Universidade Estadual Paulista "Júlio de Mesquita Filho"

Faculdade de Odontologia de Araçatuba

LARYSSA DE CASTRO OLIVEIRA

IN VITRO DENTIN PERMEABILITY AND TUBULE OCCLUSION OF IN-OFFICE DESENSITIZING MATERIALS

ARAÇATUBA-SP
2021

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IN VITRO DENTIN PERMEABILITY AND TUBULE OCCLUSION OF IN-OFFICE DESENSITIZING MATERIALS

Dissertação apresentada à Faculdade de Odontologia, Campus de Araçatuba, da Universidade Estadual Paulista "Júlio de Mesquita Filho", como parte integrante dos requisitos para obtenção do título de Mestre, pelo Programa de Pós-Graduação em Odontologia, área de Concentração em Dentística.

Orientadora: Profa. Ass. Dra. Ticiane Cestari Fagundes

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Dedicatória

A Deus

"Porque eu bem sei os pensamentos que tenho a vosso respeito, diz o Senhor; pensamentos de paz, e não de mal, para vos dar o fim que esperais. Então me invocareis, e ireis, e orareis a mim, e eu vos ouvirei. E buscar-me-eis, e me achareis, quando me buscardes com todo o vosso coração. Jeremias 29:11-13" Ao Senhor toda honra e toda glória.

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Resumo

Oliveira LC. Permeabilidade da dentina e oclusão tubular *in vitro* de materiais dessensibilizantes de consultório [dissertação]. Araçatuba: Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba; 2021.

RESUMO

Objetivo: O objetivo desse estudo foi investigar a permeabilidade dentinária e a oclusão tubular de materiais dessensibilizantes de uso em consultório. Métodos: Blocos de dentina bovina foram obtidos e imersos em EDTA 0,5 M para promover a abertura dos túbulos dentinários. Os materiais testados foram: verniz placebo (PLA); verniz fluoretado (FLU); verniz de NaF 5% + 5% trimetafosfato de sódio nanoparticulado (TMP); sistema adesivo universal (SBU); verniz contendo partículas S-PRG (SPRG); solução de Biosilicato (BIOS) e solução de amelotina (AMTN). Os materiais foram aplicados e os espécimes submetidos ao desafio erosivo-abrasivo. A permeabilidade dentinária foi avaliada em T0 (inicial), T1 (após a aplicação dos materiais) e T2 (após o desafio erosivo-abrasivo). As imagens confocais foram usadas para avaliar o comprimento e o número dos túbulos ocluídos e as imagens de microscopia eletrônica de varredura (MEV) para avaliar o número de tubulos abertos. Os dados de permeabilidade e MEV foram analisadas pelo teste ANOVA duas medidas repetidas e pós teste de Tukey. O comprimento e número de túbulos dentinários ocluídos foram analisadas pelo teste ANOVA um critério e pós teste de Tukey, Kruskal-Wallis e pós teste de Dunn's, respectivamente. Os testes de correlação de Spearman e Pearson também foram realizados. O nível de significância foi de 5%. Resultados: O grupo AMTN mostrou os menores valores de permeabilidade em T1 e a seguinte ordem decrescente ocorreu em T2: AMTN=SBU>BIOS=SPRG>TMP>FLU>PLA. O grupo SBU teve o maior comprimento médio de túbulos dentinários ocluídos. O grupo AMTN teve maior número de túbulos dentinários ocluídos do que PLA e FLU e os menores valores de túbulos dentinários abertos foram observados para os grupos AMTN e SBU. Uma correlação significativa foi encontrada entre as análises realizadas. Significância: O sistema adesivo universal e a proteína amelotina foram mais efetivos em reduzir a permeabilidade dentinária através da oclusão dos túbulos dentinários.

Palavras-chave: Dentina. Dessensibilizantes Dentinários. Hipersensibilidade da Dentina. Permeabilidade da Dentina.

Abstract

Oliveira LC. In vitro dentin permeability and tubule occlusion of in-office desensitizing materials [dissertação]. Araçatuba: Universidade Estadual Paulista, Faculdade de Odontologia de Araçatuba; 2021.

ABSTRACT

Objectives: The aim of this study was to investigate the dentin permeability and tubule occlusion of in-office desensitizing materials. Methods: Bovine dentin blocks were obtained and immersed in 0.5 M EDTA to open dentinal tubules. The materials tested were: placebo varnish (PLA); fluoride varnish (FLU); NaF 5% + 5% nanoparticulate sodium trimetaphosphate varnish (TMP); universal adhesive system (SBU); S-PRG filler-containing varnish (SPRG); Biosilicate solution (BIOS) and amelotin solution (AMTN). The materials were applied, and specimens were submitted to an erosive-abrasive challenge. Dentin permeability was evaluated at T0 (initial), T1 (after application of materials) and T2 (after erosive-abrasive challenge). Confocal images were used to evaluate length and number of dentinal tubules occluded and images from scanning electron microscopy (SEM) to evaluate opened dentinal tubules. Permeability and SEM data were evaluated by two-way repeated measures ANOVA and Tukey tests. The length and number of dentinal tubules occluded were evaluated by one-way ANOVA and Tukey, Kruskal-Wallis and Dunn's tests, respectively. Spearman and Pearson correlation tests were also used. The significance level was 5%. Results: AMTN group showed the lowest permeability value in T1 and the following decreasing order occurred in T2: AMTN=SBU>BIOS=SPRG>TMP>FLU>PLA. SBU group had the highest mean value of dentinal tubules occluded lengths. AMTN group had greater number of dentinal tubules occluded than PLA and FLU and the lowest values of opened dentin tubules were observed for AMTN and SBU groups. A significant negative correlation was found between the analysis. Significance: Universal adhesive system and the AMTN solution were more effective to reduce dentin permeability by occluding dentin tubules.

Keywords: Dentin. Dentin Desensitizing Agents. Dentin Hypersensitivity. Dentin Permeability.

