Universidade Estadual de Campinas
Faculdade de Odontologia de Piracicaba

LARISSA MARCELINO

# LONGEVIDADE DA UNIÃO DE UM ADESIVO AUTOCONDICIONANTE À DENTINA DESMINERALIZADA TRATADA COM O PEPTÍDEO DE AUTOMONTAGEM $P_{11}$-4

# BOND LONGEVITY OF A SELF-ETCHING ADHESIVE AND DEMINERALIZED DENTIN TREATED WITH $P_{11}$-4 SELF-ASSEMBLING PEPTIDE

Piracicaba
2023

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Materiais Dentários.

Orientadora: Profa. Dra. Regina Maria Puppin Rontani

Coorientadora: Profa. Dra. Carmem Silvia Costa Pfeifer

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Este exemplar corresponde à versão final da tese defendida pela aluna Larissa Marcelino e orientada pela professora Dra. Regina Maria Puppin Rontani.

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## RESUMO

O objetivo do estudo foi avaliar o desempenho de um sistema adesivo autocondicionante associado ao $P_{11}$-4 em dentina afetada por cárie, em diferentes tempos de armazenamento em água deionizada, bem como avaliar a molhabilidade de superfície e longevidade de união resina/dentina. Os grupos de estudo foram: Dentina hígida; Dentina desmineralizada; $P_{11}$-4; $Ca^{2+}$ + $PO_4^{3-}$; $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$; e $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer. Foi realizada a Avaliação da Molhabilidade (n = 6), Teste de Resistência de União à Microtração (n = 10), Avaliação da Nanoinfiltração e EDS (n = 3) e Avaliação da Morfologia da Superfície (n = 3). Os valores do ângulo de contato foram submetidos aos testes de Levene e Shapiro-Wilk para variância e normalidade, respectivamente. Em seguida, as diferenças de molhabilidade foram avaliadas estatisticamente usando ANOVA unidirecional e teste post hoc de Games-Howell no Jamovi 2.2. Os dados de resistência de união à microtração foram apresentados com estatística descritiva e analisados utilizando um modelo linear generalizado. O teste Exato de Fisher foi aplicado para falhas prematuras e padrões de fratura. Todas as análises, com nível de significância de 5%, foram realizadas em R. Os resultados demostraram que $P_{11}$-4 aumentou significativamente a molhabilidade da dentina desmineralizada. No entanto, não houve interação significativa entre o tempo de armazenamento em água deionizada e o tratamento de superfície ao avaliar a resistência de união à microtração. O tempo de armazenagem diminuiu significativamente a resistência de união à microtração para todos os grupos ($p<0,05$), enquanto não houve diferença significativa entre os grupos de tratamento ($p>0,05$). A avaliação da nanoinfiltração demonstrou infiltração homogênea de nitrato de prata ao longo da camada hibrida nos grupos dentina hígida e desmineralizada, os demais grupos apresentaram interrupção da camada hibrida no tempo de armazenamento de 24 horas. Em 6 meses de armazenamento, todos os grupos apresentaram interrupção da camada hibrida. A análise de EDS revelou maior quantidade de prata nos grupos armazenados por 24 horas, exceto para o grupo $P_{11}$-4 + $Ca^{2+}$ + $PO_4^3$. Ao longo de 6 meses de armazenamento, o grupo $P_{11}$-4 apresentou a menor quantidade de prata. A avaliação da morfologia de superfície dos grupos armazenados por 24 horas e 6 meses não demonstraram alteração ou deposição de material na superfície da dentina desmineralizada. O $P_{11}$-4 melhorou a molhabilidade da dentina desmineralizada, tornando-a mais hidrófila, com menos infiltração de prata, porém não produziu melhora na longevidade de união.

**Palavras-Chave:** Dentina. Remineralização dentária. Biomimética. Peptídeo de automontagem.

## ABSTRACT

The aim of the study was to evaluate the performance of a self-etching adhesive system associated with $P_{11}$-4 on caries-affected dentin, at different storage times in deionized water, as well as to evaluate surface wettability, and bond longevity resin/dentin. One hundred and eighty-four human third molars were sectioned, originating slices of dentin and randomly distributed according to the study group: Sound dentin; Demineralized dentin; $P_{11}$-4; $Ca^{2+}$ + $PO_4^{3-}$; $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$; e $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer. Contact angle values underwent Levene's and Shapiro-Wilk tests for variance and normality, respectively. Then, wettability differences were statistically assessed using one-way ANOVA and Games-Howell post hoc test in Jamovi 2.2. Microtensile bond strength data were presented with descriptive statistics and analyzed using a generalized linear model. Fisher's Exact test was applied to premature failure and fracture patterns. All analyses, with a 5% significance level, were conducted in R. The results demonstrated that P11-4 significantly increased the wettability of demineralized dentin. However, there was no significant interaction between storage time in deionized water and surface treatment when evaluating microtensile bond strength. Elapsed time significantly decreased microtensile bond strength for all groups ($p<0.05$), while there was no significant difference between treatment groups ($p>0.05$). The evaluation of nanoleakage showed homogeneous infiltration of silver nitrate along the hybrid layer in the sound and demineralized dentin groups, the other groups showed interruption of the hybrid layer in the 24 hours storage time. At 6 months of storage, all groups showed disruption of the hybrid layer. EDS analysis revealed a higher amount of silver in the groups stored for 24 hours, except for the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^3$ group. Over 6 months of storage, the $P_{11}$-4 group had the lowest amount of silver. The evaluation of the surface morphology of the groups stored for 24 hours and 6 months showed no change or deposition of material on the demineralized dentin surface. $P_{11}$-4 improved the wettability of demineralized dentin, making it more hydrophilic, producing a more regular hybrid layer, with less silver infiltration, but it did not produce an improvement in bond strength.



**Keywords:** Dentin. Tooth Remineralization. Biomimetics. Self-assembly peptide.

**SUMÁRIO**

## 1. INTRODUÇÃO

A cárie dentária é uma doença multifatorial, mediada por biofilme, modulada por dieta, não transmissível e dinâmica, resultando em perda mineral dos tecidos duros dentais. Como consequência desse processo, desenvolve-se uma lesão de cárie (Machiulskiene et al., 2020). Se não tratada, a cárie progride para a dentina, tecido subjacente ao esmalte, acelerando a destruição dos tecidos dentários (Bertassoni, Orgel, Antipova, Swain, 2012).

