



UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
Campus de Araçatuba

LETÍCIA CABRERA CAPALBO

**EFEITO DE SOLUÇÕES CONTENDO HEXAMETAFOSFATO DE
SÓDIO E QUERCETINA, ASSOCIADOS OU NÃO AO FLUORETO,
SOBRE O DESGASTE EROSIVO DENTINÁRIO *IN VITRO***

ARAÇATUBA - SP

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Tese 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 Doutora 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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Orientador: Prof. Juliano Pelim Pessan

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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)
2019/2021	Curso de Pós-Graduação em Ciência Odontológica – área Saúde Bucal da Criança, nível de Mestrado, na Faculdade de Odontologia de Araçatuba- UNESP
2021/2022	Curso de Pós-Graduação em Ciência Odontológica – área Saúde Bucal da Criança, nível de Doutorado, 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

Letícia Cabrera Capalbo

Dedicatória

Dedicatória

Aos meus pais, Marcos e Lúcia:

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Dedicatória

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Agradecimentos Especiais

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distância e espero que a vida permita que a gente se reencontre muitas e muitas vezes!

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Epígrafe

Epígrafe

“Não existem sonhos impossíveis para aqueles que realmente acreditam que o poder realizador reside no interior de cada ser humano. Sempre que alguém descobre esse poder, algo antes considerado impossível, se torna realidade.”

Albert Einstein

Resumo

Capalbo, LC. **Efeito de soluções contendo hexametáfosfato de sódio e quercetina, associados ou não ao fluoreto, sobre o desgaste erosivo dentinário *in vitro***. 2022. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2022.

O objetivo do presente estudo foi investigar o efeito de soluções contendo fluoreto (F), hexametáfosfato de sódio (HMP) e quercetina (QC), sozinhos ou em associação, sobre a erosão dentinária e sobre a inibição de metaloproteinases da matriz (MMPs) -2 e -9, em protocolos *in vitro*. Blocos de dentina radicular bovina (4 × 4 × 2 mm; $n = 96$), selecionados por dureza superficial, foram aleatoriamente divididos em 8 grupos experimentais ($n = 12/\text{grupo}$) e tratados 2×/dia (um minuto) com as seguintes soluções: (1) água deionizada (controle negativo); (2) 1100 ppm F ("F"); (3) 1,0% HMP ("HMP"); (4) 0,03% QC ("QC"); (5) F+HMP; (6) F+QC; (7) HMP+QC; e (8) F+HMP+QC. Os blocos foram submetidos a desafios erosivos 4×/dia, por 5 dias (exposição dinâmica a ácido cítrico 50 mmol.l⁻¹, pH 3,2, 90 s). Em seguida, foram analisados quanto à perda dentinária (perfilometria) e à perda de dureza integrada em profundidade (área sob a curva, ΔKHN). O potencial antiproteolítico das soluções contendo F, HMP e/ou QC foi analisado por zimografia. Os dados de perda dentinária ($\log_{10}$) foram submetidos a ANOVA um critério, seguido do teste de Tukey. Os resultados de ΔKHN (dados brutos) foram submetidos a ANOVA dois critérios, medidas repetidas, seguido do teste Holm-Sidak ($p < 0,05$). O menor desgaste erosivo foi observado no grupo F+HMP+QC. Nas menores profundidades (5-30 μm), os blocos tratados com a solução contendo F+HMP+QC apresentaram os maiores valores de ΔKHN . A análise zimográfica mostrou que todos os tratamentos promoveram atividade antiproteolítica total da MMP-2, com exceção da QC administrada sozinha (inibição de 77%). Para MMP-9, todas as soluções contendo HMP e a associação de F+QC apresentaram atividade antiproteolítica total. Conclui-se que a adição de HMP e QC a soluções contendo F levou a uma maior proteção contra a erosão dentinária, tanto em superfície (perda dentinária) quanto em relação ao conteúdo mineral do tecido remanescente (ΔKHN), além de promover uma completa inibição da atividade de MMPs -2 e -9 *in vitro*.

Palavras-chave: Erosão Dentária. Fosfatos. Quercetina. Dentina. Inibidores de Metaloproteinases de Matriz.

Abstract

Capalbo, LC. **Effect of sodium hexametaphosphate and quercetin, associated or not with fluoride, on dentin erosion *in vitro***. 2022. Tese (Doutorado em Ciência Odontológica, área de Saúde Bucal da Criança) - Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba 2022.

The aim of the present study was to investigate the effect of solutions containing fluoride (F), sodium hexametaphosphate (HMP) and quercetin (QC), alone or in association, on dentin erosion and on the inhibition of matrix metalloproteinases (MMPs) - 2 and -9, using *in vitro* protocols. Bovine root dentin blocks ($4 \times 4 \times 2$ mm; $n = 96$), selected by surface hardness, were randomly divided into 8 experimental groups ($n = 12/\text{group}$) and treated 2×/day (one minute) with the following solutions: (1) deionized water (negative control); (2) 1100 ppm F ("F"); (3) 1.0% HMP ("HMP"); (4) 0.03% QC ("QC"); (5) F+HMP; (6) F+QC; (7) HMP+QC; and (8) F+HMP+QC. Blocks were submitted to erosive challenges 4×/day for 5 days (dynamic exposure to 50 mmol.l⁻¹ citric acid, pH 3.2, 90 s). They were then analyzed for dentin loss (profilometry) and integrated hardness loss in depth (area under the curve, ΔKHN). The antiproteolytic potential of solutions containing F, HMP and/or QC was analyzed by zymography. Dentin loss results ($\log_{10}$ transformed) were submitted to one-way ANOVA, followed by Tukey's test. ΔKHN data (raw) were submitted to two-way, repeated-measures ANOVA, followed by the Holm-Sidak test ($p < 0.05$). The lowest dentin erosive wear was promoted by F+HMP+QC. At the lowest depths (5-30 μm), blocks treated with F+HMP+QC showed the highest values of ΔKHN . Zymography analysis showed that all treatments completely inhibited MMP-2 activity, except for QC administered alone (77% inhibition). For MMP-9, all the solutions containing HMP or the association of F+QC promoted total antiproteolytic activity. It was concluded that the addition of HMP and QC to F solutions led to greater protection against dentin erosion, both at the surface (dentin loss) and in relation to the mineral content of the remaining tissue (ΔKHN), in addition to promoting a complete inhibition of MMPs -2 and -9 activity *in vitro*.

Keywords: Tooth Erosion. Polyphosphates. Quercetin. Dentin. Matrix Metalloproteinase Inhibitors.

