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DESENVOLVIMENTO DE PROTOCOLO IN VITRO DO PROCESSO EROSIVO DO ESMALTE E EFEITO DE ENXAGUATÓRIO BUCAL FLUORETADO ASSOCIADO AO TRIMETAFOSFATO NANOPARTICULADO CONTRA A EROSÃO

Araçatuba - SP

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Desenvolvimento de protocolo *in vitro* do processo erosivo do esmalte e efeito de enxaguatório bucal fluoretado associados ao trimetafosfato nanoparticulado contra a erosão

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquista Filho", Campus de Araçatuba para obtenção do título de Doutor em Ciência Odontológica, área de concentração Saúde Bucal da Criança.

Orientador: Prof. Tit. Alberto Carlos Botazzo Delbem

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COC	Colégio Colombiano de Odontólogos
IAPD	International Association of Paediatric Dentistry
ASSOSALUD	Asociacion Nacional de Profesionales de la Salud

DEDICATORIA

A nosso Deus pai dos céus

Porque o senhor você escuta e concede a nossas necessidades, e forjar minha alma e coração com grandes provas, para servir e lograr ser o líder que o senhor espera que eu seja.

A nossa Senhora

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“Todo caminho inicia com um primeiro passo, mas também tem que acabar, agora fecho esta etapa de minha vida para iniciar uma nova”.

Palavras do autor.

EPIGRAFE

A ciência não é apenas compatível com a espiritualidade, é uma fonte profunda de espiritualidade.

Carl Sagan

Mancilla, JOFC. Desenvolvimento de protocolo *in vitro* do processo erosivo inicial do esmalte e efeito de enxaguatório bucal fluoretado associado ao trimetafosfato nanoparticulado contra a erosão. 2018. 49 f. Tese (Doutorado em ciência Odontológica, Área de Saúde Bucal da Criança) – Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2018.

RESUMO

O objetivo do presente estudo foi avaliar o efeito de enxaguatórios bucais fluoretados, suplementados ou não com trimetafosfato de sódio (TMP) micrométrico ou nanoparticulado, sobre a erosão do esmalte dental, utilizando uma boca artificial. Material e Métodos: 120 blocos de esmalte bovino foram aleatoriamente distribuídos em 5 grupos, de acordo com os seguintes enxaguatórios: Placebo (sem flúor ou TMP), 100 ppm F, 225 ppm F, 100 ppm F + 0,2% TMP microparticulado e 100 ppm F + 0,2% de TMP nanoparticulado. Os blocos foram subdivididos em 2 condições de experimento (1 ou 3 dias). Cada ciclo erosivo consistiu de 7 exposições a ácido cítrico (a cada 4 s), alternadas com 6 exposições a saliva artificial (a cada 7 s), três vezes ao dia. O tratamento com os enxaguatórios bucais foi realizado após o primeiro e último ciclo erosivo de cada dia, durante 1 min. Os blocos foram analisados por perfilometria, dureza de superfície e em secção longitudinal, bem como por energia livre de superfície (γ_s). Os dados foram analisados por ANOVA a 2 critérios, teste de Student-Newman-Keuls e coeficiente de correlação de Pearson ($p<0,05$). Resultados: De forma geral, um efeito protetor significativamente maior foi observado para os enxaguatórios bucais contendo TMP em relação à dureza e ao desgaste do esmalte, com um efeito adicional para o uso de nanopartículas. Houve uma moderada correlação entre a dureza de superfície e em secção longitudinal (r = -0,533; p<0,001). Em acréscimo, uma redução da γ_s e de seu componente apolar (γ_s^{LW}) e sítios doadores de elétrons (γ^{-}) foram observados nos grupos tratados com enxaguatórios contendo TMP. Além disso, diferenças significativas foram observadas para a maioria das variáveis analisadas com relação à duração do protocolo erosivo (1 e 3 dias). Conclusões: o tratamento com enxaguatórios bucais contendo TMP reduziu significativamente o desgaste erosivo do esmalte e a perda mineral deste em comparação aos enxaguatórios sem TMP, e tais efeitos podem ser relacionados a mudanças nos parâmetros de γ_s analisados. A boca artificial utilizada no estudo mostrou-se adequada para o estudo do desgaste erosivo do esmalte em condições *in vitro*.

Palavras-chave: Erosão dentária; Fluoretos; Fosfatos; Esmalte dentário, Dureza, Energia; Boca artificial

Mancilla, JOFC. Development of an *in vitro* protocol for the assessment of initial enamel erosion and effect of a fluoridated mouthrinse associated with nanosized trimetaphosphate against erosion. 2018. 49 f. Tese (Doutorado em ciência Odontológica, Área de Saúde Bucal da Criança) – Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista, Araçatuba, 2018.

ABSTRACT

The aim of the present study was to evaluate the effect of fluoride mouthrinses, supplemented or not with sodium trimetaphosphate (TMP), on the dental erosion of the enamel using an artificial mouth. Material and Methods: 120 blocks of bovine enamel were randomly distributed in 5 groups according to the following mouthrinses: Placebo (without fluoride or TMP), 100 ppm F, 225 ppm F, 100 ppm F + 0.2% TMP micrometric and 100 ppm F + 0.2% nanosized TMP. The blocks were subdivided into 2 experimental treatments (1 or 3 days). Each erosive cycle consisted of 7 exposures to citric acid (each 4 s), alternated with 6 exposures to artificial saliva (each 7 s), three times a day. Treatment with mouthrinses was performed after the first and last erosive cycle of each day for 1 min. The blocks were analyzed by profilometry method, surface hardness and longitudinal section analyses, as well as surface free energy (γs). Data were analyzed by two way ANOVA, Student-Newman-Keuls test and Pearson's correlation coefficient ($p < 0.05$). Results: In general, a significantly greater protective effect was observed for mouthrinses containing TMP in relation to the hardness and wear of the enamel, with an additional effect for the use of nanoparticles. There was a moderate correlation between surface hardness and longitudinal section ($r = -0.533$; $p < 0.001$). In addition, a reduction of γs and its apolar component (γs^{LW}) and electron donor sites (γ^{-}) were observed in the groups treated with TMP containing mouthrinses. In addition, significant differences were observed for most of the variables analyzed in relation to the duration of the erosive protocol (1 and 3 days). Conclusions: The treatment with mouthrinses with TMP reduced significantly the erosive enamel wear and the mineral loss compared to non-TMP mouthrinses, and such effects may be related to changes in the γs parameters. The artificial mouth used in this study was adequate for the enamel erosive studies under *in vitro* conditions.

Keywords: Tooth erosion, fluorides, phosphates, dental enamel, hardness, energy, Artificial Mouth.

SUMARY

In vitro effects of fluoride mouthrinses containing micrometric or nanosized trimetaphosphate against enamel erosion using artificial mouth

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Running title: *In vitro* evaluation of mouthrinses with TMP in artificial mouth

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In vitro effects of fluoride mouthrinses containing micrometric or nanosized trimetaphosphate against enamel erosion using artificial mouth

Abstract

Objectives: To assess the effect of fluoridated mouthrinses supplemented or not with micrometric or nanosized sodium trimetaphosphate (TMP) on enamel erosion, using an artificial mouth. Material and Methods: 120 bovine enamel blocks were randomly assigned into 5 groups, according to the following mouthrinses: Placebo (without fluoride or TMP), 100 ppm F, 225 ppm F, 100 ppm F + 0.2% micrometric TMP, and 100 ppm F + 0.2% nanosized TMP. Blocks were further divided into two test conditions (1- or 3-day). Erosive cycles were performed 3×/day, and treatment with the mouthrinses was performed twice/day. Blocks were analyzed by profilometry, surface and cross-sectional hardness, and surface free energy (γ_s). Results: overall, a higher protective effect was observed for the TMP-containing mouthrinses regarding enamel hardness and wear, with an additional effect for the use of nanoparticles. Moreover, a reduction of γ_s and its apolar component (γ_s^{LW}) and electron donor sites (γ_s^{-}) were observed in the groups with TMP. Conclusion: Treatment with TMP-containing mouthrinses significantly reduced enamel erosive wear and enamel mineral loss, and such effects were related with changes in γ_s parameters. The artificial mouth was shown to be suitable for the study of enamel erosive wear under *in vitro* conditions.