A dentina é caracterizada como um compósito nanoestruturado e hierarquicamente organizado. É formada pela dentina peritubular, que constitui a parede dos túbulos dentinários com elevado conteúdo mineral, e a dentina intertubular, situada entre os canalículos, e que consiste em uma rede orgânica tridimensional composta por fibrilas de colágeno do tipo I, onde os cristais de hidroxiapatita são depositados (Bertassoni, Orgel, Antipova, Swain, 2012). Outros componentes da matriz orgânica dentinária são as metaloproteinases da matriz (MMPs), que são enzimas endógenas capazes de degradar os componentes da matriz extracelular, ativadas durante processos patológicos, como a cárie, e etapas do procedimento restaurador, como por exemplo o condicionamento com o ácido fosfórico (Mazzoni et al., 2015).

A progressão da lesão cariosa em direção à dentina produz diversas modificações estruturais, resultando na diminuição das propriedades mecânicas e físicas deste tecido (Tjäderhane et al., 2013), o que torna o processo de adesão mais complexo (Bertassoni, Orgel, Antipova, Swain, 2012). Com o desenvolvimento da odontologia adesiva, o conceito de mínima intervenção foi amplamente difundido e estabelecido, buscando limitar a preparação das cavidades durante a remoção da cárie. A remoção seletiva do tecido cariado apresenta vantagens, principalmente quando a lesão está próxima da polpa dentária, preservando os tecidos e evitando a necessidade do tratamento endodôntico (Schwendicke, Dörfer, Paris, 2013). Essa abordagem consiste em remover a dentina infectada, preservando o máximo da dentina afetada, para servir como substrato para o procedimento adesivo restaurador (de Almeida Neves et al., 2011).

A dentina afetada apresenta dureza e resistência de união reduzidas, e a camada híbrida formada é mais porosa quando comparada à dentina sadia,

independentemente do sistema adesivo ou compósito resinoso utilizado, resultando em menor estabilidade da restauração (Perdigão, Reis, Loguercio, 2013). Tal fato pode ser prejudicial, uma vez que os procedimentos restauradores visam uma união estável entre o colágeno e os materiais poliméricos (Breschi et al., 2017). Neste contexto, a remineralização do colágeno dentinário poderia apresentar resultados satisfatórios em termos de durabilidade clínica (Zhong B et al.2015).

Na tentativa de recuperar as propriedades mecânicas perdidas pela progressão da cárie, a remineralização da dentina afetada tem sido estudada com o objetivo de restabelecer a funcionalidade do tecido através de diferentes abordagens e materiais remineralizadores (Bertassoni et al., 2009). Uma estratégia promissora é a intervenção biomimética, que visa aprimorar as propriedades e a estabilidade do substrato por meio de modificações químicas do tecido dentinário (Tjäderhane 2015). A auto-organização de peptídeos demonstra grande potencial nesse contexto devido a capacidade de formar nanoestruturas funcionais (Davies, Aggeli., 2011).

A família de peptídeos $P_{11}$ foi projetada para se auto-organizar espontaneamente em estruturas hierarquicamente organizadas em resposta a gatilhos específicos, formando β-folhas que, quando organizadas hierarquicamente, criam fibrilas entrelaçadas, resultando em fibras de colágeno (Kyle et al., 2010). Especificamente, o peptídeo de automontagem $P_{11}$-4 (PPA) (CH3CO-QQRFEWEFEQQ-NH2), comercialmente disponível como Curodont™ Repair (Barbosa-Martins et al., 2018), tem o potencial de formar fibrilas em pH baixo e ser monomérico em um pH mais alto (Kyle et al., 2010). Em pH <7, sofre automodificação espontânea, do estado líquido para um fluido Newtoniano opticamente isótropo, rapidamente, dando início ao processo de automontagem (Aggeli et al., 2003). Uma das suas características é a presença de cargas negativas em sua superfície, apresentando sítios para ligação de íons $Ca^{2+}$, controlando a deposição e o crescimento dos cristais de hidroxiapatita (Kind L et al., 2017), responsáveis por remineralizar o esmalte dental (Kirkham et al., 2007).

A aplicação do $P_{11}$-4 na dentina desmineralizada, em conjunto com um sistema adesivo convencional, demonstrou aumento considerável da resistência de união, atribuído à dependência do pH. A natureza ácida do ácido fosfórico a 37% (pH<1) desencadeou a automontagem rápida do peptídeo, criando uma estrutura de ligação

para os íons cálcio e fosfato dispersos na superfície dentinária, reforçando quimicamente toda a estrutura (Barbosa-Martins et al., 2018).

O uso do sistema adesivo autocondicionante (AC) na dentina é muito vantajoso por apresentar monômeros ácidos em sua composição, diminuindo consideravelmente a profundidade de desmineralização. Esse condicionamento e preparo do substrato ocorrem simultaneamente, permitindo melhor penetração do adesivo e eliminando a necessidade do uso separado do ácido fosfórico, tornando a técnica mais simplificada e diminuindo a camada de substrato desmineralizado desprotegida (Van Meerbeek et al. 2011).

O sistema adesivo autocondicionante de duas etapas, mais especificamente o Clearfil SE Bond, é composto por um frasco contendo um primer de monômeros ácidos (pH 2) que remove parcialmente a smear layer. Após a aplicação do adesivo contido no segundo frasco, ocorre uma ligação química entre a hidroxiapatita e os grupos carboxílicos dos monômeros funcionais (Gianinni et al., 2015). Ao contrário do sistema adesivo convencional, o AC mantém uma quantidade de cristais de hidroxiapatita ao redor das fibrilas de colágeno suficiente para protegê-las da degradação hidrolítica e enzimática. Por outro lado, o colágeno exposto após o condicionamento com ácido fosfórico, e não coberto pelo adesivo, fica muito vulnerável aos processos de degradação hidrolítica e enzimática (Van Meerbeek et al. 2011).

A dentina tratada com o PPA em conjunto com o sistema adesivo autocondicionante Clearfil SE Bond não apresentou diferença significativa em relação à dentina desmineralizada. No entanto, quando comparado à dentina sadia, apresentou um valor menor de resistência de união. A possível explicação para esses resultados é o fato de que a ausência da aplicação do ácido fosfórico deixou o processo de automontagem mais lento e menos estável, além de haver a formação de uma camada hidrofóbica após a aplicação do peptídeo, dificultando o condicionamento. Quando o PPA entrou em contato com o primer ácido, ele retornou para a fase dímero, prejudicando a molhabilidade e a penetração do adesivo (Barbosa-Martins et al., 2018). No entanto, Barbosa-Martins et al, 2018, não realizaram um estudo longitudinal para avaliar se esse protocolo do PPA associado ao sistema autocondicionante, que vem sendo indicado como mais vantajoso para

adesão à dentina é eficaz de fato. Aparentemente, o sistema AC apresenta maior estabilidade de união à dentina, do que o sistema convencional (Van Meerbeek et al, 2011).