Lista de abreviaturas e símbolos

LISTA DE ABREVIATURAS E SÍMBOLOS

AS	Artificial saliva/ <i>Saliva artificial</i>
ANOVA	Analysis of variance/ <i>Análise de variância</i>
Ca⁺²	Calcium ion/ <i>Íon cálcio</i>
CaCl₂	Calcium Chloride/ <i>Cloreto de cálcio</i>
CaF₂	Calcium Fluoride/ <i>Fluoreto de cálcio</i>
Ca(NO₃)₂	Calcium Nitrate/ <i>Nitrato de cálcio</i>
°C	Degrees Celsius/ <i>Graus Celsius</i>
DOM	Demineralized Organic Matrix/ <i>Matriz orgânica desmineralizada</i>
HCl	Hydrochloric Acid/ <i>Ácido Clorídrico</i>
F	Fluoride/ <i>Fluoreto</i>
g	Grams/ <i>Gramas</i>
H₂O	Water/ <i>Água</i>
HMP	Sodium hexametaphosphate/ <i>Hexametáfosfato de sódio</i>
KCl	Potassium Chloride/ <i>Cloreto de potássio</i>
KHN	Knoop Hardness Number/ <i>Número de Dureza Knoop</i>
L	Liter/ <i>Litro</i>
Log₁₀	Logarithm with base 10/ <i>Logaritmo na base 10</i>
mg	Milligram/ <i>Miligrama</i>
mL	Milliliter/ <i>Mililitro</i>
mm	Millimeter/ <i>Milímetro</i>
mmol.l⁻¹	Millimol per liter/ <i>Milimol por litro</i>
MIC	Minimum Inhibitory Concentration/ <i>Concentração inibitória mínima</i>
min	Minute/ <i>Minuto</i>
mol.l⁻¹	Mol per liter/ <i>Mol por litro</i>
MMPs	Matrix Metalloproteinases/ <i>Metaloproteinases da matriz</i>
NaF	Sodium Fluoride/ <i>Fluoreto de sódio</i>
NaH₂PO₄	Sodium dihydrogen phosphate/ <i>Fosfato Monossódico</i>
NaN₃	Sodium azide/ <i>Azida de sódio</i>
µm	Micrometer/ <i>Micrômetro</i>
p	Probability/ <i>Probabilidade</i>
pH	Potential of hydrogen/ <i>Potencial Hidrogeniônico</i>
ppm	Part per million/ <i>Parte por milhão</i>

Listas de abreviaturas e símbolos

r.p.m.	Revolutions per minute/ <i>Rotações por minuto</i>
SD	Standard Deviation/ <i>Desvio Padrão</i>
SH	Surface hardness/ <i>Dureza de superfície</i>
s	Seconds/ <i>Segundos</i>
TMP	Sodium Trimetaphosphate/ <i>Trimetafosfato de sódio</i>
UNESP	Universidade Estadual Paulista/ <i>São Paulo State University</i>
w/v	Weight per volume/ <i>Peso por volume</i>
ZnCl₂	Zinc Chloride/ <i>Cloreto de Zinco</i>
ΔKHN	Integrated hardness loss in depth/ <i>Perda de dureza integrada em profundidade</i>

Sumário

SUMÁRIO

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**Effect of sodium hexametaphosphate and quercetin, associated or not
with fluoride, on dentin erosion *in vitro***

LC Capalbo¹, ACB Delbem¹, R Dal-Fabbro¹,
KK Inácio², RC Oliveira², JP Pessan¹

¹Department of Preventive and Restorative Dentistry, School of Dentistry,
Araçatuba, São Paulo State University (UNESP), Araçatuba, SP, Brazil

²Department of Biological Sciences, Bauru School of Dentistry, University of São
Paulo, Bauru, SP, Brazil

Short title: Solutions containing F, HMP and/or quercetin on dentin erosion

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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

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Abstract

Objective: to investigate the ability of solutions containing sodium hexametaphosphate (HMP), fluoride (F) and quercetin (QC), alone or in association, to prevent dentin erosion and to inhibit MMPs -2 and -9 activity, using *in vitro* protocols. **Methods:** Root dentin blocks ($n=96$) were prepared and divided into 8 experimental groups ($n=12/\text{group}$), according to the solutions to be tested: Placebo; 1100 ppm F ("F"); 1.0% HMP ("HMP"); 0.03% QC ("QC"); F+HMP; F+QC; HMP+QC; and F+HMP+QC. Erosive challenges were performed 4×/day for 5 days. Specimens were treated for one minute, twice a day, with the respective solutions. Next, dentin loss (profilometry) and integrated loss of hardness in depth (ΔKHN) were determined. The antiproteolytic potential was assessed by gelatin zymography. Dentin wear results ($\log_{10}$ -transformed) were submitted to one-way ANOVA, followed by Tukey's test. ΔKHN data (raw) were submitted to two-way, repeated-measures ANOVA, followed by Holm-Sidak's test ($p<0.05$). **Results:** Dentin erosive wear was significantly lower for F+HMP+QC than for all other treatments. At the shallowest depths (5–30 μm), blocks treated with F+HMP+QC had the highest ΔKHN values. All treatments completely inhibited MMP-2 activity, except for QC administered alone (77% inhibition). For MMP-9, all HMP-containing solutions or the association of F+QC promoted total antiproteolytic activity. **Conclusion:** The addition of HMP and QC to F solutions led to greater protection against dentin erosion, both at the surface (dentin loss) and in relation to the mineral content of the remaining tissue (ΔKHN), in addition to promoting a complete inhibition of MMPs -2 and -9 activity *in vitro*.

Clinical Significance: Considering the promising results, the association of F, HMP and QC must be considered as a valuable strategy not only for high-risk patients, but also for people in general, considering its benefits on dentin erosion.

1. Introduction

Dental erosion is defined as the chemical loss of mineralized tooth substance caused by the exposure to acids not derived from oral bacteria, while erosive tooth wear is the cumulative surface loss of mineralized tooth substance due to physical or chemophysical processes (dental erosion, attrition, abrasion) [1]. A recent review estimated a global prevalence of dental erosion ranging from 30-50% and 20-45%, respectively for deciduous and permanent teeth, with figures as high as 100% in some studies [2]. Measures used to prevent or minimize tooth erosion effects include the application of topical fluorides [3], especially at high concentrations, as it provides additional minerals (CaF_2) to enamel surfaces, which reduce their rate of dissolution upon acid challenges [4]. However, the effects of fluoride on dentin erosion can be limited owing to the presence of organic matrix [3].

Differently from what is observed for tooth enamel, dentin hydroxyapatite crystals are formed in a collagen matrix, which has important implications for dental caries and erosion. After the mineral content of the dentin surface is dissolved, a network of collagen fibers is exposed, which can be degraded by proteolytic enzymes (collagenases) or by latent enzymes in dentin [matrix metalloproteinases (MMPs)] [5]. For this reason, the use of antiproteolytic agents have been widely studied in recent years regarding its effects on proteases, such as MMPs and cysteine cathepsins [6]. Recently, polyphenolic flavonoids have been extensively studied, and their potential to increase dentin mechanical properties and to inhibit its proteases, thus controlling its biodegradation, has been proven [6].