Keywords: Tooth erosion, fluorides, phosphates, dental enamel, hardness, energy.

1. Introduction

Dental erosion is a chemical process by which the mineral structure of the tooth is gradually dissolved by acids not involving bacteria, which subsequently leads to the softening of the remaining tooth surface (enamel or dentine)[1–4]. The harmful effects of acidic foods and drinks may be counteracted, at some extent, by saliva (due to its dilution effect and buffers and mineral content[5–7], as well as by Ca and P ions present in the medium (foods or drinks) at the time of exposure[1,3,8,9]. The duration and frequency of exposure to acids are also important factors that may influence enamel dissolution[10,11], In turn, when the enamel softened by acids is subjected to mechanic forces, this condition is classified as erosive tooth wear[1,9,12–14].

The effects of fluoride in reducing enamel erosion and/or to promote enamel rehardening after exposure to acids, have been intensively studied over the last decade, with results showing a limited effect of conventional fluoride formulations[15]. On the other hand, the use of stannous fluoride[16,17], titanium tetrafluoride[16,18], or the association of fluoride with phosphate salts[15,16], has shown promising results under *in vitro*[19] and *in situ*[20] conditions.

A common issue regarding *in vitro* investigations on dental erosion is the wide variation of study protocols, related to the type of acid, pH, length of exposure to the acid, mode of exposure (under static or dynamic conditions) number of challenges per day, interval between challenges, total duration of the experiment, among others[10,11], which may affect the results obtained and make it difficult (if not impossible) to compare the outcomes of different investigations[1,13,21]. All the above indicate the need for a better standardization of *in vitro* protocols, which should be ideally designed to mimic as precisely as possible the pattern of beverage consumption by individuals of all ages[22].To ensure a better reproduction of these protocols, the use of artificial mouth is feasible[23,24] as they allow the control of some of the above-mentioned variables during the erosive cycles.

In vitro studies provide useful data for a better understanding on the effects of the erosive challenge, as well as the impact of measures aiming to reduce mineral loss and/or promote remineralization of the partially demineralized surface after acid exposure[10,11,25–27]. Despite enamel wear (assessed by profilometry) is regarded as the main response variable in erosion studies, the inclusion of other analytical approaches may provide additional insights for a better understanding of the complex interplay among erosive and protective factors[28,29]. In this sense, while surface and cross-sectional hardness have been often used for this purpose[30–33], no study has yet assessed the surface free energy of enamel in erosion models. This analysis could provide important data on the changes of enamel polarity and electron donor sites, which may be also instructive in understanding the effects of both the erosive challenge and the therapeutic agent assessed.

Therefore, this study evaluated the effect of fluoridated mouthrinses, supplemented or not with micrometric or nanosized TMP on enamel erosion, hardness and surface free energy after 1 or 3 days of erosive challenge, using an artificial mouth set to mimic the consumption of acidic drinks by young subjects[22]. The null hypothesis was that the effect of TMP-containing mouthrinses on the response variables would not be different from their counterpart without TMP mouthrinses.

2. Materials & Methods

2.1. Experimental design

Bovine enamel blocks (n=120) were selected by surface hardness and randomly assigned to the following five experimental mouthrinses (n=24): Placebo (without F or TMP); 100 ppm F (100 ppm F); 225 ppm F (225 ppm F); 100 ppm F plus 0.2% micrometric TMP (100 F TMPmicro) and 100 ppm F plus 0.2% nanosized TMP (100 F TMPnano). The sample size of 12 enamel blocks was calculated based on previous study[34] considering as primary outcome the enamel wear (µm), adopting the mean difference between the groups and standard deviation of 0.60 and 0.40, respectively, α-error of 5% and a β-error of 20%. Blocks were further divided into two groups, according to the length of exposure to the erosive challenges (1 or 3 days). The erosive challenge was produced by alternate dripping cycle with citric acid and artificial saliva, in an artificial mouth, three times per day. The blocks were exposed to mouth rinses for one minute at the end of the first and last cicling of erosive treatment for day. The factors studied were mouthrinses and length of the protocol. Enamel wear[28,29,35,36], surface and cross-sectional hardness[33,35,37], and surface free energy[38,39] were analyzed as response variables.

2.2. Enamel blocks preparation

Enamel blocks (4 × 4 × 4 mm) were obtained from the buccal-cervical region of bovine incisors, previously stored in 2 % formaldehyde solution for 30 days. The teeth were extracted from ~3 year old bovine and same race (Nelore Cattle). The surface of the blocks was ground flat with water-cooled silicon carbide paper disks (600, 800, and 1,200 grit, Extec, Enfield, CT, USA) with a final polish using a felt disk (Extec) in a grinding polisher (Vector-Phoenix Beta, Buehler, Lake Bluff, Il, USA) wearing out ~200 µm of enamel surface[33]. Between each grit and at the end, the blocks were cleaned by ultrasound (Unique USC 1400, Indaiatuba, SP, Brazil) at 40 Hz, in deionized water for 20 minutes, at room temperature. As inclusion criteria, blocks should be flat, with no scratches, cracks or hypoplasia. After polishing, Blocks (n=120) with initial SH numbers (KHN) ranging from 320.0 up to 380.0 KHN[35,40–42] ($p=0.677$) were selected. Two layers of acid-resistant nail varnish (Risquê, Cosmetic Ind.,Barueri,SP,Brazil) were applied in the middle of each block, in order to maintain reference surfaces for the analysis of enamel wear by contact profilometry.

2.3. Synthesis and Characterization of the nanosized TMP

To synthesize nanosized TMP, 70 g of pure (micrometric) sodium trimetaphosphate ($Na_3O_9P_3$, Aldrich, purity 95% CAS 7785- 84-4) was ball milled using 500 g of zirconia spheres (diameter of 2 mm) in 1 L of isopropanol. After 48 h, the resulting powder was separated from the alcoholic media and ground in a mortar. The powder crystallinity was characterized by X-ray diffraction (XRD) using a Rigaku Dmax 2500 PC difractometer in the range of $10° \leq 2\theta \leq 80°$, with a scanning rate of 2° sweep speed/min[35]. The milling process produced particle size of ~22.7 nm with XDR pattern of TMP without altering its crystalline structure.

2.4. Mouthrinses formulation and determination of pH, F and P concentrations

The mouthrinses were prepared using sodium methyl-p-hydroxybenzoate, sodium saccharin, glycerol and water. Sodium fluoride (Merck KGaA Co, Darmstadt, Germany) was added to the formulations, except in the case of the placebo group, up to a final concentration of 100 or 225 ppm F. The mouthrinses containing 100 ppm F also contained 0.2% of micrometric TMP (TMPmicro) or nanosized TMP (TMPnano). The mouthrinses were checked for pH, F and P concentrations prior to the beginning of the erosive cycle. The pH values were measured using a pH electrode (Gel-Filled Plastic pH Electrode 13-620-290, Fisher Scientific accumet® Electrodes, Ottawa, Ontario, Canada) calibrated with standard solutions of pH 4.0 and 7.0[43]. Fluoride was determined by ion–specific electrode method (9609–BN, Thermo Orion, Beverly, MA, USA), which ranged from 0.5 to 8.0 ppm F for calculation of the concentration in ppm F[43]. TMP was indirectly determined through colorimetric assessment of P[44], preceded by the acid hydrolysis[45].

The mean (SD) values of fluoride (ppm) in placebo, 100 ppm F, 225 ppm F, 100 F TMPmicro and 100 F TMPnano, were 4.4 (0.1), 103.2 (1.1), 227.7 (4.7), 101.8 (2.1) and 99.1 (1.5), respectively. The mean (SD) values of TMP were: -0.85 (0.0), -0.79 (0.0), -0.76 (0.1), 597.5 (30.5) and 581.1 (28.6). The mean of pH were: 6,09 (0,04), 5,99 (0,02), 6,01 (0,04), 6,50 (0,03) and 5,96 (0,03). All analyses were performed in triplicate.