Assim, outros estudos são necessários para complementar as questões levantadas por Barbosa-Martins et al., 2018 e compreender o desempenho do sistema AC associado ao PPA, uma vez que a adição do $P_{11}$-4 promove uma alteração na superfície dentinária por seu alto potencial de remineralização biomimética. Isso a torna mais irregular, e, consequentemente, aumenta a molhabilidade, deixando a superfície tratada com características mais próximas a de uma dentina hígida, melhorando o processo de adesão. Portanto, o objetivo do estudo foi avaliar o desempenho de um sistema adesivo autocondicionante associado ao $P_{11}$-4 em dentina afetada por cárie, em diferentes tempos de armazenamento em água deionizada, bem como avaliar a molhabilidade de superfície e longevidade de união resina/dentina.

## 2. ARTIGO

Este trabalho foi apresentado no formato alternativo de tese de acordo com as normas estabelecidas pela deliberação 002/06 da Comissão Central de Pós-Graduação da Universidade Estadual de Campinas. O artigo 1 será submetido à publicação no periódico Applied Surface Science.

**Bond longevity of a self-etching adhesive and demineralized dentin treated with $P_{11}$-4 self-assembling peptide**[1]


## ABSTRACT

The objective was to evaluate the performance of a self-etching adhesive system associated with the self-assembling peptide P11-4 in caries-affected dentin, in terms of surface wettability and resin/dentin bond longevity. One hundred and eighty-four third molars were distributed in groups: Sound dentin, Demineralized, P11-4, Ca2+ + PO43-, P11-4 + Ca2+ + PO43-, and P11-4 + Ca2+ + PO43- + Primer. Wettability (n = 6), Microtensile Bond Strength at 24 hours and 6 months (n = 10), Nanoleakage, Surface Composition and Morphology (n = 3) were evaluated. P11-4 increased the wettability of demineralized dentin. Bond strength for all groups decreased at 6 mouths ($p<0.05$), while there was no difference between treatment groups ($p>0.05$). All the treated groups were effective in interrupting silver nitrate penetration in comparison with the controls. The EDS revealed the highest amount of silver in 24 hours for all groups compared with 6 months, while the P11-4 group had the lowest amount of silver. There was no silver deposition on the demineralized dentin surface. P11-4 improved the wettability of demineralized dentin, producing a hybrid layer with less silver deposition, but there was no improvement in bond strength longevity.


**Key Words:** demineralized dentin. self-etching adhesive system. $P_{11}$-4. Nanoleakage. dentin remineralization

## INTRODUCTION

[1] Artigo formatado e submetido à revista Applied Surface Science

When dentin is affected by caries disease, the tissue undergoes rapid demineralization, leading to increased porosity and softness compared to sound dentin (1). The lost mineral component is instantaneously replaced by water, resulting in altered mechanical properties, such as bond strength and hardness (2). The compromised state of the dentin affected by caries poses challenges for adhesion, irrespective of the selected adhesive system (2). Nevertheless, self-etching adhesive system have emerged as a favorable option due to their less aggressive interaction with the dentin (3). Despite the advantages of using self-etching adhesive systems, bond strength in caries-affected dentin remains lower than in sound dentin. This can be attributed to factors such as cyclic masticatory forces, thermal stimuli, and hydrolysis of monomers and collagen fibers, which degrade the resin-dentin bonded interface (1).

To address this issue, dentin remineralization has emerged as a promising alternative to restore the substrate before applying the restorative treatment, aiming to achieve a structure resembling sound dentin (4), with the aim to improve bond strength and restoration longevity (5). Among remineralization strategies, biomimicry seeks to emulate the natural process of mineralization of structures (6). The self-assembling peptide P11-4 (CH 3 CO-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH 2) has the ability of spontaneously arrange into hierarchical structures, forming intertwining fibrils that generate collagen fibers at high pH, reverting back to individual, disorganized peptides at low pH (7, 8). For example, if the pH drops below 7, P11-4, transforms into a hydrogel (7). This property presents the intriguing possibility to apply the low viscosity hydrogel in areas of tissue porosity, including carious lesions and exposed dentin (9), with the goal of attracting calcium ions to perform hydroxyapatite crystallization (7).

Studies have demonstrated the synergistic effect resulting from the combination of P11-4 with a conventional adhesive system (10,11,12). However, so far, there is no longitudinal investigation evaluating the association of P11-4 with a self-etching adhesive system, indicated by its advantage in adhesion to dentin due to greater bond stability compared to the conventional system (3). Therefore, this study aims to evaluate the performance of a self-etching adhesive system associated with P11-4 in caries-affected dentin, to evaluate surface wettability and resin/dentin bond longevity. The hypotheses tested are that pretreatment with P11-4 improves the wettability of

dentin affected by caries, increases the resin/dentin bond strength, improves the stability of the hybrid layer and consequently improves longevity of union.

## MATERIALS AND METHODS

### Sample preparation

One hundred and eighty-four non-carious human third molars were included in this study after approval of the study protocol by the Ethics Committee (Protocol: 52360821.7.0000.5418). Following extraction, the teeth were stored in deionized water at -4ºC for a maximum period of 6 months. The teeth were sliced transversally, being transformed in dentin slices. The tooth slices were randomly distributed into the respective groups, as illustrated in the flowchart.