Alternatives to enhance the effects of fluoride in vehicles for home and professional use have been studied, which include supplementation with calcium and/or phosphate salts. For tooth enamel, one of the most studied strategies is the addition of sodium hexametaphosphate (HMP) to fluoridated products, which has been shown to significantly increase their protective and therapeutic effects on enamel de- and remineralization [7–10] and on erosive tooth wear [11–13], both under *in vitro* and *in situ* conditions.

In addition to the aforementioned strategies focusing on mineralization, the use of phytochemicals has been widely studied as potential antiproteolytic and antimicrobial agents. Quercetin (QC) is a flavonoid found in many vegetables, fruits, seeds, nuts, tea, grape juice, and red wine, which has received considerable attention due to its anti-proliferative, anti-inflammatory, and anti-carcinogenic activities, its potential to modulate gene expression [14,15], and its positive effects both for prophylaxis and treatment of

patients with COVID-19 when associated with other drugs [16]. In adhesive dentistry, pretreatment of the dentin surface with a QC/ethanol solution has been shown to effectively inhibit the activity of MMPs in the hybrid layer, delaying the collapse of the collagen network, thus improving the longevity of dentin adhesion [17]. Also, QC was recently shown to promote a protective effect against dentin erosion greater than that of 12,300 ppm NaF, Chlorhexidine or Epigallocatechin Gallate (EGCG), possibly due to its effects on the preservation of the exposed dentin matrix [18]. The effects of QC associated with other actives, however, has not yet been investigated.

Considering the promising results of the association between F and HMP on enamel erosive wear [11], as well as the effects of QC alone on dentin erosion [18], it would be interesting to evaluate the association of F, HMP and QC on the prevention of dentin erosion. Thus, the aim of the present study was to investigate the effect of solutions containing F, HMP and QC, alone or associated, on dentin erosion and on the inhibition of MMPs -2 and -9, *in vitro*. The study's null hypotheses were that the addition of HMP and/or QC to F solutions would not (1) enhance the protective effect on dentin erosion, both for dentin loss and mineral loss, and (2) impact the enzymatic inhibition of MMPs -2 and -9 compared with their respective controls (containing F alone).

2. Materials and methods

2.1 Experimental design

Bovine dentin blocks ($n = 96$) were cut, sequentially polished, and selected by surface hardness (SH) [19]. The blocks were randomly divided into 8 experimental groups according to the solutions tested: Placebo ("PLA" - without F, HMP, or QC); 0.24% F ("F"); 1.0% HMP ("HMP"); 0.03% QC ("QC"); F+HMP; F+QC; HMP+QC; and F+HMP+QC. Half of the surface of each block was protected with acid resistant nail varnish (control area). The blocks were treated with the respective solutions for 1 min, twice a day (before first and third erosive challenges) and submitted to 4 daily erosive challenges (90 s) for 5 days. Following, profilometry and cross-sectional hardness analyzes were performed. The antiproteolytic potential of all the solutions on MMP-2 and MMP-9 was analyzed by zymography.

2.2 Blocks preparation

Dentin blocks ($n = 96$, $4 \times 4 \times 2$ mm) were obtained from bovine permanent mandibular central incisors roots, immediately below the cementum-enamel junction,

from buccal and/or lingual surfaces. Polishing was performed with 320, 600, 800 and 1200 grinder polisher (CARBIMET PaperDiscs, 30-5108-320, BUEHLER), as previously described [20]. The surface of each block was divided in two areas of $4 \times 2 \times 2$ mm: (1) control, protected with acid-resistant nail varnish; and (2) erosion, exposed to treatments with the respective solutions and to erosive challenges. Sample size was based on a pilot study with $n = 6/\text{group}$, according to which 9 blocks would be required to detect significant differences in mean dentin loss of blocks treated with a 1100 ppm F solution (positive control) and F+HMP+QC (test solution containing the three actives), considering mean difference = 1.0, standard deviation = 0.5, and power of 80% ($\alpha=0.05$). Due to the possibility of losses during the processing of the specimens, 12 blocks were included in each group.

2.3 Treatment and erosive challenges

The concentration of HMP in the solutions was determined based on a previous *in vitro* study assessing the effect of the association of 1100 ppm F + 1.0% HMP on enamel demineralization [7], and from a recently concluded study investigating the potential of the same association on dentin caries [21]. As for QC concentration, it was based on previous studies attesting its efficacy (dentin erosive wear) [18] and safety (minimum inhibitory concentration and cytotoxicity assays) [23]. Experimental solutions were freshly prepared using F, HMP and QC from Sigma-Aldrich Co., St. Louis, MO, USA. To prepare solutions containing QC, it was previously dissolved in ethanol and water-bath heating at 37 °C for 15 min, and then added to deionized water under agitation [23]. The concentration of fluoride and HMP in each solution were determined as described by Amaral et al. [24] and Fiske and Subbarow [25].

Blocks were treated with each solution (4 mL; $n = 12/\text{group}$) under agitation (35 r.p.m.; Shaker SK-300, São Paulo, Brazil) twice a day, before the first and third erosive challenges every day. Next, all blocks were submitted to 4 daily erosive challenges for 5 days, which consisted of immersion in 4 mL of 50 mmol.L⁻¹ citric acid (Synth®, Brazil), pH 3.2, 90 s/cycle, under stirring at 35 r.p.m. Blocks were immersed in artificial saliva (1.5 mmol.L⁻¹ Ca(NO₃)₂.4H₂O; 0.9 mmol.L⁻¹ NaH₂PO₄.2H₂O; 150 mmol.L⁻¹ KCl; 0.1 mol.L⁻¹ Tris buffer; pH 7.0; no agitation) [26] between erosive challenges (2 hours) and overnight. After treatments and immersion in citric acid or artificial saliva, blocks were washed with deionized water and gently dried with absorbent paper.

2.4 Determination of dentin loss

The acid-resistant nail varnish layer was carefully removed from the control surface with a spatula before analysis. Dentin wear was determined by profilometry (Surftest SJ 401, Mitutoyo American Corporation). The rugosimeter's tip ran along the sound surface of the specimens towards the eroded area. Five readings were taken 0.5 mm apart at the central area of each block, to calculate the average dentin loss for each specimen [27].

2.5 Dentin hardness analysis and estimation of mineral loss

Dentin blocks were cross sectioned at the center, and half of each block were included in acrylic resin and subsequently polished. For cross-sectional hardness, the micro hardness Micromet 5114 hardness tester (Buehler, Lake Bluff, USA and Mitutoyo Corporation, Kanagawa, Japan) and the Buehler OmniMet software (Buehler, Lake Bluff, United States) was employed, under a 2 g load, for 10 s. A sequence of 9 prints at distances of 5, 10, 15, 20, 25, 30, 40, 50 and 70 μm from the external dentin surface was performed in the center of each block half, for both control and test areas. Integrated hardness area ($\text{KHN} \times \mu\text{m}$) was calculated using the trapezoidal rule (GraphPad Prism, version 3.02), considering two different depths (5-30 μm and 30-70 μm). The difference of each depth of the sound dentin and the value of the integrated loss of hardness in depth was calculated (ΔKHN = loss of hardness in depth in the dentin, area under the curve) [11].