2.5. Erosive protocol - Artificial Mouth

The artificial mouth consists of two vessels of 5 L for acid solutions and artificial saliva, and a thermostat regulator, which was set at 37° C. The equipment works by means of continuous dripping system by pumps, delivering the solutions to individual niches where 12 specimens were positioned simultaneously. The control panel was programmed according to cycling's number and times both for 0.05 M acid citric (Labsynth Co., Diadema, SP, Brazil), pH 3.2 adjusted with saturated NaOH (PA-ACS, PM 40.00, Dinâmica Química Contemporânea, Indaiatuba, SP, Brazil)[24,46], and artificial saliva (1.5 mmol/L $Ca(NO_3)_2$-$4H_2O$, 0.9 mmol/L NaH_2PO_4-$2H_2O$, 150 mmol/L KCl, 0.1 mol/L Tris buffer, 0.03 ppm F, pH 7.0; Sigma-aldrich Co., San Luis, Missouri, USA)[33,36]. The pump speed of each solution was set in mL/min. The configuration of the erosive cycling were based in previous study conducted with young subjects, in which the consumption of their preferred acidic drink (orange juice or soft drink) was recorded regarding the length of each sip, total number of sips and length of each interval between sips[22].

Each erosive cycle comprised 7 exposures to citric acid (dripping for 4 seconds- 8 µL / block), alternated with 6 exposures to artificial saliva (dripping for 7 seconds 14.6 µL / block). Between cycles, artificial saliva was continuously dripped during 120 min (15mL / block) , totaling 3 cycles per day. Treatment of the blocks was performed after the first and last cycles of the day, for 1 minute, by immersion in 4 mL of mouthrinse in agitation to 37 ^{o}C. Before and after each treatment, blocks were washed in deionized water during 10 seconds and dried with a soft napkin. At end of the day, the blocks were stored in artificial saliva at 37° C, statically.

2.6. Enamel Wear Analysis

At the end of each experimental period (1 or 3 days), the nail varnish was carefully removed from the control areas of each block with acetone-soaked cotton wool, prior to analysis of surface wear by profilometry (Surftest SJ 401 – Mitutoyo Corporation, Kanagawa, Japan). The configuration of the equipment was: 80 µm range at a speed of 0.5 mm/s, 0.8 ʎC for vertical surface accuracy, and a Ra Slope between 2.91 µm – 3.02 µm[28,29]. Profilometric traces were taken from the no-eroded surfaces (control) across the eroded surfaces (length = 2 mm). Five readings were performed on each block, and enamel wear was determined as the distance (µm) between the mean line on the graph corresponding to the baseline and the peak wear[23,28].

2.7. Hardness Analysis

The enamel surface hardness (SH) was determined using a Micromet 5114 hardness tester (Buehler, Lake Bluff, IL,USA and Mitutoyo Corporation, Kanagawa, Japan) and the software Buehler OmniMet (Buehler, Lake Bluff, IL,USA) with a Knoop diamond indenter under a 25 g load for 10 s. The Final SH (KHN) was determined with the same parameters described previously. Five impressions separated from each other by a distance of 100 µm were made in the central region of each block[37] at 300 µm from the interface between the control and eroded areas. The same procedure was done for the determination of initial SH.

Following, blocks were sectioned longitudinally at the center, and half of each block was embedded in autopolymerizing incolor acrylic resin (Vipiflash, Vipi Co., Pirassununga, SP, Brazil) and subsequently polished. Cross-sectional hardness was determined with a Knoop diamond indenter, with a load of 5 g during 10 s[33] in a hardness tester (Micromet 5114, Lake Bluff, IL, USA). A sequence of nine indentations was made at distances of 5, 10, 15, 20, 25, 30, 40, 50 and 70 µm from the outer edge of the enamel in the non-eroded (control) and eroded parts of enamel. The integrated area (KHN × µm) of the control area was calculated using the trapezoidal rule (GraphPad Prism, version 3.02; GraphPad Software Inc, La Jolla, CA, USA) and subtracted from the integrated eroded area, resulting in integrated loss of hardness in depth of enamel (ΔKHN)[38].

2.8. Surface Free Energy Analysis

In order to physically/chemically characterize the specimens prior to the beginning of the study (sound enamel, n=12), as well as after the completion of each experimental phase (1 or 3 days), all the blocks were analyzed by contact angle measurements, using the sessile drop method to determine the surface free energy (γ_s). Measurements were performed by an automatic goniometer (DSA 100S, Krüss, Hamburg, Germany) using three probing liquids: water (polar), diiodomethane (apolar), and ethylene glycol (polar with acid and base components). The blocks were air dried for 45 min in order to stabilize the enamel surface enamel[46,47]. After, 0.3 μL of each liquid was automatically dispensed on a different quadrant of enamel surface of each block using a glass syringe (500 μL) and a needle of 0.5 mm gauge[38,39]. After 1s, the contact angles (right and left) were measured using the images captured by a CCD camera and Tangent method (Drop Shape Analysis DSA4 Software, version 2.0-01, Krüss). Each drop was measured 5 times during 5 s at 20° C[46,47] and relative humidity of 36 ±1. The γ_s (mN/m) and its apolar (γ_s^{LW}: Lifshiz van der Waals) and polar (γ_s^{AB}: acid/base) components as well as acid (γ_s^{+}, receptor component) and base (γ_s^{-}, donor component) polar parameters were calculated according to the model of van Oss[48], Chaudhury[49] and Good[50]. The free energy of interaction (ΔG_{sws}) between water and enamel also was calculated to determinate the hydrophobicity/hydrophilicity of enamel surface. When $\Delta G_{sws}>0$, surface was considered hydrophilic, and if $\Delta G_{sws}<0$, surface was considered hydrophobic[48,51,52].

2.9. Statistical Analysis

Statistical analyzes were performed using SigmaPlot software version 12.0

(SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) and the limit of significance was set at 5%. The values of enamel wear (µm), final SH and ΔKHN, and surface free energy were considered as outcome measures and the mouthrinses and length of erosive protocol (1 or 3 days), as the variation factors. The data were submitted to two-way analysis of variance, after exhibits normal and homogeneous distribution, followed by the Student-Newman-Keuls test. Pearson's correlation was applied to compare SH and ΔKHN data.

3. Results

No significant differences were observed among the groups regarding enamel wear after 1-day of erosive challenge ($p>0.05$). However, final surface hardness (SH) was significantly higher for groups treated with both TMP-containing mouthwashes compared with others groups ($p<0.001$), without difference between TMP micro and nano ($p=0.995$) (Table 1). Furthermore, significant differences in ΔKHN were observed among all groups ($p<0.05$), with 100 F TMPnano promoting the lowest values among all ($p<0.05$). After 3 days of acid challenge, the placebo mouthwash promoted the highest enamel wear, SH and ΔKHN ($p<0.001$), while the 100 F TMPnano promoted the lowest values ($p<0.05$). A significant difference between 1-day and 3-day was observed for all the results ($p<0,001$), except for each TMP-containing mouthwash regarding ΔKHN data ($p>0.05$). There was a moderate correlation between SH and ΔKHN (Pearson's $r = -0.533$; $p<0.001$).

Data on surface free energy (γ_s) and apolar component (γ_s^{LW}) were higher for placebo, 100 ppm F and 225 ppm F when compared to sound enamel ($p<0.05$), with no significant difference among these after 1-day erosion (Table 2). TMP groups and sound enamel showed no significantly different values among each other ($p>0.05$). Values of the polar component (γ_s^{AB}) were not significantly different among the groups ($p>0.05$), except for 100 F TMPmicro. After 3-days (Table 2), all groups presented higher values of γ_s compared with sound enamel ($p<0.05$), except 225 ppm F. Placebo and 100 ppm F showed higher values of γ_s^{LW} ($p<0.001$) compared with other groups, with no significant between each other ($p=0.058$). Sound enamel, 225 ppm F and TMPs groups presented no difference for of γ_s^{LW} ($p>0.05$). Except for sound enamel and 100 TMPm, the values of γ_s^{AB} were higher compared to 1-day ($p<0.05$).