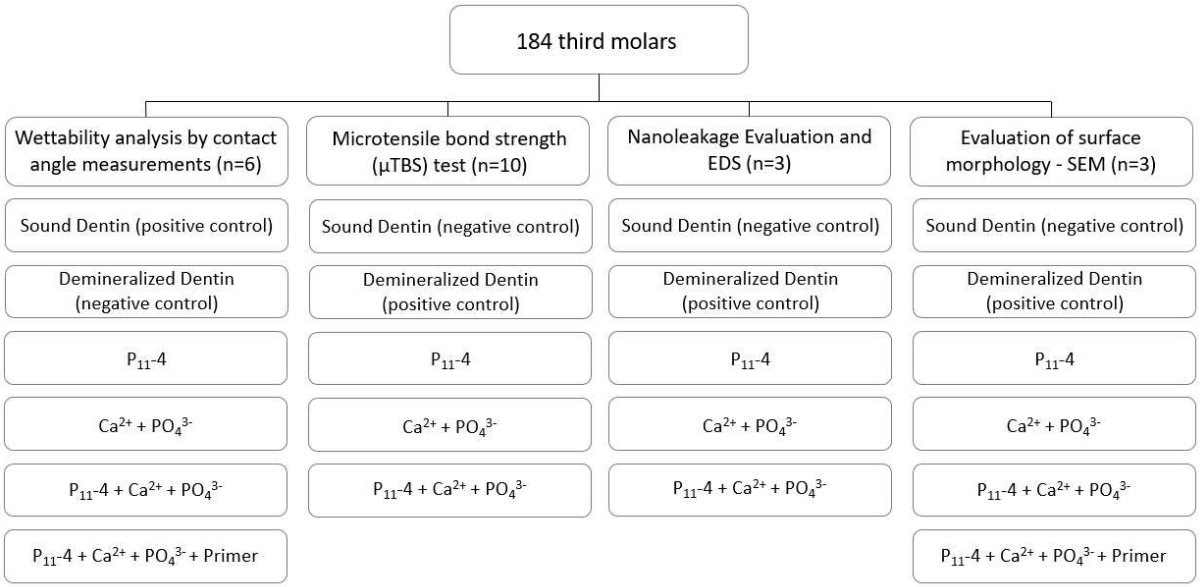


**Figure 1 -** Experimental design.

### Caries-affected dentin production – biological method

To create demineralized dentin, the external surfaces of the tooth slices were coated with nail polish varnish (Risqué, Coty Brasil Comércio S.A, Goiás, Brazil), leaving only the dentin surface exposed (13). The slices were mounted with orthodontic wire in glass container lids (14) and immersed in 800ml of 1% T Chloramine (Sigma Aldrich, São Paulo, Brazil) at room temperature for 7 days (15). Following the disinfection period, the slices were thoroughly rinsed with sterile water and transferred

to another glass flask containing 600ml of Brain Heart Infusion (BHI) culture medium, supplemented with 0.5% yeast extract, 0.5% glucose, 1% sucrose, and 2% *S. mutans* (UA159). The flask was then incubated in a microaerophilic incubator (10% $CO_2$) at 37°C. The concentration of the *Streptococcus mutans* suspension was determined by measuring the absorbance at 550nm (A550), and bacterial suspensions were adjusted to 0.05 before inoculation to initiate the cariogenic challenge (16).

On the first day of the experiment, *S mutans* strains were inoculated, and the culture medium was renewed every 48 hours for a total of 14 days. Following the caries lesion production process, the infected portion of the carious dentin was carefully removed with the help of a scalp (Golgran, São Caetano do Sul, São Paulo, Brazil). To regularize the preparation, a spherical tungsten-carbide drill (#8; JET; Beavers Dental, Morrisburg, Canada) operating at low speed was used. The removal was deemed complete when the excavated dentin surface appeared darkened and slightly hardened, in comparison to the previously removed tissue, and required gentle pressure during the excavation process (17).

**Dentin Surface Treatment**

The treatments were used according to the study groups:

**Positive control** - Sound dentin.

**Negative control** - Demineralized dentin by the biological model (DDB).

**$P_{11}$-4:** DDB treated with 10 µl solution applied (5 min).

**$Ca^{2+}$+ $PO_4^{3-}$** solution: DDB treated with 10 µl solution applied (1 min) calcium/phosphate saturated.

**$P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$**: DDB treated with 10 µl solution of $P_{11}$-4 was applied for 5 min, the excess solution was removed with absorbent paper, and then 10 µl **$Ca^{2+}$+ $PO_4^{3-}$** solution was applied for 1 min.

**$P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer**: DDB treated with 10 µl of P11-4 solution was applied for 5 min, the excess solution was removed with absorbent paper, and then 10 µl of $Ca^{2+}$+ $PO_4^{3-a}$ solution was applied for 1 min. Then the excess solution was removed

with absorbent paper, and the primer of the adhesive system was actively applied using a micro brush, following the manufacturer's guidelines.

All solutions and materials used in the experimental groups are presented on Table 1, considering their composition and applying ways.

**Table 1 -** Materials, manufactures, components and application modes.

| Materials (manufacturers) | Components* | Application mode |
|---|---|---|
| **$P_{11}$-4 Curodont™ Repair** Credentis AG, Dorfstrasse, Windisch, Switzerland | $P_{11}$-4 peptide – amino acid sequence (CH3CO-Q-Q-R-F-E-W-E-F-E-Q-QNH2). | Apply 10 µl of $P_{11}$-4 for 5 min |
| **$Ca^{2+}$ and $PO_4^{3-}$ solution** | 1.5 mM calcium, 0.9 mM phosphate, 150 mM potassium chloride (KCl), 20 mM tris base buffer, pH 7.0 | Apply 10 µl of $Ca^{2+}$ and $PO_4^{3-}$ solution for 1 min |
| **Clearfil SE Bond** | Primer: MDP, HEMA, dimethacrylate monomer, water, catalyst<br>Bond: MDP, HEMA, dimethacrylate monomer, microfiller, catalyst | 1. Apply Primer for 20 seconds and dry with mild air flow<br>2. Apply Bond, and air flow gently<br>3. light curing for 10 seconds |
| **Filtek™ Z250 XT** 3M ESPE; St Paul, MN, USA | BisGMA, UDMA, BisEMA, camphorquinone, treated silanized ceramic, TEGDMA, Treated silica | 1. Incremental insertion 2mm<br>2. Light cure for 20s |

***Composition of all materials were described as manufacturer presented on the commercial material.**

## Wettability Assessment

Thirty-six dentin slices (n=6) were randomly assigned to different study groups. To evaluate surface hydrophilicity, the specimens were placed on the table of the Digidrop goniometer (Labometric Lda, Leiria, Portugal), with their surfaces

perpendicular to the attached syringe. A drop of approximately 2 µl of deionized water was dispensed onto the center of each dentin slice. The contact angles of the water droplets on dentin surfaces were measured using a Charge-Coupled Device (CCD) camera system, with the image immediately sent to the computer for analysis, using the GBX Digidrop software (GBX Company, Bourg de Péage, France). The mean and standard deviation of the contact angles were calculated to assess the change in surface hydrophilicity resulting from the treatment of demineralized dentin (10).