2.6 Gelatin Zymography

To assess the effect of HMP, QC and F as potential MMP inhibitors, purified human MMP-2 and MMP-9 (Sigma-Aldrich Co., St. Louis, MO, USA) were diluted in sample buffer at a 4:1 ratio and electrophoresed under nonreduced conditions on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) containing 1 mg/mL gelatin from porcine skin (Sigma-Aldrich Co., St. Louis, MO, USA) as previously described by Gonçalves et al [28] (Figure 1). molecular-weight SDS-PAGE standards (Bio-Rad Laboratories, Hercules, CA, USA) were Prestained low-range used as molecular-weight markers. After electrophoresis, gels were washed for 1 hour in 2% Triton X-100 and incubated for 18 hours at 37 °C in activation solution (50 mmol.L⁻¹ Tris HCl, 5 mmol.L⁻¹ CaCl₂, 0.001 mmol.L⁻¹ ZnCl₂, 0.02% (w/v) NaN₃, pH 7.4) containing HMP, F and QC

(associated or not) or deionized water (placebo – negative control). After incubation, gels were stained in 0.1% Coomassie Brilliant Blue R-25 for 30 min and destained (30% methanol, 10% acetic acid diluted in deionized water). Gelatinolytic activity was detected as clear bands. Gels were scanned (Densitometer DS-6000, Locus Biotechnologia, Cotia, Brazil) and the intensities of the separate bands were analyzed using the software ImageJ (Research Services Branch, National Institutes of Health, Bethesda, MD, USA). Bands from the active fraction (~ 66 kDa for MMP-2 and ~77 kDa for MMP-9) were taken for analysis. Inhibition rates of F, HMP and/or QC were expressed as percentage according to the activity of MMPs without pretreatment (negative control). An overview of the zymographic analysis is shown in Figure 1.

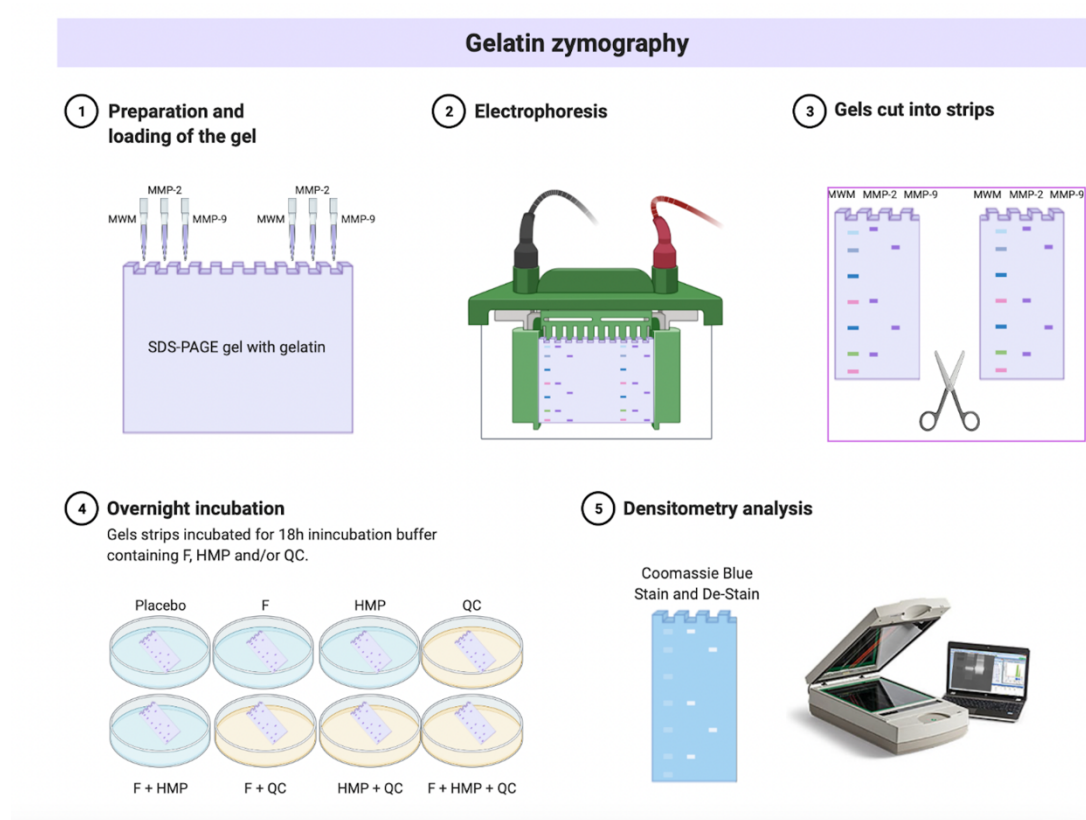


Figure 1. Schematic representation of the gelatin zymography protocol. MWM: molecular weight marker; MMP-2: matrix metalloproteinase 2; MMP-9: matrix metalloproteinase 9.

2.7 Statistical Analysis

SigmaPlot 12.0 software was used for statistical analysis, and a 5% level of significance was set. Data analysis considered the solutions as independent variables, and dentin loss and Δ KHN as dependent variables. All data passed normality (Shapiro-

Wilk) and homogeneity (Barlett) tests. Dentin wear results ($\log_{10}$ -transformed) were submitted to one-way ANOVA, followed by Tukey's test. Data on integrated loss of hardness in depth (Δ KHN, raw data) were submitted to two-way, repeated-measures ANOVA, followed by Holm-Sidak's test.

3. Results

3.1 Determination of dentin loss

Significant differences were observed among all solutions, except between HMP and HMP+QC. Dentin wear was significantly higher for PLA than for all other groups. Also, the use of F+HMP+QC led to the lowest wear values among all study groups (Figure 2).