Regarding electron-receptor sites (γ_s^{+}: acid-Lewis), no significant differences were verified among the groups and between the length of erosive challenge ($p=0.171$) (Table 3). For the 1-day erosion protocol, values of electron-donor sites (γ_s^{-}: base-Lewis) were higher for placebo and 100 ppm F compared to other groups ($p<0.05$), without significant difference between each other ($p=0.792$). Furthermore, sound enamel and 225 ppm F groups were not significantly different ($p=0.854$), and the 100 F TMPnano group presented lowest values of γ_s^{-} ($p<0.05$). Except for sound enamel ($p=0.845$), the values of γ_s^{-} were lower after 3-days of erosion compared with 1-day ($p<0.001$). Free energy of interaction (ΔG_{sws}) presented positive values among sound enamel, placebo, 100 ppm F and 225 ppm F ($p>0.05$), after 1-day of erosion (Table 3). Negative values were observed for TMP groups ($p>0.05$). For the 3-day protocol, all groups showed negative values of ΔG_{sws} ($p<0.001$).

4. Discussion

This in vitro study evaluated the effect of mouthrinses containing 100 ppm F associated with micrometric or nanosized TMP on enamel erosion during, 1-day or 3-days, using an artificial mouth in order to closer mimic the pattern of beverage intake by young adults. The results showed that TMP-containing mouthrinses exhibited a significantly greater reduction of enamel erosion (Table 1). Thus, the null hypothesis was rejected.

The erosive challenge produced by the artificial mouth was based on the usual habits of consumption of acidic beverages previously determined in a population comprising young adults, regarding the mean number of sips, length of each sip and length of each interval among sips[22]. Despite significant differences among the mouthrinses were not detected by contact profilometry for the 1-day protocol, it was noteworthy that surface (SH) and cross-sectional (ΔKHN) hardness were shown to be suitable for analyzing minor changes on enamel surface[53] and into depth in the enamel[30–32], respectively. It is important to know that the values of enamel wear determined after 1-day of erosive challenge were below the limit of detection from the contact profilometry that is 0.4 um (Attin, 2006). However, hardness analysis were able to determine a clear dose-response relationship between fluoride concentration in the mouthrinses without TMP (*i.e.*, placebo, 100 ppm F and 225 ppm F) and enamel demineralization (SH and ΔKHN) (Table 1). TMP-containing mouthrinses decreased by 22% the surface softening of the enamel compared with 225 ppm F group. Meanwhile, the great impact was in the extension of the demineralization in depth. Cross-sectional hardness analysis shows an increment of 36% with TMPmicro and 67% with TMPnano. Probably, the effect of nanoparticle is related with its higher reactivity with enamel. Notwithstanding, significant differences in enamel mineral loss among groups were only observed for cross-sectional hardness. Moreover, higher values of γ_s for placebo, 100 ppm F and 225 ppm F groups (compared with sound enamel) might indicate an increase in enamel surface roughness, being γ_s^{LW} was the component that most contributed to this trend. The increase of apolar energy has been associated with slight roughness changes, with γ_s^- decreasing to zero for poly(methyl methacrylate), it characterizing as hydrophobic[54]. Differently, enamel more affected by erosion (placebo, 100 ppm F and 225 ppm F) present a greater exposition of phosphate groups leading to small increases in the γ_s^- and positive ΔG_{sws} values, indicating a hydrophilic surface. As enamel blocks were storage overnight in artificial saliva, after erosive challenfe, the γ_s^- should be reduced. For groups treated with TMP-containing mouthrinses, on the other hand, the lack of changes in γ_s^{LW} and the decrease of the γ_s^- component may indicate calcium phosphate deposition (from artificial saliva), leading to smoothing of the irregularities of enamel caused by the acid challenge[54]. Based on previous data with a similar cycle phosphate (sodium hexametaphosphate), it is likely that TMP adsorption on enamel by exposure to the mouthrinse leads to an increase in the γ_s^- component on enamel surface[38]. Upon exposure to artificial saliva, these values are reduced due to precipitation of calcium phosphate[19,38]. It is known that dental substrate does not favor deposition of calcium phosphate[38] even after treatment with fluoride[19,55].

A lower mineral loss for the groups treated with the TMP mouthrinses is related to their ability to adsorb on enamel, acting as a barrier against acid diffusion into the enamel[30–32]. On the other hand, the effect of fluoride mouthrinses against enamel demineralization depends on CaF_2 deposition[56]. However, CaF_2 is not able to block acid diffusion to deeper layers of enamel[57], and the critical pH for its dissolution is reached during the erosive cycle[4]. As the addition of TMP in the mouthrinses does not enhance CaF_2 deposition on hydroxyapatite[56], the adsorption of TMP on enamel occurs between OH^- from hydroxyapatite and TMP[58], thus reducing mineral loss (Table 1). Furthermore, TMP may favor the precipitation of hydroxyapatite with high Ca/P ratio and low solubility[56,58]. Although the results from contact profilometry were below of detection limit, hardness analysis (ΔKHN) showed that reducing the particle size of TMP enhance its capacity to bind on enamel surface and inhibit acid diffusion. Their high ratio of surface area to volume, and consequently a greater percentage of atoms on the surface make these nanoparticles more reactive than microparticles[33,35,59]. The studies show higher retention of calcium and phosphate in enamel[42,60] and greater mineral gain in depth of enamel[59]. The maintenance a harder enamel in depth may have contributed to the lower enamel wear after 3-days of erosive challenge especially considering that ΔKHN results did not differ between the 1- and 3-day protocols.

Based on the above, the lower enamel wear of the TMP-treated groups after 3-days of erosive challenges is related to the capacity of TMP in reducing enamel softening. The similar results between 100 F TMPmicro and 225 ppm F mouth rinse denote that the product precipitated on enamel in the presence of fluoride (225 ppm F) during the 3-days protocols erosion minimizes enamel wear, which seems to occur more on the outer surface than in depth of enamel[4,15,18]. In addition to reducing enamel wear, these precipitates can smooth the irregularities in the eroded area, since the γ_s^{LW} component decreased[54] and the calcium phosphates deposited with fluoride reduce γ_s^- (Table 3)[19,38]. In contrast, enamel treated with placebo and 100 ppm F showed higher γ_s^{LW} and lower γ_s^- values than sound enamel, suggesting an eroded area with more irregularities and with higher hydrophobicity[54]. As mouthrinses containing TMP enhanced the precipitation of calcium phosphate[19,38], γ_s^- is shown with reduced values and γ_s^{LW} with unchanged values, consistent with a eroded area less irregular.

The use of the artificial mouth for *in vitro* studies allows a better standardization of the erosive cycles, and consequently the the erosive effects on the tooth surfaces[23]. Nonetheless, variations among different artificial mouth models[17,24,61], as well as other variables may significantly impact the results obtained. These variables may include (but are not restricted to) the type of acid, pH and temperature of the solution, formulation and temperature of the artificial saliva, length of each challenge, number of challenges per cycle, total number of cycles per day, and flow rate of the solutions used in the model. All the above strengthen the need for an updated erosive protocol, that takes into account all variables that may be relevant from a clinical standpoint, to be reproducible in *in vitro* studies. These factors are decisive for the good use of the artificial mouth and achieve successful studies, either with citric acid[28,61] or hydrochloric acid[17]. It is noteworthy that two previous investigations not using an artificial mouth adopted similar parameters to those used in the present study regarding the pH and type of acid, and the exposure time to the studied solutions[33,62], what reinforces that that the parameters used in the present study are reliable[22].

5. Conclusion

Treatment with TMP-containing mouthrinses significantly reduced enamel erosive wear and enamel mineral content compared with mouthrinses without TMP, with an additional effect for the use of nanoparticles. The effects of TMP seem to be related with changes in the parameters of enamel surface free energy, what adds to the body of evidence regarding the effects of fluoridated formulations containing TMP. The parameters adopted for the acid challenges in the artificial mouth, based on clinical observations, indicate that this approach is suitable for the study of enamel erosive wear under *in vitro* conditions.

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Disclosure Statement

The corresponding author has a patent for a product used in the study, by the National Institute of Industrial Property - INPI/SP, on 06/16/2010 under number C1 0801811-1, and granted on April 11, 2017.

