing. It has also been shown to have the capacity to completely inhibit MMPs activity [28]. Although most of the studies on the effects of TMP and HMP were carried out with tooth enamel, there is evidence that these salts also have therapeutic properties on dentin. Gonçalves *et al.* [18] demonstrated that 1.5% TMP can act as an effective inhibitor of collagen degradation mediated by MMP-2 and MMP-9, as well as proteases extracted from healthy dentin. In addition, the treatment of artificial dentin caries lesions with 1.5% TMP supplemented with $Ca(OH)_2$ induced their remineralization [19]. Surprisingly, however, the association between TMP and F was proven ineffective in promoting the remineralization of dentin lesions [19], possibly due to the use of an unsuitable F:TMP molar ratio. Therefore, in the present study, HMP concentrations of 0.5% and 1% were established based on previous studies in which HMP was added to the 250 ppm F and 1100 ppm F dentifrices, respectively [10,11,16]. As for the concentration of 0.75% HMP, since no study has evaluated the association between HMP and 500 ppm F, it was extrapolated from the aforementioned data.

In spite of containing phosphate in its structure, cyclic HMP cannot be regarded as a source of free phosphate to interact with dental hard tissues, considering that the hydrolyzation of its molecule does not spontaneously occur under physiological conditions [20,29,30]. In fact, the effect of HMP is associated with its capacity to interact with the tooth surfaces. When adsorbed, HMP becomes negatively charged, allowing the retention of Ca^{2+} and CaF^{+} ions, which leads to the formation of a protective layer that restricts acid diffusion and increases calcium and fluoride penetration into tooth hard tissues[10,16]. In this regard, it has been suggested that this protective layer is capable to retain fluoride compounds that can be released when cariogenic challenges occur, promoting the formation of more reactive compounds [31]. This mechanism has a direct impact on the incorporation of fluoride and calcium by enamel and dentine, intensifying remineralization at the subsurface. Previous

studies have shown that TMP adsorption and CaF_2 deposition on dentin produce a synergistic effect and reduce acid diffusion. In turn, these processes increase calcium phosphate precipitation and, consequently, protect dentin against erosion [32], and promote obliteration of dentine tubules [33]. Although no study has investigated the association between F and HMP on the deposition of CaF_2 in dentin so far, our findings on the remineralizing effects of such association might be related to CaF_2 deposition.

The present study allowed the observation of a clear dose-response relationship between F concentrations in the treatment solutions and their resulting remineralizing effects; the solution containing 1100 ppm F was 46,6% and 112% more effective than those containing 500 and 250 ppm F, respectively. Similar rates (51,6% and 102%, respectively) were observed in an *in vitro* study on enamel demineralization [10], thus validating the model employed in the present investigation. Nonetheless, the additional benefit of HMP on dentine remineralization was much lower than those reported on enamel demineralization. While Camara *et al.* [10] demonstrated that dentifrices containing 250 ppm F + 0,5% HMP were 189% and 43% greater than dentifrices containing 250 ppm F or 1100 ppm F, respectively, treatment with 250 ppm F + 0,5% HMP in the present study promoted a remineralizing effects 33.3% higher than 250 ppm F and 59% lower than that observed for 1100 ppm F. Although the reasons for such differences are not apparent, they might be due to the factors related to the structural differences between both tissues (enamel and dentin), so that further studies are needed to better understand the dynamics linking HMP and dentin.

Despite significant differences were observed among all groups concerning their remineralizing potential at the subsurface, it was noteworthy that the addition of HMP to the 250 ppm F solution promoted the greatest improvement (33.3%) in relation to its counterpart without HMP; the corresponding percentages for solutions containing HMP and fluoride at 500 ppm F and 1100 ppm F were 22.5% and 9.8%, respectively, compared with their counterparts without HMP. Although the causes for such a trend were not investigated in the present study, it is possible that the sequestration of Ca^{2+}

and CaF^+ by HMP at higher concentrations (negatively influencing the uptake of these ions by dentine) might have played a key role on the results observed. This reinforces the need for an appropriate HMP/F ratio in order to achieve optimum effects on de- and re-mineralization [10].

The formation of caries-like lesions in bovine dentin is an important approach for the study of new strategies to prevent or treat dentin carious lesions [34–38]. Considering the differences between enamel and dentin in relation to its matrix, hydroxyapatite crystals size and solubility, the latter is especially important from a clinical perspective, as a smaller drop in oral pH (around 6.2 – 6.3) is capable of dissolving dentin mineral, while the estimated value for enamel solubility is 5.5 [5]. Therefore, dentin is more susceptible to demineralizing events than enamel, what hinders direct extrapolation of the results obtained for enamel caries to dentine caries. In addition, the degradation of the collagen matrix makes the dentin more porous, which allows a greater penetration of H^+ ions, leading to a more severe mineral loss [32,39]. Another important aspect observed was the pattern of the caries-like lesions formed in dentin. Despite the hardness averages in longitudinal section at greater depths were close to the those in the sound (control) area, even at 330 μm depth it did not reach those values, as in enamel models. Once again, this trend might also be related to the structural differences between enamel and dentin mentioned above, especially the higher porosity of dentine.