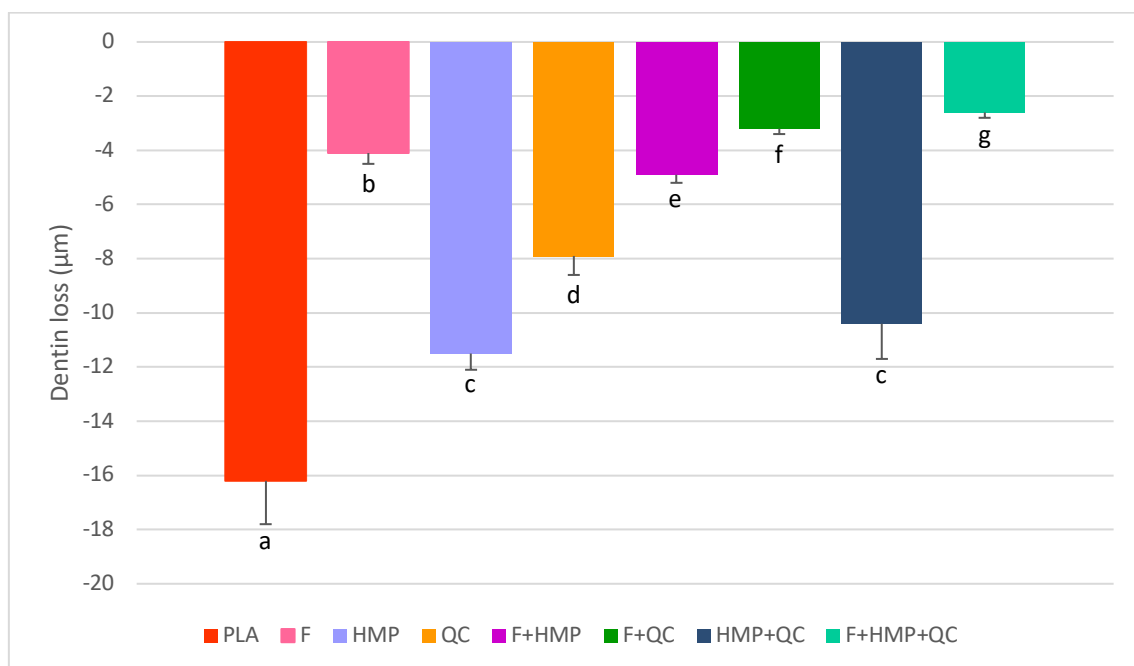


Figure 2. Mean values of dentin loss (μm) according to the experimental solutions. Different letters indicate significant differences among the groups. One-way ANOVA ($\log_{10}$ -transformed data) and Tukey's test ($p < 0.05$, $n = 12/\text{group}$). Bars indicate SD. Pla = Placebo; F = 1100 ppm fluoride; HMP = 1% sodium hexametaphosphate; QC = 0.03% quercetin.

3.2 Dentin hardness analysis

Table 1 shows mean (SD) values of integrated loss of hardness in depth (Δ KHN) according to the experimental solutions. Variance analysis revealed significant differences among treatments ($p < 0.001$) and between depths ($p < 0.001$), with significant interaction between the two variables ($p < 0.001$). The lowest Δ KHN values were observed for PLA, both at 5-30 μ m and at 30-70 μ m depths, which were significantly different from the other groups. At the shallowest depths (5-30 μ m), blocks treated with solutions containing F+HMP+QC had the highest Δ KHN values. No significant differences were observed among groups treated with F, QC, and F+HMP; among QC, F+HMP, and F+QC; and between HMP and HMP+QC. At the deepest depths (30-70 μ m), significant differences were observed only between HMP and QC; and between HMP and F+HMP.

Table 1. Mean (SD) values of dentin cross-sectional hardness (Δ KHN) according to groups in different depths

Groups	Δ KHN (5-30 μ m)	Δ KHN (30-70 μ m)
PLA	244.8 (30.7) ^{Aa}	1173.4 (57.1) ^{Ba}
F	546.0 (34.9) ^{Ab}	1371.1 (76.4) ^{Bbcd}
HMP	375.2 (52.6) ^{Ac}	1308.6 (82.8) ^{Bb}
QC	564.3 (44.3) ^{Abd}	1422.5 (60.2) ^{Bcd}
F+HMP	586.0 (55.1) ^{Abd}	1417.3 (90.8) ^{Bcd}
F+QC	628.7 (42.2) ^{Ad}	1364.3 (71.7) ^{Bbcd}
HMP+QC	391.5 (44.6) ^{Ac}	1347.8 (106.0) ^{Bbcd}
F+HMP+QC	724.3 (31.2) ^{Ae}	1374.2 (45.3) ^{Bbcd}

Different uppercase superscript letters indicate significant differences between the depths within each group. Different lowercase superscript letters indicate significant differences among the treatment groups within each depth. Two-way, repeated-measures ANOVA and Holm-Sidak's test ($p < 0.05$, $n = 12/\text{group}$). Pla = Placebo; F = 1100 ppm fluoride; HMP = 1% sodium hexametaphosphate; QC = 0.03% quercetin.

3.3 Gelatin zymography

Zymograms of gelatinolytic activity and band intensities determined by densitometry are shown in Figure 3. The enzymatic activities of MMP-2 and MMP-9 were visible by bands in PLA. The percentage of inhibition of MMPs -2 and -9 activity is shown

in Figure 4. Reduced activity of MMP-9 and both MMPs was observed for F and QC alone, respectively. Furthermore, MMP-2 proved to be slightly more sensitive to the presence of F than MMP-9 (100 and 93% of inhibition, respectively). The association of F and QC, however, promoted a complete antiproteolytic potential (for both MMPs). Also, incubation buffers containing HMP (alone or associated) completely inhibited the activity of MMP-2 and MMP-9.