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Table legend

Table 1 – Mean values (SD) enamel wear and surface hardness (SH) and integrated loss of subsurface hardness (ΔKHN) according to groups and time of erosion challenge (n=12)

Table 2 – Mean values (SD) of surface free energy (γ_s) and their components apolar (γ_s^{LW}) and polar (γ_s^{AB}) according to groups and time of erosion challenge (n=12)

Table 3 – Mean values (SD) of receptor (γ_s^{+}) and donor (γ_s^{-}) electrons from polar energy and free energy of interaction (ΔG_{sws}) according to groups and time of erosion challenge (n=12)

Table 1 – Mean values (SD) enamel wear, surface hardness (SH) and integrated loss of hardness in depth of enamel (ΔKHN) according to groups and time of erosion challenge (n=12)

Groups	**Wear (μm)**		**SH (KHN)**		**ΔKHN (KHN × μm)**	
	1-day^A	**3-day**^{B}	**1-day**^{A}	**3-day**^{B}	**1-day**^{A}	**3-day**^{B}
Placebo	0.55^a (0.18)	3.75^a (0.65)	213.4^b (15.4)	156.1^b (17.5)	3,364^b (831)	4,132^b (859)
100 ppm F	0.53^a (0.15)	2.75^b (0.59)	214.9^b (11.7)	143.4^c (10.4)	1,990^c (523)	2,471^c (394)
225 ppm F	0.46^a (0.08)	2.17^c (0.47)	246.2^c (13.6)	176.5^d (16.3)	1,465^d (454)	2,488^c (711)
100 F TMPmicro	0.46^a (0.13)	2.21^c (0.13)	300.2^d (14.6)	181.8^d (17.8)	941^e* (476)	1,247^d* (521)
100 F TMPnano	0.34^a (0.06)	1.31^d (0.61)	300.3^d (12.3)	204.1^e (17.1)	489^f* (284)	772^e* (296)

Equal lowercase letters indicate lack of significant differences among groups (within each column). Distinct uppercase letters means significant difference between the times of erosion challenge for each response variable. Asterisks (*) indicate lack of significant difference between times of erosion (two-way ANOVA, Student-Newman-Keuls's test, $p<0.05$).

Table 2 – Mean values (SD) of surface free energy (γ_s) and their components apolar (γ_s^{LW}) and polar (γ_s^{AB}) according to groups and time of erosion challenge (n=12)

Groups	**γ_s (mN/m)**		**γ_s^{LW} (mN/m)**		**γ_s^{AB} (mN/m)**	
	1-day^{A}	**3-day**^{B}	**1-day**^{A}	**3-day**^{B}	**1-day**^{A}	**3-day**^{B}
Sound	23.3^{a}* (2.0)	23.5^{a}* (2.9)	27.2^{a}* (1.9)	27.4^{a}* (2.0)	-4.0^{a,b}* (1.9)	-4.3^{a}* (2.2)
Placebo	26.8^{b} (1.2)	32.6^{b} (2.1)	30.3^{b}* (1.3)	30.6^{b}* (2.0)	-3.5^{a,b} (1.9)	2.1^{b} (1.1)
100 ppm F	25.5^{b} (1.7)	28.5^{c} (1.3)	30.5^{b} (1.3)	31.8^{b} (1.2)	-5.0^{a} (2.2)	-3.2^{c} (1.3)
225 ppm F	25.8^{b}* (1.3)	24.5^{a,d}* (2.0)	30.1^{b} (1.3)	26.6^{a} (1.2)	-4.3^{a,b} (1.7)	-2.1^{c} (1.5)
100 F TMPmicro	23.2^{a} (1.1)	25.6^{d} (1.3)	26.1^{a} (1.1)	27.8^{a} (1.3)	-2.9^{b}* (1.3)	-2.2^{c}* (0.5)
100 F TMPnano	22.0^{a} (1.9)	25.4^{d} (1.6)	27.0^{a}* (1.4)	27.7^{a}* (1.5)	-5.0^{a} (2.1)	-2.4^{c} (1.0)

Equal lowercase letters means no significant differences among groups (column). Distinct uppercase letters means significant difference between the times of erosion challenge. Asterisks (*) indicate lack of significant difference between times of erosion. (two-way ANOVA, Student-Newman-Keuls's test, $p<0.05$).

Table 3 – Mean values (SD) of electron receptor (γ_s^+) and donor (γ_s^-) sites from polar energy and free energy of interaction (ΔG_{sws}) according to groups and time of erosion challenge (n=12)

Groups	**γ_s^+ (mN/m)**		**γ_s^- (mN/m)**		**ΔG_{sws} (mN/m)**	
	1-day^A	**3-day^A**	**1-day^A**	**3-day^B**	**1-day^A**	**3-day^B**
Sound	0.2^a (0.1)	0.2^a (0.2)	29.1^{a}* (1.3)	29.2^{a}* (1.4)	5.6^{a}* (2.3)	5.9^{a}* (2.5)
Placebo	0.2^a (0.2)	0.1^a (0.1)	31.3^b (0.8)	13.2^b (1.8)	8.7^a (1.4)	-29.1^b (5.6)
100 ppm F	0.3^a (0.2)	0.2^a (0.2)	31.1^b (2.5)	9.1^c (1.9)	8.2^a (3.9)	-40.0^c (6.6)
225 ppm F	0.2^a (0.2)	0.1^a (0.1)	29.2^a (1.8)	21.3^d (2.4)	5.1^a (2.9)	-9.2^d (5.2)
100 F TMPmicro	0.1^a (0.1)	0.1^a (0.1)	23.1^c (1.5)	13.7^b (1.8)	-5.2^b (3.1)	-26.6^b (4.7)
100 F TMPnano	0.3^a (0.2)	0.1^a (0.1)	21.3^d (1.4)	11.2^e (1.6)	-8.7^b (4.0)	-33.7^e (4.8)

Equal lowercase letters means no significant differences among groups (column). Distinct uppercase letters means significant difference between the times of erosion challenge. (*) No difference between times of erosion. (two-way ANOVA, Student-Newman-Keuls's test, $p<0.05$).

6. ANEXO

6.1. Anexo A – Boca Artificial

Biopdi

Equipamentos médicos e odontológicos

Manual do Usuário
Simulador de Boca Artificial

Fabricante, responsável pela comercialização e garantia:

Biopdi

Rua Porceno Marino, 105, Jardim Gilbertoni

CEP: 13574-560 São Carlos, SP

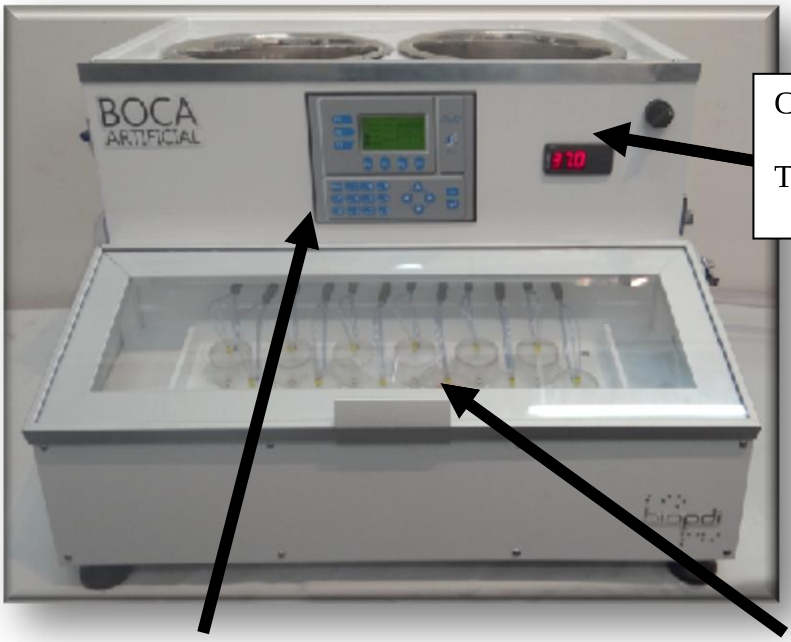
Email: contato@biopdi.com.br ou vendas@biopdi.com.br

CNPJ: 13.027.001/0001-71

6.1.1. Figura do Esquema de funcionamento da Boca Artificial

Ácido cítrico

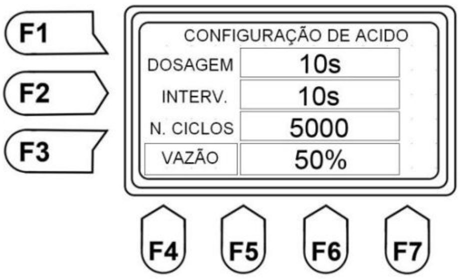
Saliva Artificial



Controle de Temperatura

Painel de controle e programação

Boxes de gotejamento de soluções



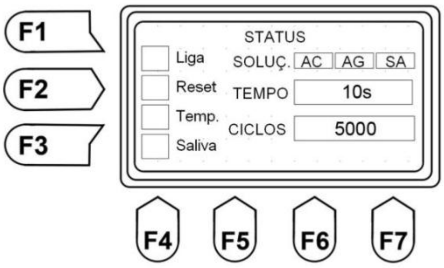
CONFIGURAÇÃO DE ACIDO

DOSAGEM 10s

INTERV. 10s

N. CICLOS 5000

VAZÃO 50%



STATUS

☐ Liga

☐ Reset

☐ Temp.