Despite all precautions to mimic the conditions found in the oral environment were taken, the *in vitro* nature of the present study has some inherent limitations related to biological events, including collagen degradation and effects of oral microorganisms [40,41]. Also, the high organic content, and consequently, the elastic properties of the dentine [38] are factors that can influence hardness measurement. Marshall *et al*. [42] showed that dentin tested under hydrated conditions provides more realistic data, which are closer to *in vivo* conditions. Therefore, this approach was adopted in the present investigation, in order to better standardize the method of hardness analysis, thus providing more reliable data.

Finally, considering the benefits of the association of F and HMP on dentin remineralization observed in the present study, such association could be assessed in different topical formulations. For daily use, toothpastes and mouthwashes must be considered to enhance the remineralization in patients at high risk. For clinical practice, formulations with high concentration of F, such as gels, foams and varnishes, can also be considered as alternative treatment options for initial dentin caries.

To sum up, it can be concluded that the addition of HMP to fluorided solutions led to enhanced remineralization of artificial caries lesions in dentin, both at the surface and in depth. Therefore, considering the promising results of the present study in dentin, alongside previous investigations regarding the protective effect of these compounds against enamel erosive wear and enamel de- and re-mineralization, the association of F and HMP must be considered as a valuable strategy not only for high-risk patients, but also for people in general, considering its benefits on both dental caries and enamel erosive wear.

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Anexos

5. ANEXOS

ANEXO A

JOURNAL OF DENTISTRY

INSTRUÇÕES AOS AUTORES

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.
Reference to a book:
[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
Reference to a chapter in an edited book:
[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
Reference to a website:
[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/ aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
Reference to a dataset:

[dataset] [5] M. Oguro, S. Imahiro, S. Saito, T. Nakashizuka, Mortality data for Japanese oak wilt disease and surrounding forest compositions, Mendeley Data, v1, 2015. https://doi.org/10.17632/ xwj98nb39r.1.

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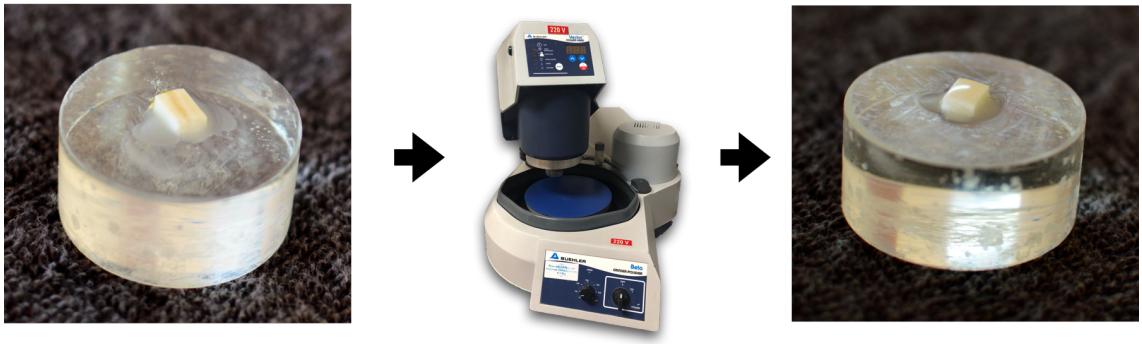
ANEXO B

PREPARO DOS BLOCOS DE DENTINA

1. Bloco de dentina bovina medindo 6×4×2 mm, obtido após secção transversal e longitudinal em cortadeira.



2. Bloco fixado com cera pegajosa em suporte de acrílico antes (esquerda) e após (direita) polimento.

ANEXO C

PREPARO E DOSAGEM DAS SOLUÇÕES

- **Água deionizada** (Placebo – controle negativo);
- **Soluções de HMP** $(NaPO_3)_6$, (Sigma-Aldrich Co., USA), nas concentrações de 0,5%; 0,75% e 1%;
- **Soluções de F** (NaF, Merck, Alemanha), nas concentrações de 250, 500 e 1100 ppm F;
- **Soluções** contendo 250 ppm F e 0,5% de HMP, 500 ppm F e 0,75% de HMP, e 1100 ppm F e 1% de HMP.

As 10 soluções foram preparadas e, em seguida, as dosagens de fósforo e flúor foram realizadas.