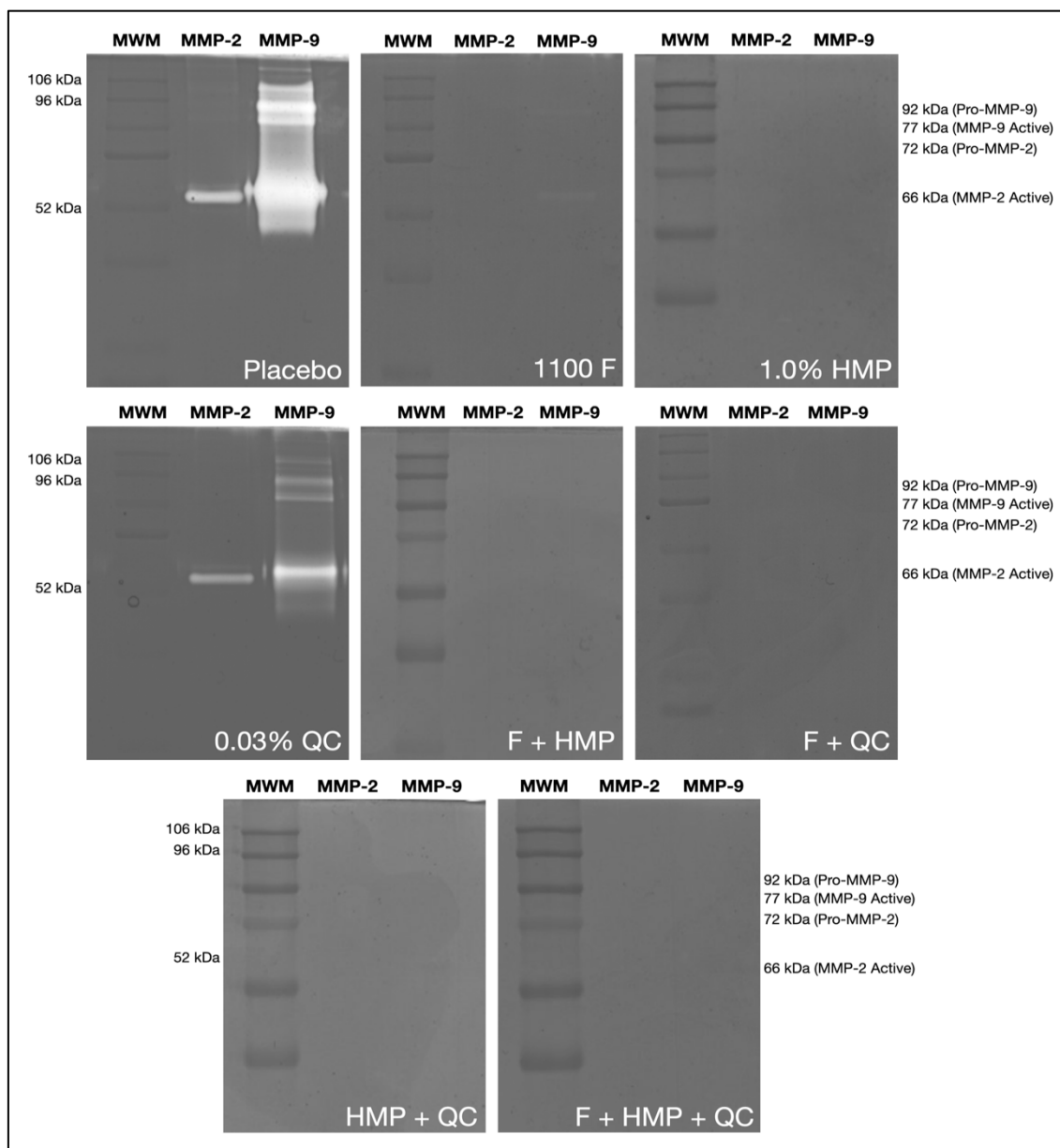


Figure 3. Representative image of zymography assay gel after electrophoresis showing the effect of F, HMP and/or QC on the gelatinolytic activity of MMPs-2 and -9. MWM: molecular weight marker; MMP-2: matrix metalloproteinase 2; MMP-9: matrix metalloproteinase 9. Pla = Placebo; F = 1100 ppm fluoride; HMP = 1% sodium hexametaphosphate; QC = 0.03% quercetin.

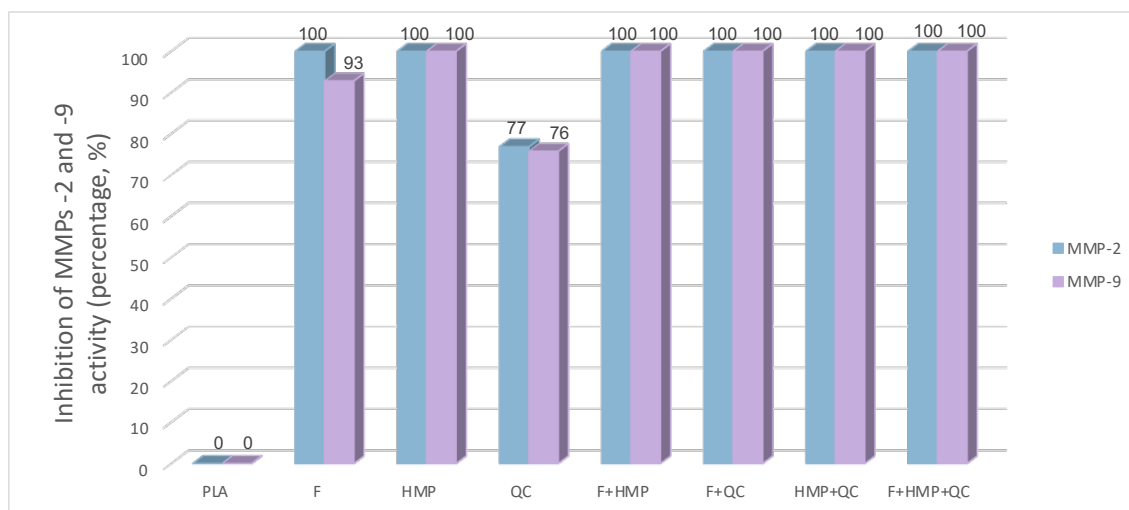


Figure 4. Percentage of inhibition of gelatinolytic enzyme activities (MMP-2 and MMP-9) according to the test solutions. MMP-2: matrix metalloproteinase 2; MMP-9: matrix metalloproteinase 9. Pla = Placebo; F = 1100 ppm fluoride; HMP = 1% sodium hexametaphosphate; QC = 0.03% quercetin.

4. Discussion

Tooth erosion is becoming an increasing problem worldwide due to its rising rates over the last decades, along with its cumulative effects throughout life [2,29]. Approaches to increase the benefits of F have been intensively investigated, yet with much to be explored. When it comes to dentin, in addition to its mineral content, the use of collagen crosslinkers and antiproteolytic agents can be considered promising strategies and have been increasingly investigated for dental erosion, dental caries, and bonding systems [6,9,11,17,18]. Nonetheless, this is the first study evaluating the potential of F, HMP and QC, when co-administered, in reducing dentin erosion and in promoting an antiproteolytic activity. Given that the addition of HMP and/or QC to F solutions enhanced the effects of F for most of pairwise comparisons (except for F+HMP regarding dentin loss and Δ KHN) and increased the enzymatic inhibition of MMP-9 only, both null hypotheses of the study were partially rejected.

Although F products have a great potential to prevent dental caries by reducing tooth demineralization and promoting its remineralization, its effects on tooth erosion are less outstanding [30], ought to the much lower pH of the erosive acids compared to those involved in dental caries (*i.e.*, bacterial acids) [30]. Considering this, the use of vehicles with high F concentrations (professionally applied) and/or the increased frequency of use of F products for self-application are the most common ways to prevent or reduce the

progression of tooth erosion [3]. In the present study, F was used at 1100 ppm and applied twice a day, aiming to attain conditions closer to the most usual for self-application by children and adults worldwide.

The use of polyphosphate salts is a proven effective strategy to increase F benefits on enamel caries and erosion, both under *in vivo* and *in situ* protocols [7,9,11,31,32]. In dentin, there are few studies assessing its benefits either on dental caries or tooth erosion [13,21,33,34]. For dentin caries, a recent work proved that the supplementation of F solutions with sodium trimetaphosphate (TMP) led to a higher remineralization rate of artificial caries lesions compared to its counterparts without TMP [33]. As for dentin erosive wear, Danelon *et al.* [13] showed that F varnishes supplemented with TMP have a higher protective effect than varnishes containing only F, for specimens submitted to erosive challenges followed or not by abrasion by toothbrushing.