**Microtensile Bond Strength Assay (µTBS)**

One hundred dentin slices (n=10) were randomly assigned to the different study groups. The application of Clearfil SE Bond Self-Etch Adhesive was carried out by a single operator following the manufacturer's instructions. For light curing, an LED curing unit (VALO™ Cordless, Ultradent, US) with an irradiance of 911 mW/cm$^2$ was used. The micro-hybrid composite resin (Filtek™ Z250 XT, A3.5, 3M ESPE) was applied in two increments of approximately 2 mm in height, forming a block with a total height of 4 mm. Each increment was photoactivated for 20 seconds. After the restorative procedure, the resin/dentin sets were stored in containers with deionized water for 24 hours and 6 months, and kept in an incubator at 37ºC for the respective storage periods.

After the designated storage period, the sets were sectioned perpendicular to the bonding interface (composite resin/adhesive/dentin) in both mesiodistal and buccolingual directions to obtain beams with a square-shaped transversal area with a maximum size of 1 mm$^2$. Each end of the beams was securely fixed using cyanoacrylate glue (Super bonder power flex, Loctite, Düsseldorf, Germany) within the microtensile device. This setup ensured that the adhesive area was perpendicular to the long axis of the tensile force, allowing for the µTBS (microtensile bond strength) test using the EZ-Test testing machine (Shimadzu Corporation, Kyoto, Japan). The bonding area was kept out of glue contact. The cross-sectional area of each beam was measured using a digital caliper (Mitutoyo; Kawasaki, Japan) to determine the accurate cross-sectional area (18). The tensile speed during testing was set to 0.5 mm/min until the point of fracture, and the resulting force value was used to calculate the bond strength (MPa).

The same procedures for obtaining the beam specimens and evaluating the microtensile bond strength were carried out on all groups after 6 months of storage in deionized water at 37ºC.

**Analysis of Failure Mode**

The fractured specimens, both those stored for 24 hours and 6 months, were analyzed and characterized by the same operator under a stereomicroscope (Carl Zeiss, Oberkochen, Germany). The type of failure was classified into four categories: adhesive failure (A), mixed failure (M), cohesive into the dentin failure (CD), and cohesive into the composite failure (CC) (18).

Following the classification based on the failure type, the specimens were fixed onto stubs using double-sided carbon tape (Electron Microscopy Sciences, Washington 19034 – USA). To ensure optimal conditions for analysis, the specimens were dehumidified for 2 hours inside a sealed plastic container containing silica gel. Subsequently, the specimens were coated with a thin layer of gold (SCD 050; Balzers, Schaan, Liechtenstein) for 120s at 40 mA. The specimens were then analyzed using a scanning electron microscope (JSM 5600 LV; JEOL, Tokyo, Japan) at a voltage acceleration of 15 kV at 100x magnification funder observation of the same previously calibrated operator.

**Interfacial Nanoleakage Assay**

Thirty dentin slices of third molars (n=3) were prepared and treated based on the respective study groups described in the Microtensile Bond Strength Assay section. These specimens were randomly divided into two groups for evaluation after different storage times in an incubator with deionized water at 37ºC, namely 24 hours and 6 months.

From each group, three slices were carefully sectioned from the middle portion, ensuring the inclusion of the resin-dentin interface (n=3). Subsequently, these slices were immersed in a 50% ammoniacal silver nitrate solution (w/w) for 24 hours, while being protected from light. After this period, the specimens were rinsed with deionized water and immersed in a photographic development solution for 8 hours under

fluorescent light. This process served to reduce silver ions, forming metallic silver grains that occupied the voids along the bonded interface (19).

Then, the slices were embedded in acrylic resin and polished with sandpaper with grit #600, #800, #1200, #2400, and #4000 (Carborundum abrasives, Guarulhos, São Paulo, Brazil). Between each change in the sandpaper granulation, all slices were rinsed with deionized water in an ultrasonic bath for 30 min (Ultrasound Ultrason 1440 D-Odontobrás Ind. E Com. Med. Odont. Ltda, Rio Preto, SP, Brazil) to remove debris.

The specimens were deproteinized with 10% NaOCl for 5 min, rinsed in an ultrasonic bath for 30 min, and dehumidified for 1 day in a sealed plastic container containing silica gel. The specimens were coated with carbon (Balzers-SCD 050 Sputter Coater), analyzed using SEM (JSM-5600LV; JEOL, Tokyo, Japan), and observed in electron backscattered mode at 15 kV.

**Energy dispersive X-Ray Spectroscopy (EDS)**

The same dentin slices used by the nanoleakage assay were evaluated for the EDS assay. The amount of silver nitrate absorption in the hybrid layer was recorded using an energy-dispersive X-ray spectroscopy (EDS) detector coupled with SEM. Three representative and different areas of each slice of the bonding interface were chosen for measurements, and the atomic percentage of silver (in % Ag) was quantified by the Easy Macro software (VANTAGE digital microanalysis system) available at SEM (12). It evaluated the distribution of Ag deposits on hybrid layer region and qualitatively descripted.

**Evaluation of surface morphology using SEM**

Fifteen dentin slices from human third molars (n=3) were prepared and treated according to the study groups outlined in the flowchart. After the completion of the treatments, the dentin slices were carefully divided into identical parts by the use of a double-sided diamond saw (American Burs, Palhoça, Santa Catarina, São Paulo) mounted on a handpiece with constant refrigeration. This division allowed the separation of the samples based on the two storage times, 24 hours, and 6 months. With the assistance of a diamond tip #3216 in high rotation and constant irrigation, two channels were created on each part, marking the ¼ position of the sample (20).

Following the storage period in deionized water, the samples were frozen by at -80ºC for 20 minutes. Subsequently, a surgical chisel was positioned within the channels, and the samples were fractured accordingly. Each fractured specimen was then subjected to a gradual dehydration process using ethanol at different concentrations (50%, 70%, 90% and 100%) for 20 min each. After the dehydration process, all samples were soaked in alcohol within the drying chamber. In this chamber, the samples were cooled down to 4ºC, and a $CO_2$ pressure was applied, gradually increasing to reach a maximum of 1650 pounds.

All samples were fixed on stubs with double-sided carbon tape (Electron Microscopy Sciences, Washington 19034 – USA) and dehumidified for 2 hours in a closed plastic container containing silica gel. The stubs were coated with gold (SCD 050; Balzers, Schaan, Liechtenstein) for 120s at 40 mA, and analyzed in a scanning electron microscope (SEM) (JSM 5600 LV; JEOL, Tokyo, Japan) at a voltage acceleration of 15 kV at 150x, 500x, and 1500x magnifications by the same previously calibrated operator. For the other specimens (6 months storage), the same procedure was performed.