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LISTA DE ABREVIATURAS, SÍMBOLOS E SIGLAS

#	número (number)
%	percentagem (percentage)
<	menor (less than)
=	igual (equal)
>	maior (greater)
±	mais ou menos (more or less)
≥	maior ou igual (greater or equal)
AMTN	solução de amelotina (amelotin solution)
ANOVA	análise da variância (analysis of variance)
Asup	área de superfície dentinária exposta (exposed dentinal surface area)
BIOS	solução de Biosilicato (Biosilicate solution)
C	controle (control)
CaF_2	fluoreto de cálcio (calcium fluoride)
CLSM	microscopia confocal à laser (Confocal Laser Scanning Microscopy)
cm	centímetros (centimeter)
cm^2	centímetro quadrado (square centimeter)
D	deslocamento da bolha (bubble displacement)
DH	hipersensibilidade dentinária (dentin hypersensitivity)
EDTA	ácido etilenodiamino tetra-acético (ethylenediamine tetraacetic acid)
et al.	e colaboradores (and others)
FLU	verniz fluoretado (fluoride varnish)
g	grama (grams)
h	hora (hour)
H_2O	água (water)
HTC	dentina hipersensível / tratada / desafiada (hypersensitive / treated / challenged dentin)
L	comprimento do capilar (capillary length)
LCO	Laryssa de Castro Oliveira
Lp	condutância hidráulica (hydraulic conductance)
M	mol (mol)
mg	miligrama (milligram)
min	minuto (minute)

ml	mililitro (mililiter)
mm	milímetro (millimeter)
mmol/L	milimoles por litro (millimoles per liter)
mW	MEGAWATT
n	número amostral (sample size)
nm	nanometro (nanometer)
ºC	grau Celsius (degree Celsius)
p	probabilidade de significância (significance probability)
pH	potencial hidrogeniônico (potencial of hydrogen)
PH	pressão hidrostática (hydrostatic pressure)
PLA	verniz placebo (placebo varnish)
psi	libra-força por polegada quadrada (pounds per square inch)
Q	taxa de filtração (filtration rate)
s	segundo (second)
SBU	sistema adesivo universal (universal adhesive system)
SEM	microscopia eletrônica de varredura (scanning electron microscopy)
SPRG	verniz com partículas S-PRG (S-PRG filler-containing varnish)
STC	dentina hígida / tratada / desafiada (sound / treated / challenged dentin)
T	tempo (time)
T0	análise inicial de permeabilidade (inicial evaluation of permeability)
T1	análise de permeabilidade após o tratamento (post-treatment permeability evaluation)
T2	análise final de permeabilidade (final permeability evaluation)
TCF	Ticiane Cestari Fagundes
TMP	verniz de trimetafosfato de sódio nanoparticulado (nanoparticulate sodium trimetaphosphate varnish)
Vs	volume padronizado do túbulo capilar (standardized volume of the capillary tube)
w/w	peso (weight)
x	vezes
µg	micrograma (microgram)
µl	microlitro (microliter)
µm	micrometro (micrometer)

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In vitro dentin permeability and tubule occlusion of in-office desensitizing materials

Abstract

Objectives. The aim of this study was to investigate the dentin permeability and tubule occlusion of in-office desensitizing materials.

Methods. Bovine dentin blocks were obtained and immersed in 0.5 M EDTA to open dentinal tubules. The materials tested were: placebo varnish (PLA); fluoride varnish (FLU); NaF 5% +

5% nanoparticulate sodium trimetaphosphate varnish (TMP); universal adhesive system (SBU); S-PRG filler-containing varnish (SPRG); Biosilicate solution (BIOS) and amelotin solution (AMTN). The materials were applied, and specimens were submitted to an erosive-abrasive challenge. Dentin permeability was evaluated at T0 (initial), T1 (after application of materials) and T2 (after erosive-abrasive challenge). Confocal images were used to evaluate length and number of dentinal tubules occluded and images from scanning electron microscopy (SEM) to evaluate opened dentinal tubules. Permeability and SEM data were evaluated by two-way repeated measures ANOVA and Tukey tests. The length and number of dentinal tubules occluded were evaluated by one-way ANOVA and Tukey, Kruskal-Wallis and Dunn's tests, respectively. Spearman and Pearson correlation tests were also used. The significance level was 5%.

Results. AMTN group showed the lowest permeability value in T1 and the following decreasing order occurred in T2: AMTN=SBU>BIOS=SPRG>TMP>FLU>PLA. SBU group had the highest mean value of dentinal tubules occluded lengths. AMTN group had greater number of dentinal tubules occluded than PLA and FLU and the lowest values of opened dentin tubules were observed for AMTN and SBU groups. A significant negative correlation was found between the analysis.

Significance. Universal adhesive system and the AMTN solution were more effective to reduce dentin permeability by occluding dentin tubules.

Keywords: Dentin Desensitizer; Dentin Hypersensitivity; Dentin Permeability; Dentinal Tubule Occlusion.

1 INTRODUCTION*

Dentin hypersensitivity (DH) is defined as a short sharp pain caused by thermal, tactile, chemical or osmotic stimuli not linked to a pathological process [1-3]. At present, specialized desensitizing agents exist and act chemically, obliterating the dentinal tubules or reducing nervous excitability, however, no single agent can be considered ideal for long-term relief [4,5]. The treatments can be performed in-office, where one application usually results in immediate pain relief [5,6]. Over the years, new ways to decrease the HD pain, through tubular occlusion, were commercialized such as fluoride-based and bonding materials [7]. Furthermore, some materials are in development based on inorganic phosphate salts [8,9], proteins from tooth structure [10] or with bioactive purpose [11,12].

Varnishes with high fluoride concentration have been identified as the first choice for therapy against HD because they involve the deposition of large amounts of calcium fluoride (CaF2), however, due to the limitations regarding the duration of its effects, the addition of an inorganic phosphate salts has been proposed as a method to increase the action on the dental tissue [8,9]. Among them, sodium trimetaphosphate (TMP) has shown a remineralization action on dentin tissue when associated with fluorides through adsorption to the dentin surface, promoting obliteration of dentinal tubules, protection of the collagen matrix and deposition of calcium phosphate-apatite [8,9].

Regarding bonding agents, universal dental adhesive system has been used as an option for promoting a desensitizing effect by sealing the dentinal tubules [13]. The acidic monomers of self-etch adhesives promote the simultaneous dissolution of smear layer and creates a hybrid layer without exposing the collagen fibrils, eliminating the risk of collapse of the collagen network and promoting a desensitizing effect on DH [14-16].

Recently, a product that combines fluoride varnish with bonding agents and a surface pre-reacted glass-ionomer (S-PRG) filler that provide recharging abilities was launched in the market [17]. In addition to fluoride, S-PRG fillers release multiple ions such strontium, borate, aluminum, silicate and sodium which may have the capability to inhibit demineralization and promote remineralization, being considered as a bioactive material [7,18].

Other material that consists of bioactive fillers is the bioactive glasses and glass-ceramics which have been demonstrated to be effective in bone regeneration [11,19]. Potential

* Normalizada de acordo com a revista Dental Materials

obliterating effect on dentinal tubules, in addition to remineralization and prevention of dentin demineralization are properties that have been attributed to Biosilicate [19,20].

Scientific and technological advances have brought other alternative to treat HD, that is a protein specifically expressed during the maturation stage of dental enamel formation, known as amelotin (AMTN). AMTN was localized closer to enamel than ameloblasts at the cell-matrix interface, suggesting an involvement in mineralization and dentogingival attachment, rather than direct cell adhesion [21]. Limited results showed its effect in dentin structure especially in relation to tubule obliteration, but some studies have demonstrated an extensive calcium phosphate precipitation and hydroxyapatite mineralization in a dose-dependent manner [10,22]. Despite the wide range of available products, the literature that assess the properties of this commercially and experimental in-office materials through occluding dentinal tubules are scarce of studies.