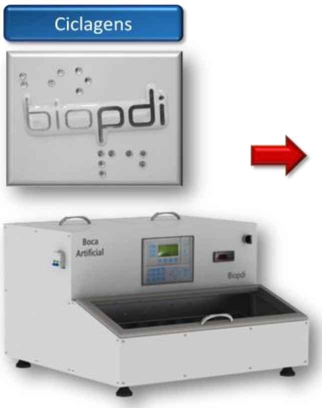
☐ Saliva

SOLUÇ. AC AG SA

TEMPO 10s

CICLOS 5000

6.1.2. Protocolo de configuração da Boca Artificial para os analises



Mancilla et al., 2017

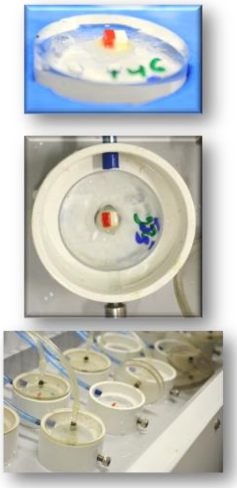
Protocolo x dia:
- Dosagem 4s Ácido Cítrico;
- vol. 1,5 mL/min
- Intervalo 7 vezes

Saliva Artificial
- vol. 1,5 mL/min
- Ciclos: 7s x 6 vezes.
- ciclagem total : 28s + 42s
- = 70s

- Descanso de 2h,
- gotejamento SA
- vol. 1,5 mL/min
- Temperatura: 37°C
- 3 ciclos x dia

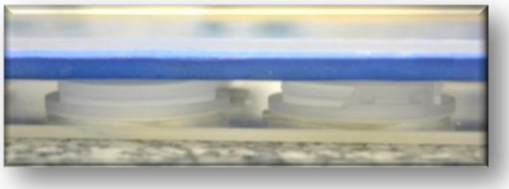
Configuração do painel da Boca Artificial

Posicionamento das amostras na Boca Artifical



Protocolo para analises de 3 - dias

Protocolo para 3 dias:
- Repetir protocolo de um dia
- Final do ciclo deixar em descanso em Saliva Artificial (4mL)
- Estufa Bacteriológica a 37Cº até a próxima ciclagem.



6.2. Anexo B– Preparação de amostras e Soluções

6.2.1. Preparação das amostras (dentes bovinos Incisivos)

Corte e Obtenção dos Blocos para o estudo

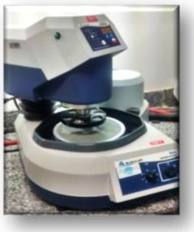
Polimento dos Blocos

Analises de Dureza Superficial Inicial (SHi)

Corte e Polimento

N=120

Grupos x 5
(n=24)
Tratamento
1 dia e 3 dias
(n=12)

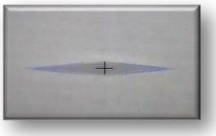


Danelon et al., 2018

SHi:

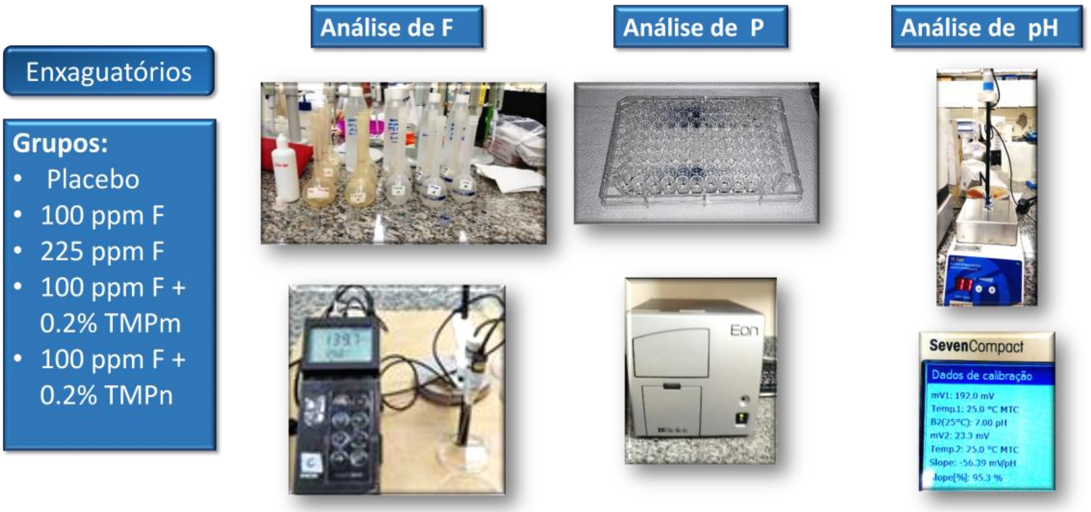
- 320-380 KHN
- Carga 25g x 10s
- 100 µm
- 5 marcações

(p=0.677)



Favretto et al., 2013

6.2.2. *Processo de Verificação de F, P e pH nos Enxaguatorios do estudo*



Enxaguatórios

Grupos:
- Placebo
- 100 ppm F
- 225 ppm F
- 100 ppm F + 0.2% TMPm
- 100 ppm F + 0.2% TMPn