**Statistical Analysis**

Contact angle values underwent Levene's and Shapiro-Wilk tests for variance and normality, respectively. Then, wettability differences were statistically assessed using one-way ANOVA and Games-Howell post hoc test in Jamovi 2.2. Microtensile bond strength data were presented with descriptive statistics and analyzed using a generalized linear model. Fisher's Exact test was applied to premature failure and fracture patterns. All analyses, with a 5% significance level, were conducted in R.

**RESULTS**

Among the different groups tested, the $P_{11}$-4 group exhibited the most substantial increase in wettability, closely followed by the group treated with $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer. Both groups demonstrated enhanced wettability, evident from the reduced contact angle of deionized water on the demineralized dentin surface. Conversely, the sound and demineralized groups showed the highest contact angle values, indicating reduced wettability. There was no statistically significant difference

between the demineralized dentin group and the group $P_{11}$-4 and $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer.

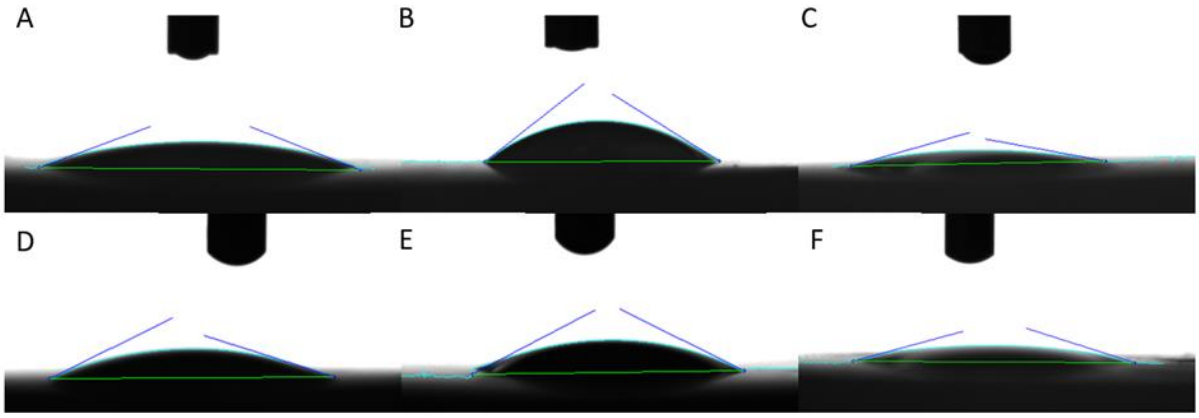


**Figure 2:** A- Sound Dentin, B - Demineralized, C - $P_{11}$-4, D - $Ca^{2+}$ + $PO_4^{3-}$, E - $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$, F - $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer.

**Table 2:** Means and standard deviations of contact angle between water drop and dentin substrate.

| Treatments | θ ± SD |
|---|---|
| Sound | 36.6 ± 14.7 a |
| $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ | 29.1 ± 5.45 a |
| $Ca^{2+}$ + $PO_4^{3-}$ | 23.7 ± 3.99 a |
| Demineralized | 22.8 ± 7.82 ab |
| $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ +Primer | 11.1 ± 2.97 b |
| $P_{11}$-4 | 10.1 ± 2.61 b |

Different letters indicate statistically differences between values. The data were submitted to the Levene test to evaluate the homogeneity of the variances and to the Shapiro-Wilk test to evaluate the normality of the data distribution. The statistical difference was fixed at α=5%.

Concerning uTBS, there was no significant interaction between study factors (time elapsed x treatments). Results indicated that after 6 months the uTBS values in all four treatments was significantly lower compared to the values observed at 24h ($p<0.05$, Figure 3). At 24 hours, there was no significant difference between all four

treatments ($p>0.05$), however, all treatments showed significantly lower bond strength than sound dentin group ($p<0.05$). After 6 months, the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ treatment showed less resistance than the others ($p<0.05$) and all treatments showed less resistance than healthy teeth ($p<0.05$), Figure 3.

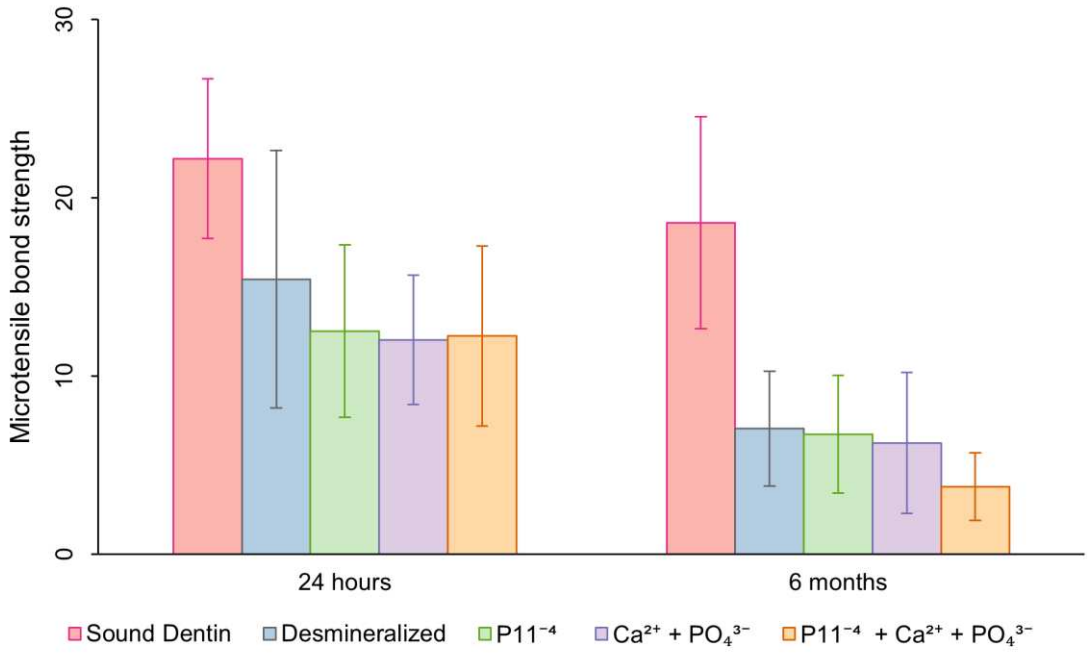


**Figure 3.** Microtensile bond strength as a function of treatment and time (mean and standard deviation)

It is observed in Figure 3 that there was no significant association between treatments and the presence of premature failures ($p>0.05$). After 24 hours, there was no premature failure. At 6 months, the percentages of premature failures ranged from 10.0% (Sound Dentin and $P_{11}$-4) to 40.0% ($Ca^{2+}$ + $PO_4^{3-}$).