In view of the foregoing, this study has the potential to provide evidence regarding the treatment of HD by using commercialized and experimental in-office desensitizing materials through dentinal tubule occlusion by dentin permeability, scanning electron microscopy and confocal laser scanning microscopy after erosive-abrasive challenges. The null hypotheses tested were: 1) no significant differences will be observed in dentin permeability reduction among groups; 2) there would be no difference between times evaluations in dentin permeability analysis, for each group separately; 3) no differences will be observed in dentinal tubule occlusion among groups 4) there is no correlation among the different analysis.

2 MATERIALS AND METHODS

This study was reviewed and approved by the local Animal Use Ethics Committee under protocol #00418-2020. One-hundred and twelve freshly extracted bovine incisors were collected and teeth with caries, cracks, or gross irregularities of dentin structure were excluded from the study.

2.1 Dentin permeability

2.1.1 Specimen preparation

For this analysis, were separated seventy (n=70) bovine incisors. From each tooth, a root dentin block was obtained with dimensions of 4x4x1 mm and polished with waterproof abrasive paper (#600, #800 e #1200 grit), in a polishing machine (AutoMet 250 PRO, Buehler, IL, USA), under running water. The thickness was checked with a digital micrometer (Mitutoyo America, Dawn, IL, USA). The specimens were ultrasonically cleaned for 5 min between each grit (Cristófoli, Campo Mourão, PR, Brazil). The obtention of blocks were carried out to minimize the direction of dentinal tubule differences, prioritizing a perpendicular direction. To simulate the open dentin tubules, present in cervical hypersensitive areas, the smear layer was removed using 0.5 M EDTA solution for 5 min [23].

2.1.2 Dentin permeability measurements

Dentin permeability was measured at three time points: T0 - after immersion in EDTA 0.5M; T1 – after materials application and T2 – after erosive-abrasive challenge and the method used was referred previously by Favretto and others [24]. A THD-03D (Odeme Medical Equipment and Dental Ltda, Joaçaba, SC, Brazil) apparatus was used with deionized water. The conductance measurement was performed through a capillary tube to the filtration chamber and through an injection system with a small portion of air, forming a bubble, and penetrating inside the capillary tube. The bubble is pushed by the flow of liquid that moves from the pressure chamber to the filtration chamber, and this linear displacement of the air bubble is measured inside the capillary tube for 5 min utilizing a digital micrometer. The pressure applied on the test surface was 10 psi. The obtained value corresponds to the bubble displacement into the capillary tube, which allows obtention of the filtration rate, which is the volume of deionized water that passed through the dentinal tubules, determined by the standardized volume (Vs) of the capillary tube (μL) multiplied by the bubble displacement in mm (D) and divided by the multiplication of the capillary length in mm (L) and time in minutes (T), $Q = (Vs \times D)/(L \times T)$.

In order to obtain the hydraulic conductance of dentin μL cm^2/min cm H_2O (Lp), the filtration rate (Q) was divided by the multiplication of the difference in hydrostatic pressure across the dentin (PH) and the exposed dentinal surface area (Asup), Lp = Q/(PH × Asup). Blocks with values 15% above or below the overall average were discarded [23-26].

2.1.3 Experimental groups and in-office desensitizing materials application

The samples of dentin were separated randomically in seven experimental groups: placebo varnish (PLA), fluoride varnish (FLU); TMP varnish (TMP); universal adhesive system (SBU); S-PRG filler-containing varnish (SPRG); Biosilicate solution (BIOS) and amelotin solution (AMTN). The composition of each material is described in Table 1.

In samples of groups PLA, FLU and TMP a thin layer was passively applied for 5 s under the clean and dry surface with a disposable applicator, remaining stable for 10 min. In samples of group SBU a thin layer was actively applied for 20 s on the clean and dry surface with a disposable applicator and polymerized using light intensity of 1200 mW/cm^2 (Radii, SDI, Victoria, Australia) for 10 s. No previous acid etching was performed.

In samples of group SPRG a thin layer of one drop active mixed with base in the base container was applied for 3 s on the clean and dry surface with specific applicator and polymerized using light intensity of 1200 mW/cm^2 (Radii, SDI, Victoria, Australia) for 10 s. The uncured layer was removed from surface with a water-moistened cotton pellet.

In group BIOS a thin layer of Biosilicate was applied for 5 s on the clean and dry surface with a disposable applicator, remaining stable for 10 min. The solution was composed of Biosilicate and distilled water (1:10 ratio) and for simulation of the professional-use products, the particles were mixed with distilled water immediately before application.

In samples of group AMTN 5 μL of solution were applied for 10 s on the clean and dry surface with a disposable applicator, remaining stable until no visible liquid left. The solution was prepared with 100 μL of pure water add to 500 μg of AMTN powder. The final result was 200 μL of solution (5μg/uL concentration).

A single researcher (LCO) performed the application of each treatment only once and the specimens were immediately stored in artificial saliva (1.649 mmol/L CaCl2 H2O, 5.715 mmol/L KH2 PO4, 8.627 mmol/L KCl, 2.950 mmol/L NaCl g/l.92 mmol/L Tris buffer, pH adjusted to 7 with HCl) for 6 h at 37 °C [27].

2.1.4 Erosive-abrasive challenge

To simulate a 1-year period and an extremely condition of a subject that already has signs of HD, the specimens were subjected to a 5-day erosive-abrasive challenge. Erosive cycles were performed four times daily, and abrasive challenges were applied after the first and last cycles. The samples were eroded by immersion in 2 ml/block of citric acid (pH=3.2) for 2 min under an orbital shaking table (Tecnal TE – 420, Piracicaba, SP, Brazil) [28] with 1 h immersion in artificial saliva between the cycles [28,29].

Abrasive challenge was performed by brushing the specimens for 15 s using an automated machine (MSET, Elquip, São Carlos, SP, Brazil), at 150 strokes/min during 2 min and axial load of 150 g [8,29]. For all groups, brushing was performed with a *slurry* made from Colgate Total 12 (Colgate-Palmolive, SP, Brazil) dentifrice and artificial saliva (1:3 w/w). Between each cycle, the specimens were cleaned with deionized water and at the end of experiment period, the samples were stored under 100% humidity until analysis.

2.2 SEM

2.2.1 Specimen preparation

The dentin blocks with 6x4x2 mm (n=21) and the solution for simulate the opened dentinal tubules were obtained using the same method described in 2.1.1. However, each specimen was previously protected using an acid-resistant varnish (Colorama, São Paulo, SP, Brazil) to create three areas (2 mm): HTC – hypersensitive / treated / challenged dentin (EDTA immersion); C – control (no treatment) and STC – sound / treated / challenged dentin (without EDTA immersion). Only C and STD areas received the acid-resistant varnish. The STC was identified by a permanent marker pen on the side of the specimen and had the acid resistant varnish removed to receive the treatments.