ANEXO D

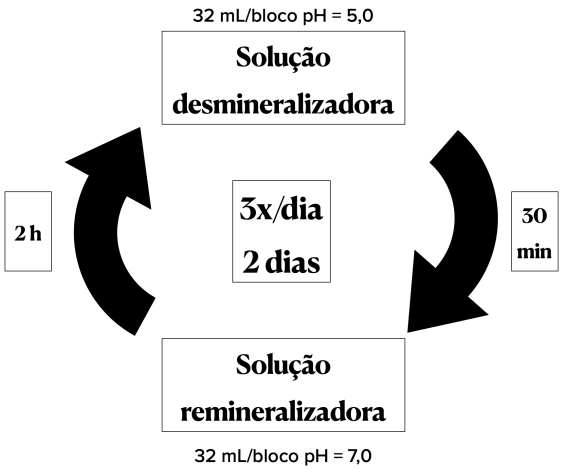
PREPARO DOS BLOCOS E INDUÇÃO DAS LESÕES ARTIFICIAIS DE CÁRIE

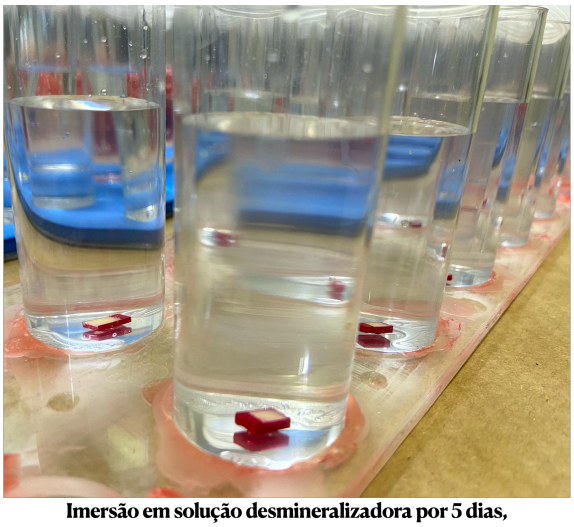
Bloco de dentina

1/3 do bloco protegido com verniz ácido-resistente

1 = Área controle 2 = Área desmineralizada

1/3 de cada bloco foi protegido com verniz ácido-resistente (imagem da direita) e em seguida foi realizada a indução das lesões artificiais de cárie.





Imersão em solução desmineralizadora por 5 dias, completando 7 dias de indução de lesão de cárie artificial

ANEXO E

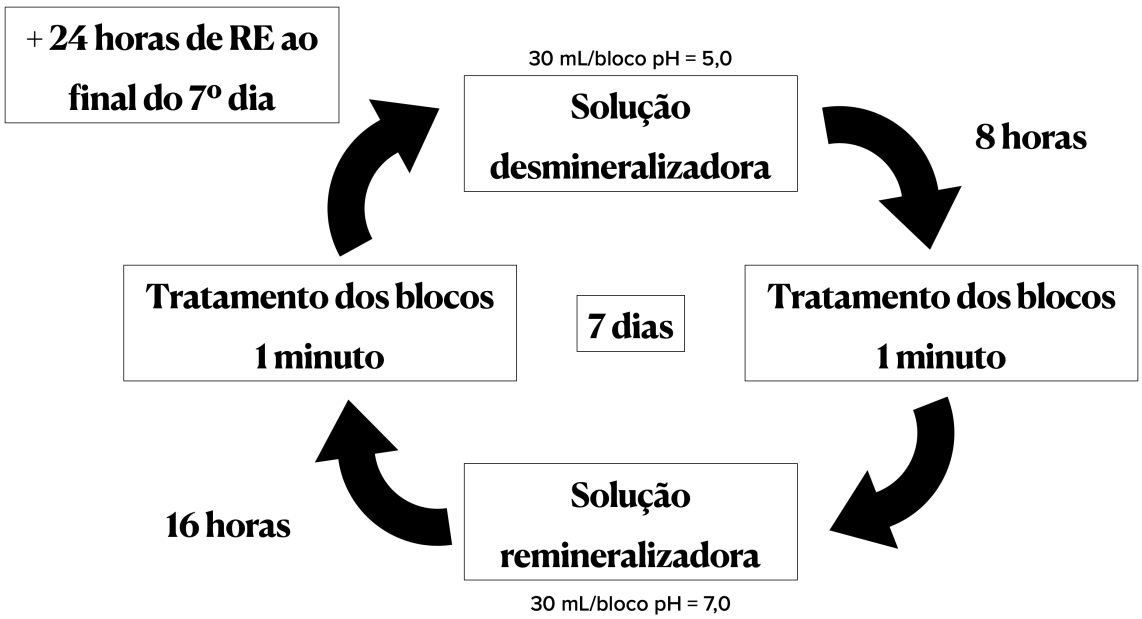
TRATAMENTOS E CICLAGEM DE pH

1/3 do bloco protegido com verniz ácido-resistente (controle)