Concerning failure site analysis, there was a significant association between the treatments and the fracture pattern ($p<0.05$). At both 24 hours and 6-month evaluations, most specimens exhibited a mixed type of failure regardless of the treatment applied. However, after 24 hours, the sound group showed a significantly lower occurrence of adhesive-type failures, with only 1.8% of the specimens displaying this type of failure. In contrast, the $Ca^{2+}$ + $PO_4^{3-}$ and $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ groups showed higher percentages of adhesive-type failures, at 9.4% and 14.8%, respectively. After 6 months, the occurrence of adhesive failure increased in both

sound and $Ca^{2+}$ + $PO_4^{3-}$ groups, with 12.5% and 12.9% of specimens showing this type of failure, respectively. However, the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ group exhibited a notably higher percentage of adhesive-type failure at 33.3% (Figure 4). This indicates that over time, the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ treatment led to a higher prevalence of adhesive-type fractures compared to the other treatments and the sound group.

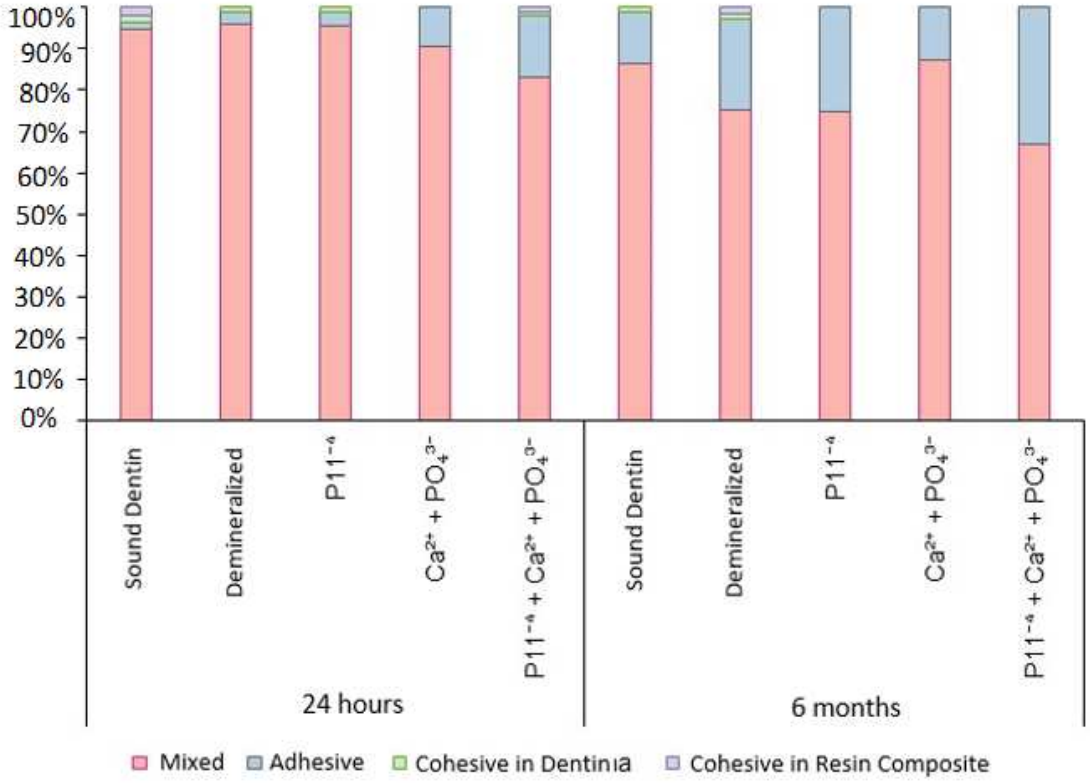


**Figure 4**. Distribution of failure pattern according to group.

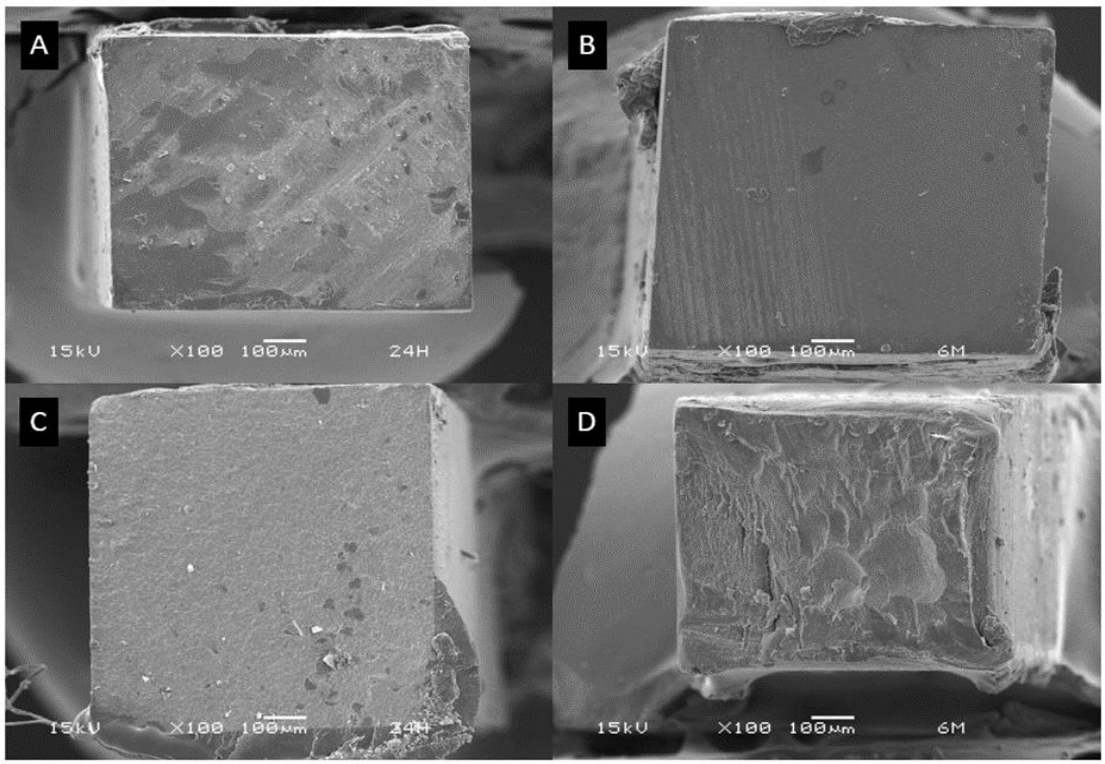


**Figure 5:** SEM image of failure modes: (A) Mixed; (B) Adhesive; (C) Cohesive in Resin Composite; (D) Cohesive in Dentin.