2.2.2 Experimental groups and in-office desensitizing materials application

The treatments were applied only on HTC and STC using the same method described for 2.1.2.

2.2.3 Erosive-abrasive challenge

The challenge was performed as described in 2.1.4.

2.2.4 SEM analysis

After the challenge period, three specimens of each group were dried in a desiccator and sputter-coated with gold in a vacuum evaporator (EIKO IB-3 ion coater, Hitachi, Tokyo, Japan). Micrographs of HTC, C and STC area were obtained using a scanning electron microscope (TESCAN VEGA3 SBU) to verify the number of opened dentin tubules at x2000 magnification. The assessments were performed using the image analysis software program ImageJ (National Institutes of Health), to standardize the counting, considering the partial obliteration as opened dentinal tubules [29].

2.3 CLSM

2.3.1 Specimen preparation

The dentin blocks with 4x2x2 mm (n=21) and the solution for simulate the opened dentinal tubules were obtained using the same method described in 2.1.1.

2.2.2 Experimental groups and in-office desensitizing materials application

For this analysis, 0.1% fluorescent rhodamine dye was previously added to the materials, in a proportion of 0.02 mg/g[30] and the application was performed using the same method described for 2.1.2.

2.2.3 Erosive-abrasive challenge

The challenge was performed as described in 2.1.4.

2.2.4 CLSM analysis

After the challenge period, three specimens of each group were examined using a laser confocal microscope (Leica TCS SP2, Mannheim, Germany) to identify mean length and number of dentinal tubules occluded under x40 magnification at a depth of 100 µm [31]. The fluorescence infiltration of materials was detected using a 543 nm excitation line [30].

Two images were obtained from each specimen and a software program ImageJ (National Institutes of Health) was used to perform the assessments [32]. The mean length of dentinal tubules occluded was performed by a single evaluator (TCF). The number of dentinal tubules occluded was evaluated by two blinded evaluators (TCF and LCO), calibrated between themselves by inter-examiner Kappa test, according to scores: I-0≤6, II-7≤12, III-13≤18, IV-19≤24; for example, when an image had a number of 4 dentinal tubules occluded, the score

attributed was I-0≤6. In cases of disagreement, a new assessment was performed until a consensus between the evaluators was reached.

2.4 Statistical analysis

For dentin permeability and SEM analysis, normal distribution and homoscedasticity of the data were checked with the Shapiro–Wilk and Brown-Forsythe tests, respectively. Once these assumptions were satisfied, data were evaluated by two-way repeated measures ANOVA and Tukey tests.

For CLSM analysis, once normal distribution and homoscedasticity were also checked. Data of mean length of dentinal tubules occluded were evaluated by one-way ANOVA and Tukey tests. Regarding the number of dentinal tubules occluded, the results obtained in scores by the two examiners were submitted to the Kappa test (inter-examiners) and by Kruskal-Wallis and Dunn tests.

Spearman test was used to correlate the number of dentinal tubules occluded analyzed by CLSM with final dentin permeability values (T2) and opened dentin tubules analyzed by SEM. Pearson test was used to correlate the mean length of dentinal tubules occluded analyzed by CLSM with dentin permeability values (T2) and opened dentin tubules analyzed by SEM. The software SigmaPlot 12.0 (Systat Software Inc, San Jose, CA, USA) was used for all calculations, considering a significance level of 5%.

3 RESULTS

3.1 Dentin permeability measurements

Table 2 shows the results of the dentin permeability test before and after materials application (T0 and T1, respectively) and after erosive-abrasive challenge (T2). At T0 there were not statistically differences among groups in permeability data ($p>0.05$). However, in T1 was observed that the AMTN group resulted in the lowest permeability values, differing statistically from the others ($p<0.05$). The highest values were observed for the PLA and FLU groups when comparing to the other groups ($p<0.05$). In T2 the data of permeability followed the following decreasing order: AMTN=SBU>BIOS=SPRG>TMP>FLU>PLA.

In comparison among the evaluation times for each group, all materials showed decrease permeability values between T0 and T2, except PLA and FLU groups which presented T0 values higher than T2. For TMP, BIOS, AMTN and SPRG groups the following pattern was observed: T1<T2<T0. Only for SBU group statistically significant difference was not observed between T1 and T2 ($p = 0.435$).

3.2 SEM analysis

Data referring to SEM analysis are described in Figure 1. Regarding HTC area, the lowest values of opened dentin tubules were observed for AMTN and SBU groups when compared to the other groups evaluated ($p<0.05$) and statistically significant differences were not observed between them ($p=0.391$). There was no statistically significant difference among FLU, TMP and BIOS groups. PLA group showed the highest value among all groups ($p<0.05$). C and STC areas presented no statistically significant difference among the groups ($p>0.05$). For each group separately, only AMTN and SBU groups did not demonstrate differences between the areas. Representative images from each area and groups were showed in Figure 2.

3.3 CLSM analysis

Data referring to CLSM analysis are described in Table 3 and Figure 3. Regarding the mean length of dentinal tubules occluded, the highest values were observed for the SBU, AMTN and SPRG groups ($p>0.05$). The PLA, FLU and TMP group showed the lowest values, when compared to the other groups ($p>0.05$). There were no statistically significant differences among BIOS, AMTN, SPRG and FLU ($p>0.05$).

Regarding the number of dentinal tubules occluded, the inter-examiner Kappa test

was considered as substantial agreement with a value of 0.71. Only the AMTN group showed a statistically significant difference with PLA and FLU groups ($p<0.05$).

3.4 Correlations

The statistical analysis highlights a significant negative correlation ($p=0.0000$ for mean lengths; $p=0.0004$ for number of dentinal tubules occluded) between the data from CLSM analysis and dentin permeability values (T2). A significant negative correlation ($p=0.0015$ and $p=0.0022$) was also observed between the data from CLSM and opened dentin tubules (SEM).

4 DISCUSSION

Dentin permeability analysis was used to provide parameters that achieve a reduction in clinical symptoms of hypersensitivity, in addition to being widely used by *in vitro* studies for these purpose [6,31,33]. Previous studies used SEM to evaluate the effect of the desensitizing agents and observe the blockage of the dentin tubules after using desensitization materials [34] and the CLSM analysis, allowed the observation of the obliteration provided by these materials, as well as the length of their penetration into the dentin tubules [35].

The first and second null hypotheses are rejected, once differences were observed in dentin permeability among the groups and between times evaluations for each group separately. Therefore, it was no observed differences among all groups in T0, proving that the method used to simulate the opened dentin tubules was effective and guaranteed the standardization of the initial permeability values. Ethylenediaminetetraacetic acid (EDTA) is a chelator capable to increased dentin permeability in all root thirds, compared to other solutions, as concluded by Bighetti Trevisan et al. [36].