Despite the favorable data using the cyclophosphate described above, no study has assessed the effects of HMP on dentin erosive wear. This polyphosphate has the capacity to interact with the tooth surfaces and, when adsorbed, it becomes negatively charged, allowing the retention of Ca^{2+} and CaF^+ ions. For enamel, this leads to the formation of a protective layer that restricts acid diffusion and increases calcium and fluoride penetration into tooth hard tissues [35]. The mechanisms above have been regarded as the reasons for the synergistic effects between HMP and F on both enamel de- and re-mineralization and erosive wear [7,9,11]. For dentin, on the other hand, such effects were not observed in the present study. In fact, when associated with F, HMP increased dentin loss and was not able to promote significant differences in ΔKHN (compared with F alone). Although not investigated in the present study, it is hypothesized that such effects might have resulted from HMP's chelating properties. Considering differences between enamel and dentin regarding mineral content and structure, the latter presents a more porous surface, thus with a larger surface area than enamel (especially considering intratubular dentin under *in vitro* conditions), what facilitates the penetration of HMP and consequent ion sequestration in deeper areas of dentin specimens, thus negatively interfering with F action.

As for QC, it is a polyphenolic flavonoid with the capacity to interact and modify dentin matrix regarding its crosslinking properties. This plays an important role on the prevention and control of dentin erosion, which makes QC a suitable alternative. Jiang *et al.* [18] showed that QC alone promoted lower dentin erosion than F, EGCG or chlorhexidine. In the present study, however, such an effect was not observed, considering that the group treated with QC alone presented significantly greater dentin

loss than the group treated with F alone. This unexpected pattern may be due to the different treatment protocols and parameters for erosive challenges, as detailed below.

In the study by Jiang *et al.* [18] dentin blocks were immersed in artificial saliva (AS) for 1 h prior to a single treatment with the solutions (2 min application with a microbrush under static conditions); blocks were immersed again in AS for 2 h prior to the first erosive challenge; and exposure to citric acid was performed under static conditions (5 min). Conversely, in the present study, blocks were not pre-treated with AS. Although immersion in AS is not able to form an acquired salivary pellicle, this may have led to changes in physical-chemical properties of dentin surfaces which, in turn, could impact the interaction between treatments and dentin [36]. Also, exposure to acid challenges immediately after the treatments in the present study may not have allowed enough time for the interaction of the actives with dentin, nor stabilization of a protective layer. Finally, exposure to acid challenges under dynamic conditions (as in the present study) is known to potentiate the dissolution of tooth minerals [37]. The factors above hinder any direct comparisons between the studies.

Dentin is a complex tissue composed of apatite, water, collagen and other proteins. When exposed to acids, peritubular/intertubular dentin minerals are initially released, enlarging dentin tubules. Eventually, a superficial layer of demineralized organic matrix (DOM) can be observed, which is known to be an important protective barrier against erosion and abrasion [38]. However, DOM can breakdown by host-derived salivary or endogenous dentin enzymes, such as MMPs, so that the use of MMP inhibitors is another potential and relevant strategy for the prevention or control of dentin caries and erosion. Accordingly, in the present study QC showed a partial protective effect against collagen matrix degradation by inhibiting 77 and 76% of MMP-2 and MMP-9, respectively, when used alone. Furthermore, QC increases tissue inhibitor metalloproteinase (TIMP-2) expression [39]. Interestingly, dentin treated with QC and exposed to erosive challenges was previously shown to present thicker DOM compared to treatment with F [18]. The preservation of DOM by QC, although not investigated in the present study, may be considered as a possible mechanism for the protection against dentin erosion (bulk loss of tissue) and mineral loss (Δ KHN) of the remaining tissue. It is also noteworthy that HMP was proven to have the capacity to completely inhibit the activity of MMPs -2 and -9 *in vitro*, in agreement with previous studies [21,40]. Incubation buffer containing F alone was effective at inhibiting MMP-2 (100%) and MMP-9 (93%) activity. When co-administered, either in pairs or all three actives together, the enzymatic inhibition of MMPs -2 and -9 was completely effective.

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 natural saliva instead of the artificial used in the present study. Also, the high organic content, and consequently, the elastic properties of the dentin [41] are factors that can influence mechanical measurement (profilometry and cross-sectional hardness). 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 wear and hardness analysis, thus providing more reliable data.

The synergistic effect of F+QC, and especially F+HMP+QC, both on dentin loss and on Δ KHN, is paramount from a clinical perspective, considering that the treatments were effective not only in leading to lower dentin loss, but also resulted in less softened dentin. The latter is extremely important, as it directly impacts on the resistance of the remaining dentin to future erosive challenges. Although the exact mechanisms were not investigated in the present study, the considerably better effect of the association of F+HMP+QC is probably due to a combination of the effects of each of the compounds, providing prevention in three different aspects: mineralization (F or F+HMP), crosslinking (QC), and MMP inhibition (F, HMP and QC).

To sum up, it can be concluded that the addition of HMP and QC to F solutions led to enhanced protection against dentin erosion, both at the surface (dentin loss) and in relation to the mineral content of the remaining tissue (Δ KHN), in addition to completely inhibiting MMPs -2 and -9 *in vitro*. Considering the promising results of the present study, the association of F, HMP and QC must be regarded as a valuable strategy not only for high-risk patients, but also for people in general, considering its benefits on dentin erosion. Further research on this topic employing *in situ* or *in vivo* protocols could bring relevant information on the actual clinical effects of the compounds when co-administered.

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Anexos

5. ANEXOS

ANEXO A

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[4] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13 March 2003).

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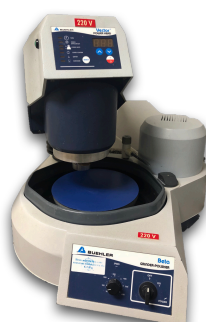
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ANEXO B

PREPARO DOS BLOCOS DE DENTINA



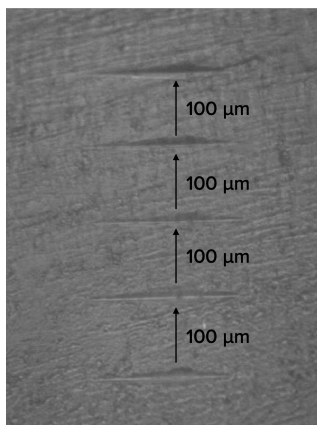
1. Bloco de dentina bovina medindo 4×4×2 mm (acima), obtido após secção transversal e longitudinal (abaixo) de raízes de incisivos inferiores bovinos.



2. Bloco fixado com cera pegajosa em suporte de acrílico antes (esquerda) e após (direita) polimento em politriz (Buehler, Lake Bluff, Illinois, Estados Unidos – ao centro) com lixas nas granulações 320, 600, 800, 1200 e discos de feltro com solução diamantada (CARBIMET PaperDiscs, Buehler, Lake Bluff, Illinois, Estados Unidos).