Análise de F

Delbem et al., 2003

Análise de P

Fiske e Subbarow 1925

Análise de pH

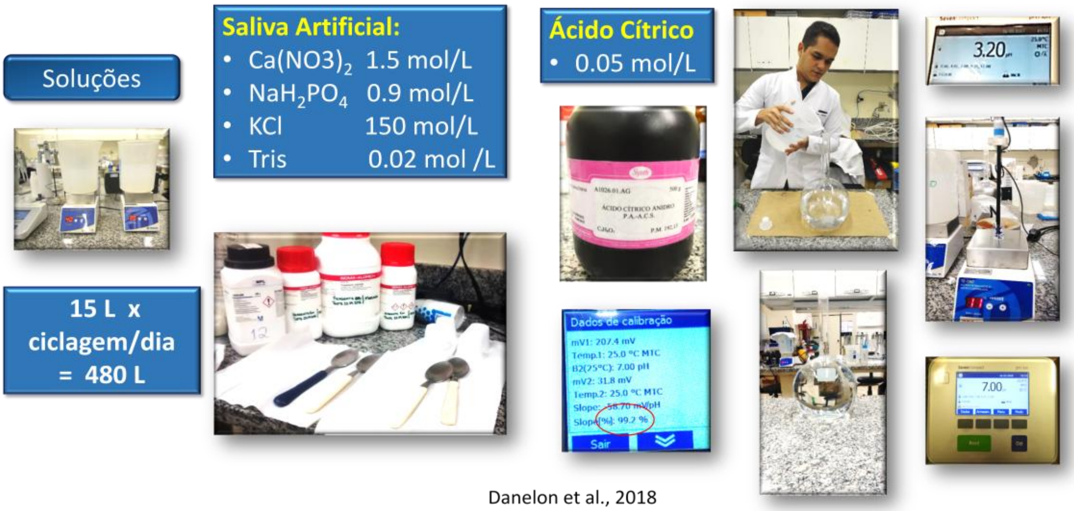
Delbem et al., 2012

6.2.3. *Preparação e analises do pH da saliva e o ácido cítrico*

Reagentes

Preparação

Analises de pH



Soluções

15 L x ciclagem/dia = 480 L

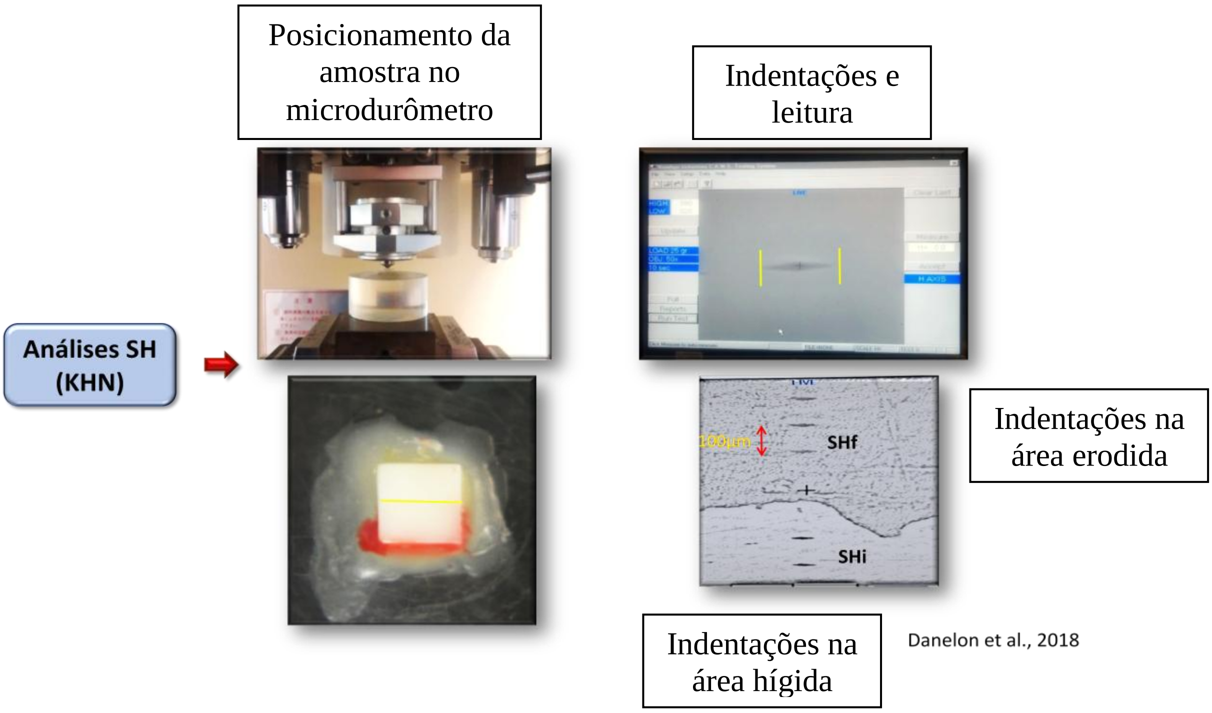
Saliva Artificial:
- $Ca(NO3)_2$ 1.5 mol/L
- NaH_2PO_4 0.9 mol/L
- KCl 150 mol/L
- Tris 0.02 mol /L

Ácido Cítrico
- 0.05 mol/L

Danelon et al., 2018

6.3. Anexo C – Analises

6.3.1. Analises da superfície erodida (KHN)



Posicionamento da amostra no microdurômetro

Indentações e leitura

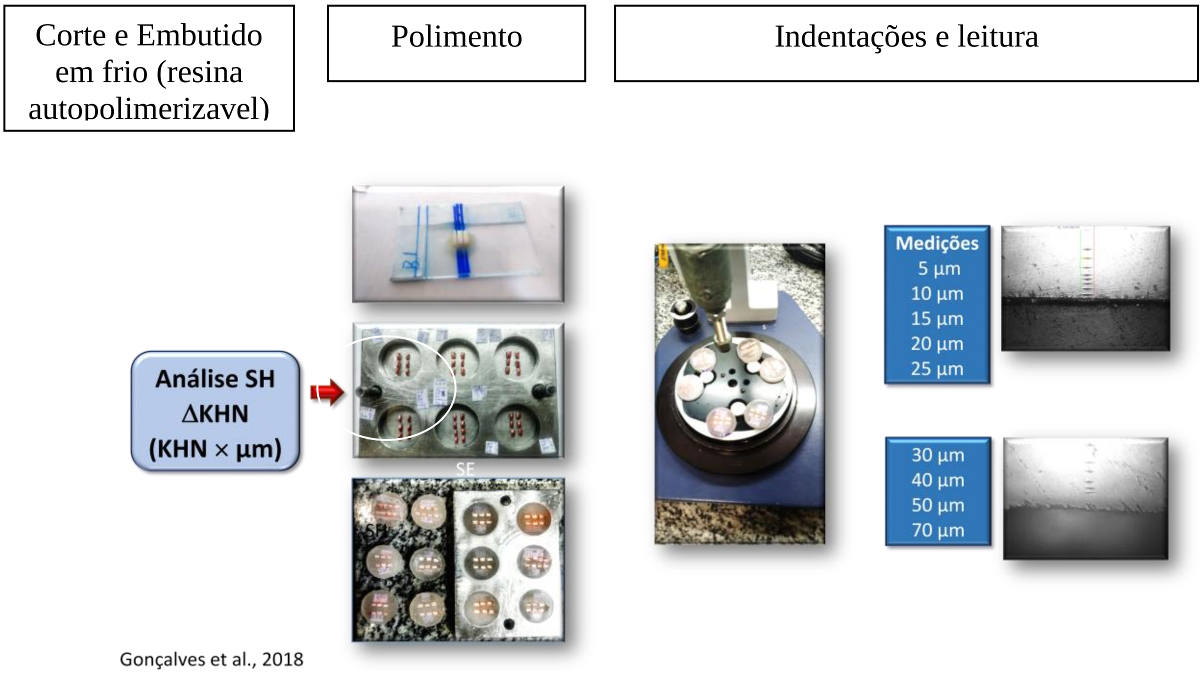
Análises SH (KHN)

Indentações na área erodida

Indentações na área hígida

Danelon et al., 2018

6.3.2. Preparação das amostras para análises de dureza interna ΔKHN (KHN × µm)



Corte e Embutido em frio (resina autopolimerizavel)

Polimento

Indentações e leitura

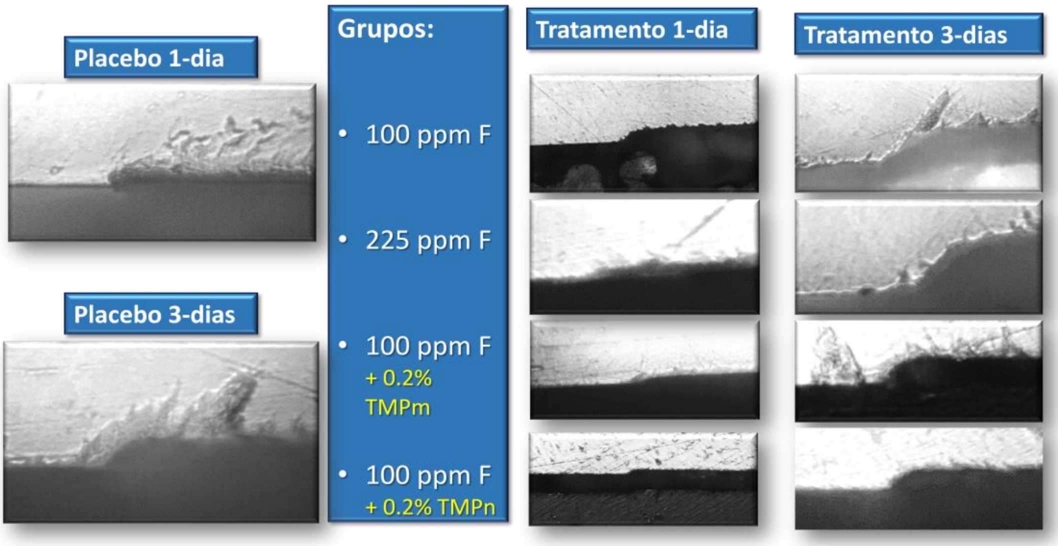
Análise SH ΔKHN (KHN × µm)