T1, PLA and FLU groups showed no significant differences between each other, as also observed in previous study by Esteves et al. [23] that evaluated dentin permeability after dentin hypersensitivity treatments and justified by the inactive permanence of the placebo varnish under the substrate surface until the moment of analysis [23]. Different active ingredients were evaluated in this study: TMP which has great capacity to increase the negative polarity of the surface, enhancing the deposition of calcium phosphate apatite [8,37]; the bioactive technology present in the BIOS and SPRG groups, which was intentionally developed to promote the occlusion of dentinal tubules through the deposition of micrometric particles (0.1-10 μm) [19,38] and the bonding agents present in the SBU and SPRG groups, which allowed the formation of a stable layer on the surface substrate after its application [13]. Thus, the common obliterating proposal of these materials provided similar results between them.

It should be noted, however, that the results showed superiority of the AMTN group in relation to the others groups, attributed to the characteristics of this protein for calcium phosphate mineralization in a dose-dependent manner [10]. It is known that the binding affinity of AMTN to hydroxyapatite mineral is higher than other enamel proteins. This effect is similar for some of the saliva proteins that are strong binders to hydroxyapatite and remain mostly intact when bound to the mineral [10]. These characteristics could justify the results observed for AMTN during T1.

Permeability after the abrasive-erosive challenge (T2) was performed to verify the durability of the treatment, simulating a 1-year period [29]. FLU group had the highest permeability value among the materials, explained by the fact that fluoride, even in the form of varnish, is easily removed with brushing or acid attack [39], as also observed in Figure 3 with the presence of some opened dentinal tubules. In another *in vitro* study, the authors also concluded that the fluoride action were ineffective, trough EDS analysis, which did not properly detect the ion because of it extreme lightweight [40]. In contrast, TMP group had lower results compared to FLU, which could be related to the synergistic effect promoted by the association between TMP and fluoride. Once adsorbed to dentin, TMP works as a nucleating agent for precipitation of calcium phosphates and when the dentin is exposed to a medium rich in calcium and phosphate (such as human or artificial saliva), there is a higher deposition [8,9].

Groups BIOS and SPRG, on the other hand, have a similar bioactive action mechanism that justifies the similar behavior against the erosive-abrasive challenge [18,41]. Bioactive glasses degrade in aqueous environments to release calcium, sodium and phosphate ions which are able to form a calcium phosphate layer on dentin surfaces and raise the pH of the environment, favoring apatite deposition [41]. The multi-ions released by the S-PRG filler played an important role in anti-demineralization, forming fluorapatite, fluoridated apatite, and/or strontium apatite incorporated at the calcium site in hydroxyapatite [17,18]. The release of ions from these materials promotes the buffering of acids and the increase in dentin remineralization, justifying the similarity of their results. Despite this, these materials were not able to achieve the best results as the groups of AMTN and SBU which presented the lowest values of dentin permeability. This result can be attributed to biomimetic mineralization promoted by AMTN which can create an enamel-like layer on the exposed dentin that resembles natural enamel to protect from external stimuli [42]. Moreover, the ability of universal adhesive system to form a stable surface layer under the dentin, blocks the tubules and counteracts the hydrodynamic mechanism of DH, while promotes a long-lasting desensitizing effect [13,16,43]. Besides that, only SBU group demonstrated thick layer even after the erosive-abrasive challenge as shown in SEM and CLSM micrographs. In fact, an essential aspect of varnishes is its viscosity, related to molecular weight which affect the liquid flow and its penetration in dentinal tubules [44]. As the literature showed, increased tubular penetration is associated with decreased viscosity and as we have materials that are still in development in this study, we might suggest that the similarity of this characteristic between AMTN and SBU groups could contribute with the results that we obtained.

Analyzing the behavior of each group over the three times evaluations, a reduction in permeability was observed for all materials between T0 and T2, except for PLA and FLU groups. These data corroborate with other studies that also concluded, through dentin permeability analysis, the ineffective action of conventional fluoride varnish after erosive-abrasive challenges [6,39,40,45]. This can be justified by the fact that CaF_2 deposits, which constitute the mechanism of action of conventional fluoride varnish, do not present good chemical resistance upon erosive conditions [40]. For PLA group, it is speculated that erosive-abrasive challenges could provide a surface change and promote wear and exposed dentinal tubules [40] as observed in the present study by SEM analysis (Figure 2).

On the other hand, only SBU maintained permeability between T1 and T2, suggesting a good tubular occlusion and corroborating with other *in vitro* studies [13,43]. The authors justify these results by the chemical interaction of the 10-MDP functional monomer, which allows a stable union with hydroxyapatite and guarantees the formation of insoluble salts that also contribute to the protection of collagen fibers [13,43,46]. The addition of this monomer was carried out to prevent differences in the demineralization depth caused by the acid etching pretreatment [47,48]. For this reason, no previous acid etching was performed, and the properties related to this material justify the results found. For the other groups (BIOS, AMTN and SPRG), the values obtained between the times showed the same pattern of behavior: T2 values were lower than T0 and higher than T1. This behavior suggests the effectiveness in controlling dentin permeability, justified by the bioactive characteristics mentioned above.

The third hypotheses were also rejected since SEM and CLSM analysis showed differences for dentinal tubule occlusion. Groups AMTN and SBU demonstrated higher values for the mean length of dentinal tubule occluded and the lower for opened dentin tubules. A previous study showed that collagen gels impregnated with AMTN could be mineralized in 5 hours in simulated body fluid buffer [48]. This mineral-inducing property and mineralization in a dose-dependent manner could be responsible for the formation of the distinct aprismatic, highly mineralized and superficial layer referred to a final enamel proposed by Holcroft and Ganss [42] and observed in the present study through SEM and CLSM micrographs. All the compositional characteristics mentioned above for the universal adhesive system can be attributed to the results obtained for the dentinal tubule occlusion observed for this group.

The other groups (BIOS and SPRG) demonstrated similarity behavior for the CLSM and SEM analysis. The crystalline character of BIOS group offers a great advantage over all

other types of bioglasses because crystallization promotes less sharp and abrasive particles resulting in dentin occlusion through hydroxyl carbonate apatite formation [11,19]. The SPRG group had the remineralization affected by ions released from S-PRG filler. Among them, silicate is known to allows the infiltration of silica and hydroxyapatite nanoparticles into demineralized dentin acting as seeds within the collagen matrix [17]. Finally, FLU and TMP groups, although inferior to the other groups, showed similar infiltrated tubule length and opened dentin tubules, which may be related to the properties mentioned before. It is also important to emphasize that PLA group, despite not having an active principle in its composition, also demonstrated the ability to infiltrate the dentinal tubules in only one sample images, justifying the high standard deviation value.