1/3 do bloco protegido com verniz ácido-resistente (área desmineralizada)

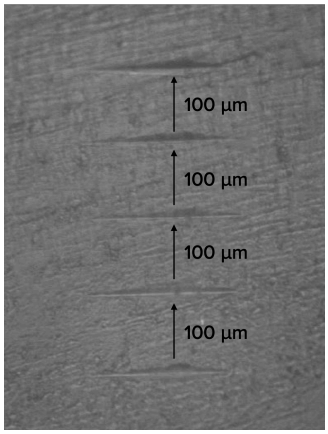
1 = Área controle 2 = Área desmineralizada 3 = Área tratada

Mais 1/3 de cada bloco foi protegido com verniz ácido-resistente e, em seguida, foram realizados a ciclagem de pH e os tratamentos.



ANEXO F

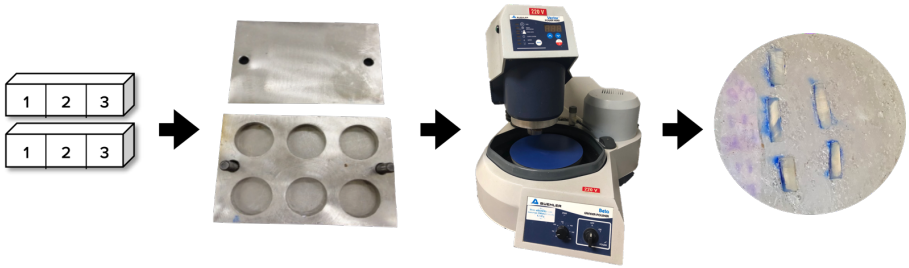
ANÁLISE QUANTO À RECUPERAÇÃO DE DUREZA DE SUPERFÍCIE



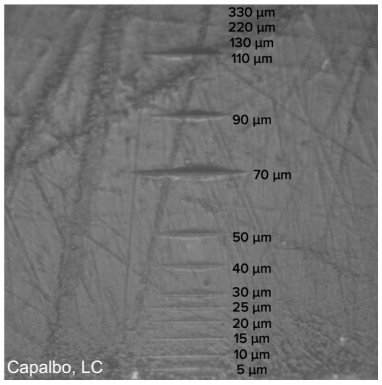
Cinco endentações realizadas em cada uma das três áreas dos blocos, com 100 µm entre elas. A carga utilizada foi de 10 g, por 10 segundos.

ANEXO G

ANÁLISE DA DUREZA DA DENTINA EM SECÇÃO LONGITUDINAL (ΔKHN)



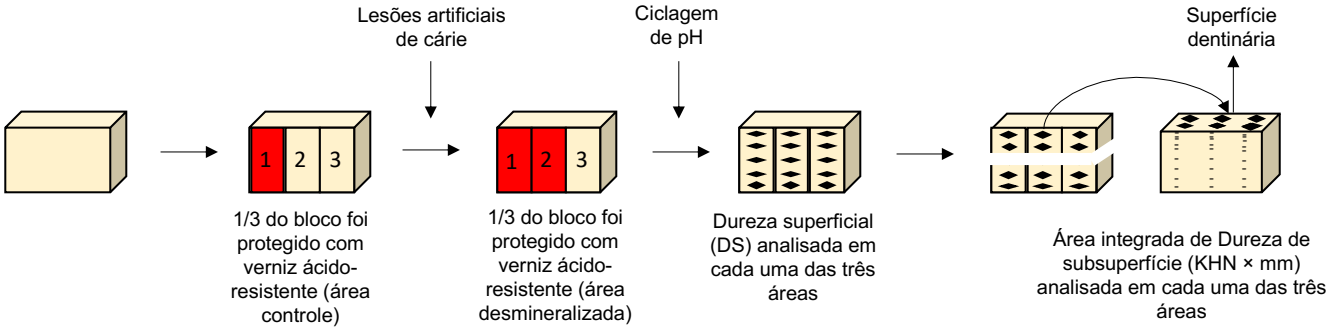
Carga: 2 g
Tempo: 10 s



Os blocos foram seccionados longitudinalmente e embutidos em resina acrílica a frio. Em seguida, foram avaliados quanto à dureza em secção longitudinal em microdurômetro, com carga de 2 g, durante 10 segundos.

ANEXO H

FLUXOGRAMA DAS ANÁLISES



Blocos de dentina preparados e divididos em três áreas. Após a indução das lesões artificiais de cárie e a ciclagem de pH com tratamento, as análises de dureza superficial e em secção longitudinal foram realizadas.