ANEXO C

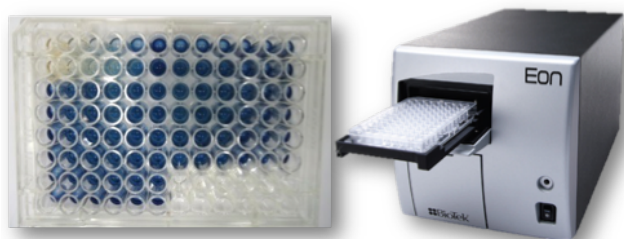
SELEÇÃO DOS BLOCOS QUANTO À DUREZA DE SUPERFÍCIE



Análise de dureza de superfície utilizando microdurômetro Micromet 5114 hardnesstester (Buehler, Lake Bluff, Estados Unidos e Mitutoyo Corporation, Kanagawa, Japão) (esquerda). À direita, imagem apresentando cinco endentações realizadas no centro dos blocos, com 100 µm entre elas. A carga utilizada foi de 10 g, por 10 segundos.

ANEXO D

PREPARO E DOSAGEM DAS SOLUÇÕES



As soluções de tratamento foram preparadas e, em seguida, foram realizadas as dosagens de:

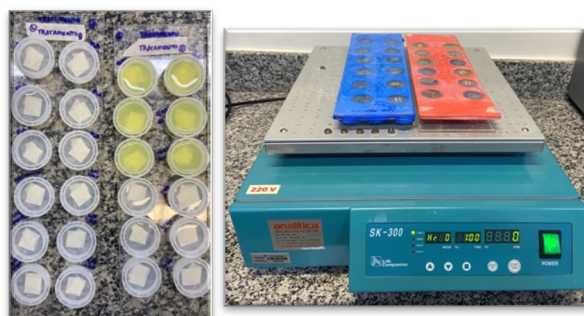
- Fósforo: à direita; método colorimétrico do molibdato;*
- Flúor: à esquerda; utilizando eletrodo específico para íon flúor (9609 BN; Orion Research Inc., Beverly, Mass., Estados Unidos).*

ANEXO E

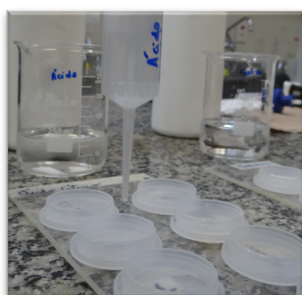
TRATAMENTOS E DESAFIOS EROSIVOS



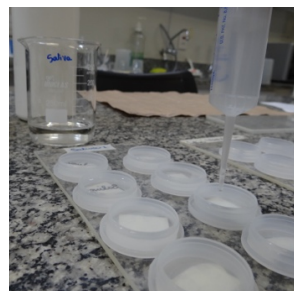
1. Aplicação de esmalte de unha ácido-resistente em metade da superfície de cada bloco de dentina (área hígida - controle).



2. Imersão dos blocos de dentina nas respectivas soluções de tratamento (esquerda) sob agitação a 35 r.p.m. em mesa agitadora (Shaker SK-300, Shaker SK-300, Jeio Tech, Coréia) por 1 min (direita),

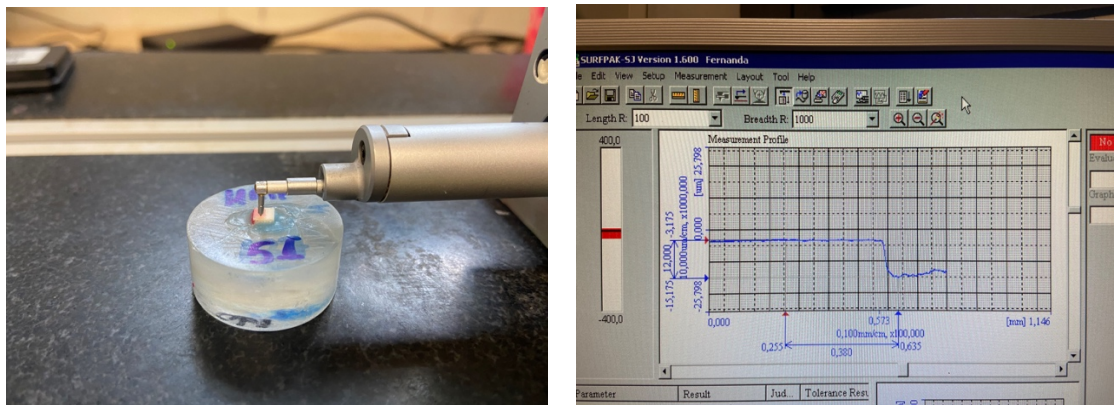


3. Desafios erosivos com imersão dos blocos de dentina em ácido cítrico (esquerda) sob agitação a 35 r.p.m. em mesa agitadora (Shaker SK-300, Jeio Tech, Coréia) por 90 s (direita), 4×/dia, por 5 dias.



4. Imersão dos blocos em saliva artificial (esquerda) por 2 horas e overnight em estufa a 37 °C (direita).

ANEXO F PERFILOMETRIA



À esquerda, rugosímetro adaptado para perfilometria (Programa Surfpak – Surftech Mitutoyo, Japão).

Cinco varreduras foram feitas da área hígida da dentina em direção à área erodida.

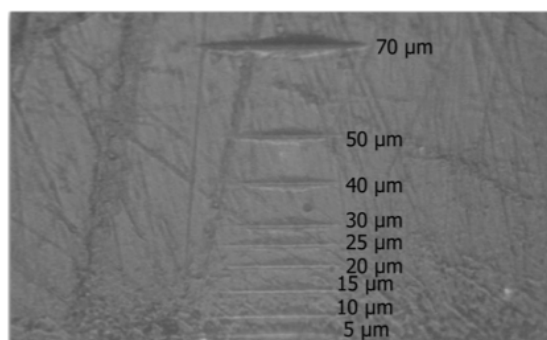
À direita, imagem do gráfico mostrando o degrau formado pela perda dentinária na área erodida.

ANEXO G

ANÁLISE DA PERDA DE DUREZA INTEGRADA EM PROFUNDIDADE (ΔKHN)



Os blocos foram seccionados longitudinalmente (1) e embutidos a frio em bases de resina acrílica com a superfície interna voltada para cima (2). Em seguida as bases foram polidas em politriz (Buehler, Lake Bluff, Illinois, Estados Unidos) com lixas nas granulações 320, 600, 800 e 1200 e discos de feltro umedecidos em solução diamantada (3) até que as faces internas dos blocos de dentina estarem expostas e livres de resina acrílica (4).



Em seguida, os espécimes de dentina foram analisados utilizando o microdurômetro Micromet 5114 hardnesstester (Buehler, Lake Bluff, Estados Unidos e Mitutoyo Corporation, Kanagawa, Japão) (esquerda), com carga de 2 g, por 10 s.

À direita, a imagem mostra as 9 impressões realizadas a partir da superfície externa do bloco nas profundidades de **5, 10, 15, 20, 25, 30, 40, 50 e 70 μm** .