The fourth hypothesis was also rejected, as we observed an inversely proportional correlation. This result means that the lower permeability values have correlation with satisfactory dentinal tubules occlusion obtained by the materials proposed in the present study. Studies that evaluate the dentinal fluid flow and the proportional with tubule radius show a sealing effect and the data corroborates with our study [50,51]. Dentinal fluid flow is proportional to the 4^{th} power of the tubule radius which means that when obliterating the radius, the fluid flow will decrease to 1/16 of its primary flow [51]. On the basis of the above, it may be concluded that to reduce or eliminate DH, it is not necessary to occlude tubules completely. Besides that, dentin is a porous, fluid-filled, mineralized tissue that contribute to penetrability and the formation of hydroxycarbonate apatite particles above and within the tubules, or a combination of the two may guarantee the satisfactory effect[33] of the proposed treatments as observed in the present study and confirmed by the correlation between the different analysis.

Direct comparison of the results of this study with clinical desensitization is limited due to different factors. First, the etiology of HD promoted in this study through the use of EDTA and the physiological oral conditions that are not possible for replication. In addition, the permanence of the proposed materials on the dentin surface without external action which could prevent this contact after its application. However, it is only from *in vitro* studies that the behavior of new materials can be evaluated so that their clinical application can be performed with plausibility and safety.

Although AMTN demonstrates favorable behavior on dentin structure, studies that assess its properties and effects on mineralization are still scarce in the literature. It is noteworthy that because the solution of AMTN used in this study is experimental, more

evaluations that assess the concentration, form of application and the time the material remains on the dentin, need to be carried out and will have great importance in the performance of the material. Moreover, as DH is a worldwile condition, further studies can also contribute to reduce the costs and achieve a mode of delivery as at-home (patient-applied) therapy. In addition, the evaluation of the long-term clinical durability of obliteration of the dentinal tubules of these materials is necessary.

5 CONCLUSIONS

Within of the limits of this study it was concluded that in relation to dentin permeability, all materials were able to reduce the permeability values after the erosive-abrasive challenge, except conventional fluoride varnish. Only the universal adhesive system was able to resist the proposed challenge. Regarding dentinal tubule occlusion, AMTN and the universal adhesive system proved to be effective in sealing the dentinal tubules. Furthermore, the negative correlation found confirms the action of desensitizing materials studied by dentinal tubule occlusion.

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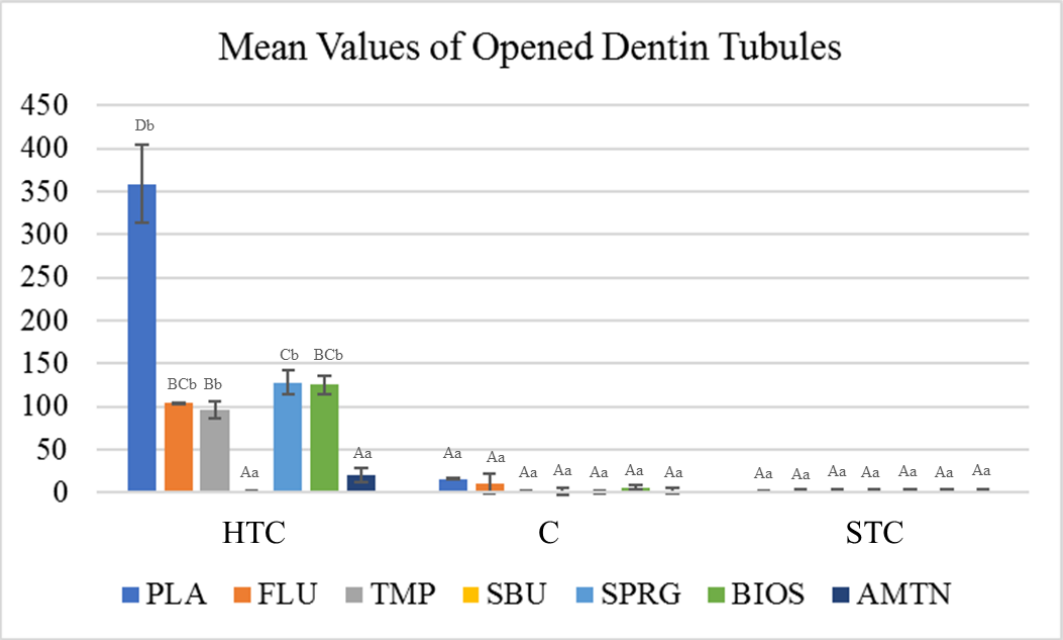
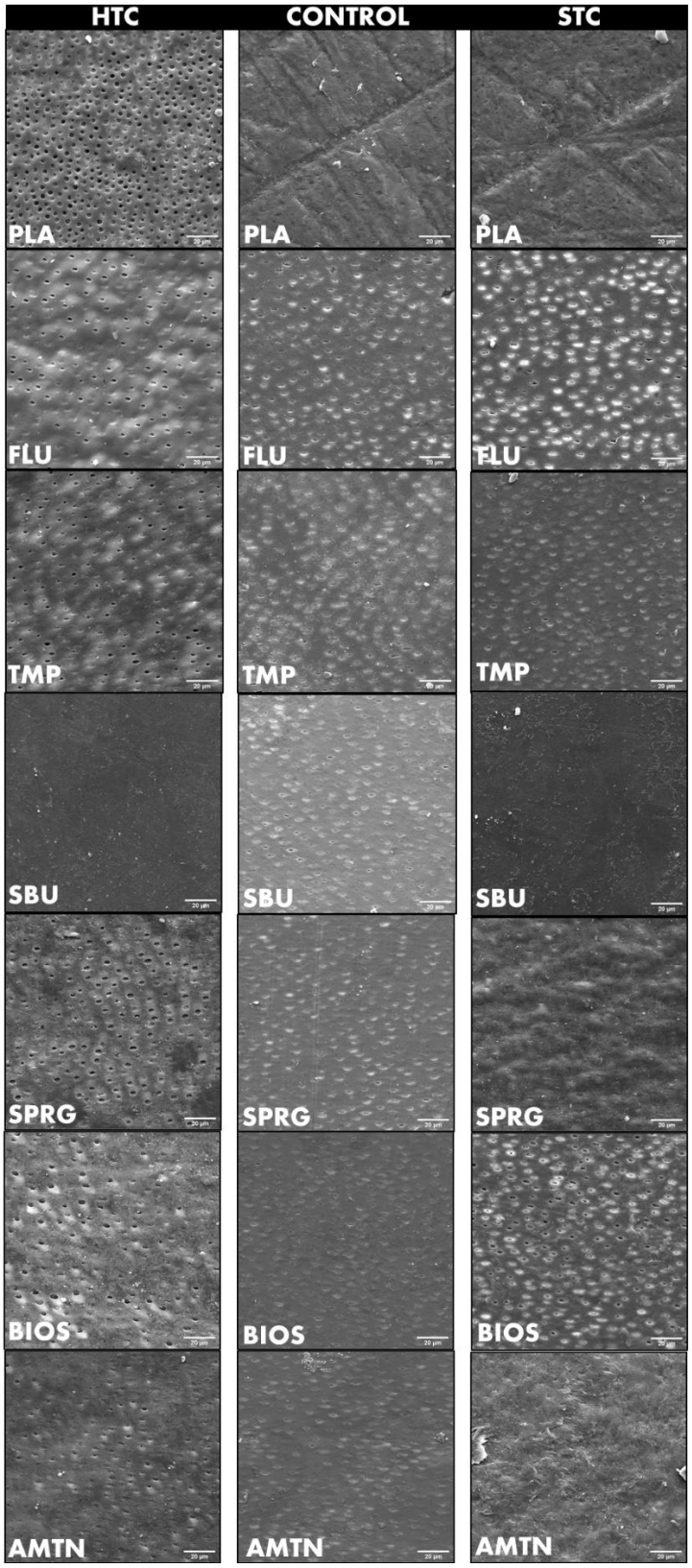
FIGURAS