Medições
5 µm
10 µm
15 µm
20 µm
25 µm

30 µm
40 µm
50 µm
70 µm

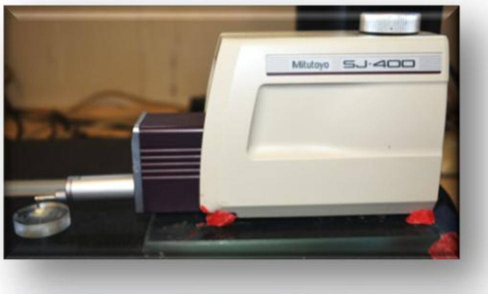
Gonçalves et al., 2018

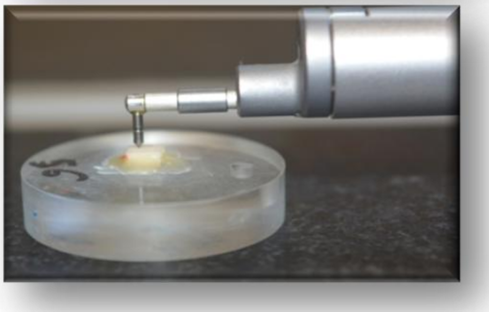
Degraus por grupos e tratamentos



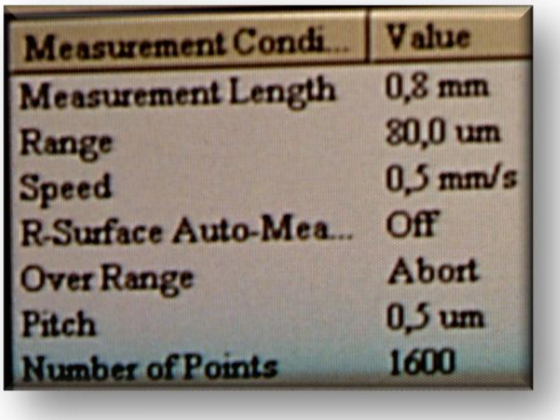
6.3.3. Analises de perfilometria

Posicionamento das amostras para leitura



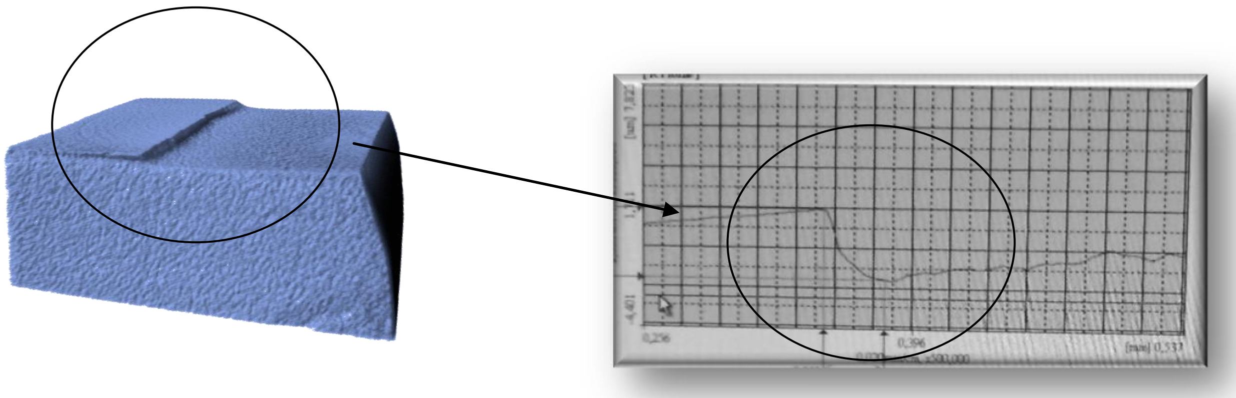


Configuração do equipamento para leitura



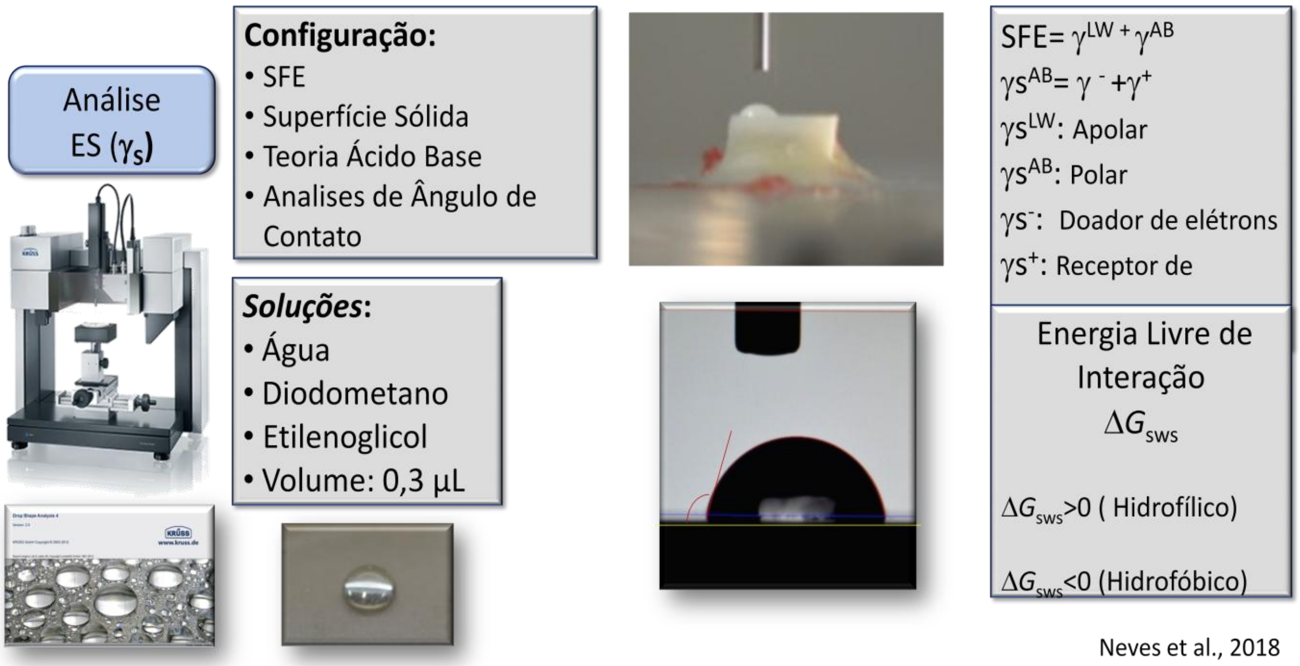
Measurement Condi...	Value
Measurement Length	0,8 mm
Range	80,0 um
Speed	0,5 mm/s
R-Surface Auto-Mea...	Off
Over Range	Abort
Pitch	0,5 um
Number of Points	1600

Leitura



6.3.4. Analises da energia livre de superfície (SFE)

Goniômetro e Software	Configuração para analises de SFE	Posicionamento das amostras e leitura	Fatores a analisar



Análise ES (γ_S)

Configuração:
- SFE
- Superfície Sólida
- Teoria Ácido Base
- Analises de Ângulo de Contato

***Soluções*:**
- Água
- Diodometano
- Etilenoglicol
- Volume: 0,3 µL

SFE= $\gamma^{LW} + \gamma^{AB}$

$\gamma s^{AB} = \gamma^{-} + \gamma^{+}$

γs^{LW}: Apolar

γs^{AB}: Polar

γs^{-}: Doador de elétrons

γs^{+}: Receptor de

Energia Livre de Interação ΔG_{sws}

$\Delta G_{sws} > 0$ (Hidrofílico)

$\Delta G_{sws} < 0$ (Hidrofóbico)

Neves et al., 2018