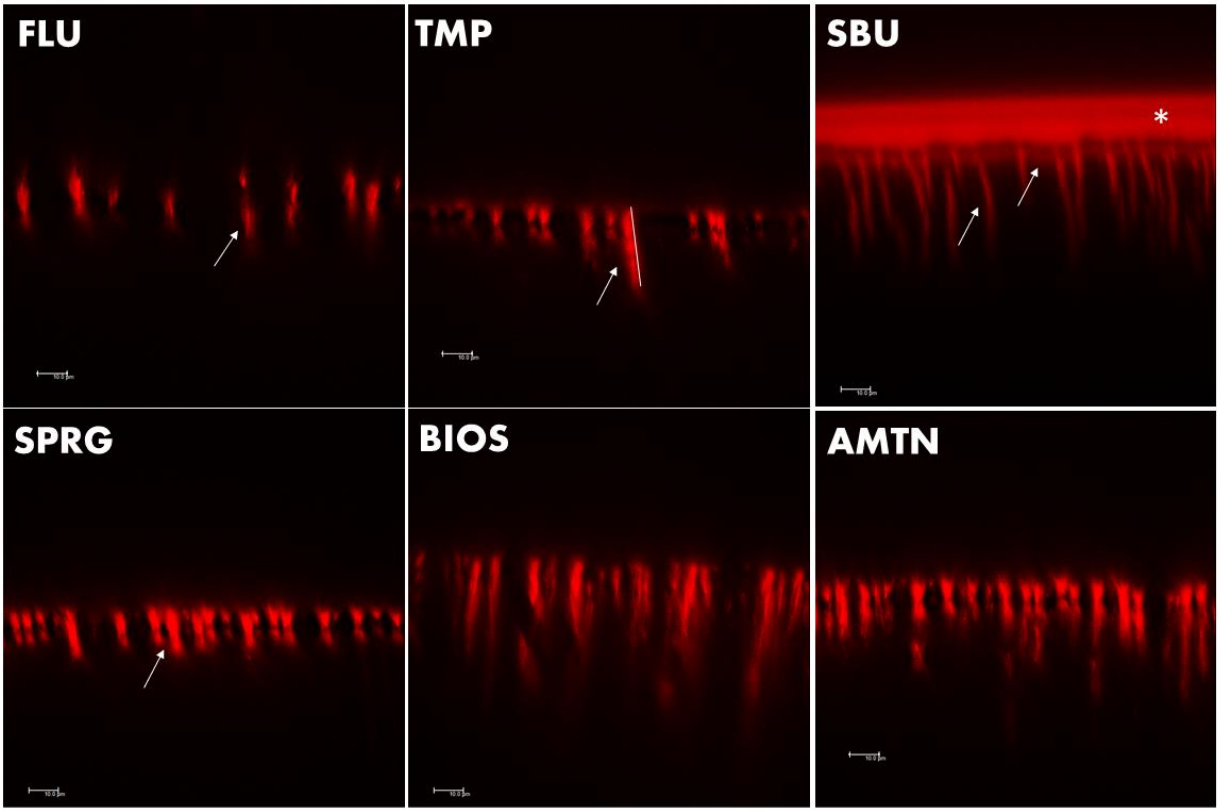
PLA, Placebo varnish; FLU, Fluoride varnish; TMP, TMP varnish; SBU, Universal adhesive system; SPRG, Varnish with S-PRG filler. BIOS, Biosilicate solution; AMTN, Amelotin solution

Fig. 1 - Mean values of number of opened dentinal tubules in each area and group.



PLA, Placebo varnish; FLU, Fluoride varnish; TMP, TMP varnish;
SBU, Universal adhesive system; SPRG, Varnish with S-PRG filler.
BIOS, Biosilicate solution; AMTN, Amelotin solution.

Fig. 2 - Representative SEM micrographs of dentin surface according to each area and group.



PLA, Placebo varnish; FLU, Fluoride varnish; TMP, TMP varnish; SBU, Universal adhesive system; SPRG, Varnish with S-PRG filler. BIOS, Biosilicate solution; AMTN, Amelotin solution.

Fig. 3 - Representative CLSM micrographs demonstrating dentinal tubule occlusion of each group after erosive-abrasive challenge. The asterisk indicates the layer remained by SBU group and the arrows and lines the infiltration of materials.

TABELAS

Table 1 - In-office desensitizing materials tested in this study.

Groups	Material	Main ingredients	Manufacturer	Batch #
PLA	Placebo varnish	Artificial resin, solvent, essence, saccharine, and deionized water.	SS White Produtos Odontológicos	-
FLU	Fluoride varnish (Duraphat)	5% NaF (22.600 ppm), colophony; solvent, shellac; mastic; saccharine and others.	Colgate-Palmolive Company	022001
TMP	TMPnano varnish	NaF 5%+5% TMPnano (22,7 nm); artificial resin, solvent, essence, saccharine, and deionized water.	SS White Produtos Odontológicos and Sigma-Aldrich	-
SBU	Universal Single Bond	BISGMA; HEMA; UDMA; DPIHFP, 10-MDP; solvent; water; silane and others.	3M ESPE	1833100782
SPRG	Barrier Coat (S-PRG filler varnish)	S-PRG filler (3,0 μm): TEGDMA; Bis-MPEPP; fluorine boron aluminosilicate; MAA; phosphonic acid and others.	Shofu INC.	121901
BIOS	Biosilicate solution	Solution 1:10 powder (P_2; O_5-Na_2; O-CaO-SiO_2 1-10 μm) and distillate water.	Laboratory of Vitreous Materials at the Federal University of São Carlos	-
AMTN	Amelotin solution	Protein derived from dental enamel in ultrapure water solution (5μg/μL)	Experimental	-

Abrevviations: TMPnano (nanoparticulate sodium trimetaphosphate) TEGDMA (triethylene glycol dimethacrylate); BISGMA (diglycidildimethacrylate A); HEMA (Hydroxyethylmethacrylate); UDMA (1,3 glycol dimethacrylate) DPIHFP (Diphenyliodonium hexafluorophosphate); 10-MDP (10-decanediol phosphate methacrylate); Bis-MPEPP (bisphenol A polyethoxy methacrylate); MAA (methacrylic acid)

Materials	Main ingredients	Manufacturer	Batch #	Application
Placebo varnish (PLA)	Artificial resin, solvent, essence, saccharine, and deionized water.	SS White Dental Products	-	A thin layer was passively applied for 5 s under the clean and dry surface with a disposable applicator, remaining stable for 10 min
Fluoride varnish (Duraphat – FLU)	5% NaF (22.600 ppm), colophony; solvent, shellac; mastic; saccharine and others.	Colgate-Palmolive Company	022001	
TMPnano varnish (TMP)	NaF 5%+5% TMPnano (22,7 nm); artificial resin, solvent, essence, saccharine, and deionized water.	SS White Dental Products and Sigma-Aldrich	-	
Universal Single Bond (SBU)	BISGMA; HEMA; UDMA; DPIHFP, 10-MDP; solvent; water; silane and others.	3M ESPE	1833100782	A thin layer was actively applied for 20 s on the clean and dry surface with a disposable applicator and light-cured for 10 s. No previous acid etching was performed
Barrier Coat (S-PRG filler varnish – SPRG)	S-PRG filler (3,0 μm): TEGDMA; Bis-MPEPP; fluorine boron aluminosilicate; MAA; phosphonic acid and others.	Shofu INC.	121901	A thin layer of one drop active mixed with base in the base container was applied for 3 s on the clean and dry surface with specific applicator and light-cured for 10 s. The uncured layer was removed from surface with a water-moistened cotton pellet
Biosilicate solution (BIOS)	The solution was composed of Biosilicate powder (P_2; O_5-Na_2; O-CaO-SiO_2 1-10 μm) and distilled water (1:10 ratio) and for simulation of the professional-use products, the particles were mixed immediately before application	Laboratory of Vitreous Materials at the Federal University of São Carlos	-	A thin layer was applied for 5 s on the clean and dry surface with a disposable applicator, remaining stable for 10 min.
Amelotin solution (AMTN)	Protein derived from dental enamel. The solution was prepared with 100 μL of pure water add to 500 μg of AMTN powder. The final result was 200 μL of solution (5μg/uL concentration).	Experimental	-	5 μL of solution were applied for 10 s on the clean and dry surface with a disposable applicator, remaining stable until no visible liquid left.

Abrevviations: TMPnano (nanoparticulate sodium trimetaphosphate) TEGDMA (triethylene glycol dimethacrylate); BISGMA (diglycidildimethacrylate A); HEMA (Hydroxyethylmethacrylate); UDMA (1,3 glycol dimethacrylate) DPIHFP (Diphenyliodonium hexafluorophosphate); 10-MDP (10-decanediol phosphate methacrylate); Bis-MPEPP (bisphenol A polyethoxy methacrylate); MAA (methacrylic acid)

Table 2 - Dentin permeability measurements (Lp; Mean ± SD) of the different in-office desensitizing materials, before and after application, and after erosive-abrasive challenge.

Times / Groups	After EDTA immersion (T0)	After application (T1)	After erosive-abrasive challenge (T2)
PLA	1.10 ± 0.06^{Ab}	0.84 ± 0.07^{Ca}	1.47 ± 0.05^{Ec}
FLU	1.09 ± 0.10^{Ab}	0.88 ± 0.04^{Ca}	1.24 ± 0.05^{Dc}
TMP	1.06 ± 0.08^{Ac}	0.68 ± 0.04^{Ba}	0.96 ± 0.07^{Cb}
SBU	1.09 ± 0.07^{Ab}	0.65 ± 0.04^{Ba}	0.69 ± 0.05^{Aa}
SPRG	1.08 ± 0.07^{Ac}	0.66 ± 0.02^{Ba}	0.85 ± 0.06^{Bb}
BIOS	1.04 ± 0.06^{Ac}	0.68 ± 0.06^{Ba}	0.84 ± 0.04^{Bb}
AMTN	1.06 ± 0.08^{Ac}	0.51 ± 0.06^{Aa}	0.75 ± 0.06^{Ab}

Distinct letters, uppercase in columns and lowercase in rows, indicate statistically significant differences ($p<0.05$).

Table 3 - Length of dentinal tubule occluded measurements (µm; Média ± SD) and scores values of number of dentinal tubules occluded, according to in-office desensitizing materials tested.

Groups	Mean length of dentinal tubules occluded	Score – number dentinal tubules occluded				
		I	II	III	IV	
PLA	4.76 ± 8.24^{D}	4	2	0	0	B
FLU	11.81 ± 3.31^{BCD}	2	3	1	0	B
TMP	7.56 ± 4.31^{CD}	2	1	3	0	AB
SBU	24.15 ± 5.49^{A}	0	4	1	1	AB
SPRG	18.66 ± 4.04^{AB}	0	2	3	1	AB
BIOS	20.7 ± 5.24^{AB}	0	3	3	0	AB
AMTN	16.46 ± 0.74^{ABC}	0	1	2	3	A

Distinct letters indicate statistically significant differences ($p<0.05$).

ANEXOS

ANEXO A – Certificado da Comissão de Ética no Uso de Animais

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Avaliação in vitro dos efeitos de diferentes protocolos para hipersensibilidade dentinária após desafio erosivo-abrasivo"**, Processo FOA nº 00418-2020, sob responsabilidade de Ticiane Cestari Fagundes Tozzi apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 02 de Outubro de 2020.

VALIDADE DESTE CERTIFICADO: 02 de Junho de 2022.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 02 de Julho de 2022.

CERTIFICATE

We certify that the study entitled **"In vitro evaluation of the effects of different protocols for dentin hypersensitivity after erosive-abrasive challenge"**, Protocol FOA nº 00418-2020, under the supervision of Ticiane Cestari Fagundes Tozzi presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on October 02, 2020.

VALIDITY OF THIS CERTIFICATE: Junho 02, 2022.
DATE OF SUBMISSION OF THE FINAL REPORT: July 02, 2022.

Prof. Associado Guilherme de Paula Nogueira
Coordenador da CEUA
CEUA Coordinator

CEUA - Comissão de Ética no Uso de Animais
Faculdade de Odontologia de Araçatuba
Faculdade de Medicina Veterinária de Araçatuba
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(CEUA)

ANEXO B – Normas da revista selecionada para publicação do artigo

Dental Materials – Guide for Authors:

https://www.elsevier.com/journals/dental-materials/0109-5641/guide-for-authors