The evaluation of nanoleakage showed continuous infiltration of silver nitrate along the hybrid layer in the sound and demineralized dentin groups, while the other groups showed interruption of infiltration through the hybrid layer for the 24 hours storage time. However, at 6 months of storage, all groups showed continuous disruption of the hybrid layer.

The EDS analysis showed a higher amount of silver for the groups stored for 24 hours, at the different points selected for analysis, except for the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ group, which showed the lowest deposition along the hybrid layer. Groups stored for 6 months showed less deposition of silver nitrate along the hybrid layer, with the lowest amount visualized in the $P_{11}$-4 group.

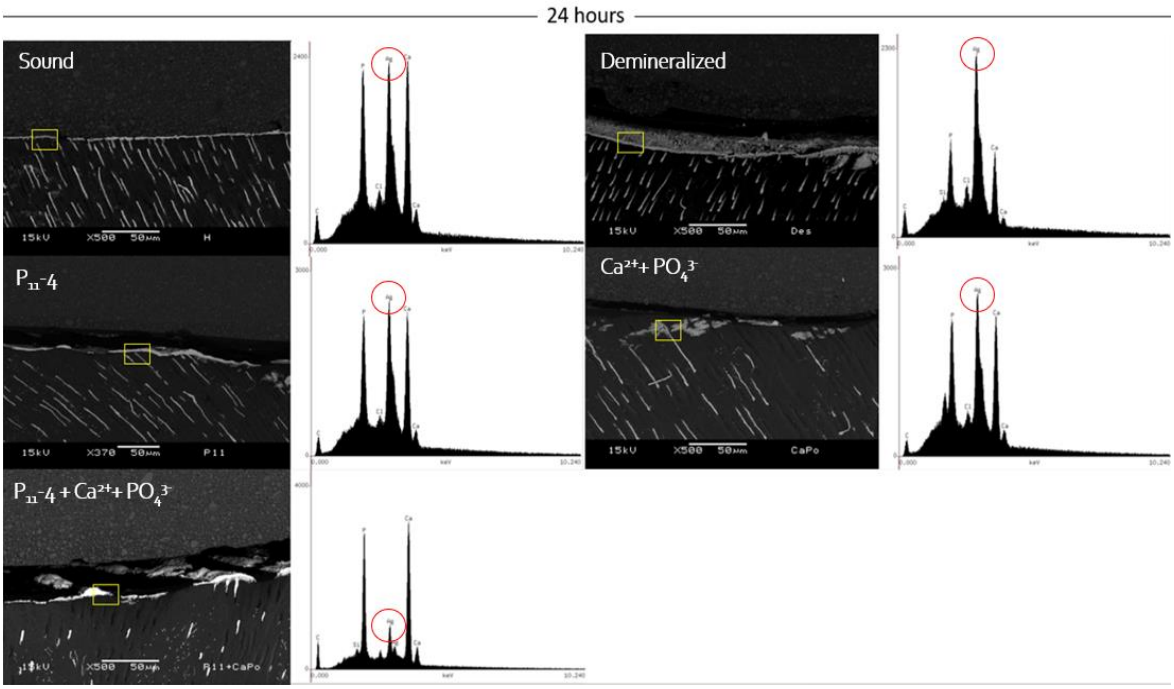


**Figure 6:** Scanning backscatter electron micrographs depicting the resin-dentin bond interface and energy dispersive X-ray spectroscopy (EDS) evaluating the Ag % in samples stored for 24 hours in deionized water. The yellow square shows the area analyzed by the EDS. The red circle shows the silver peak.

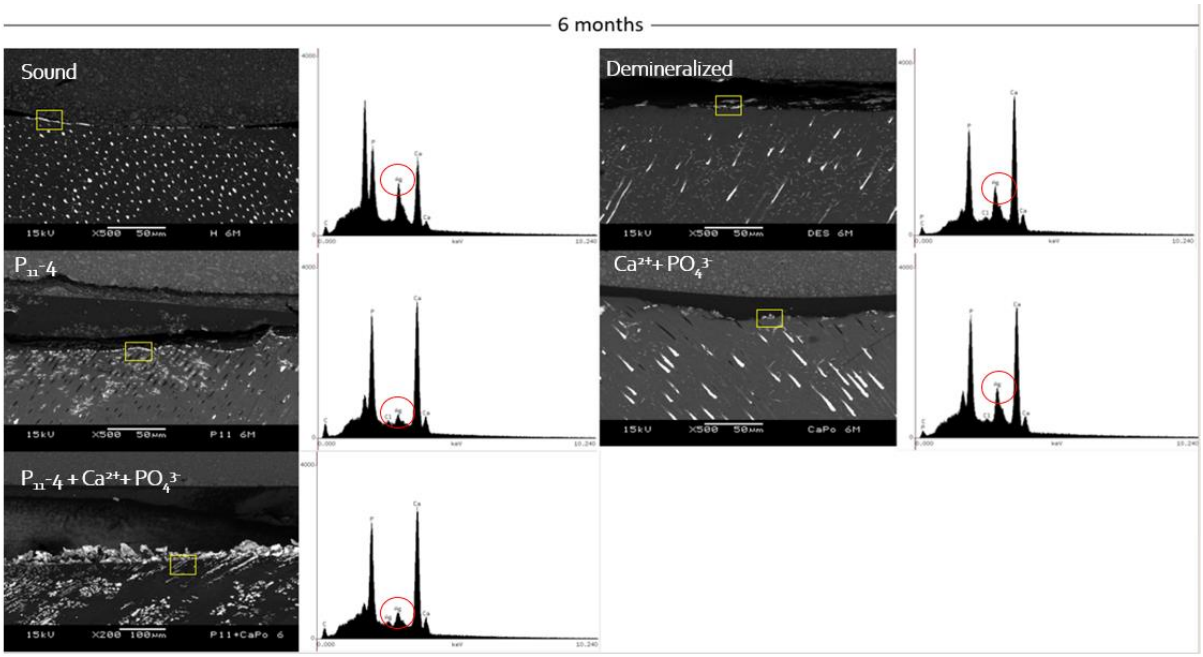


**Figure 7:** Scanning backscatter electron micrographs depicting the resin-dentin bond interface and energy dispersive X-ray spectroscopy (EDS) evaluating the Ag% in samples stored for 6 months in deionized water. The yellow square shows the area analyzed by the EDS. The red circle shows the silver peak.

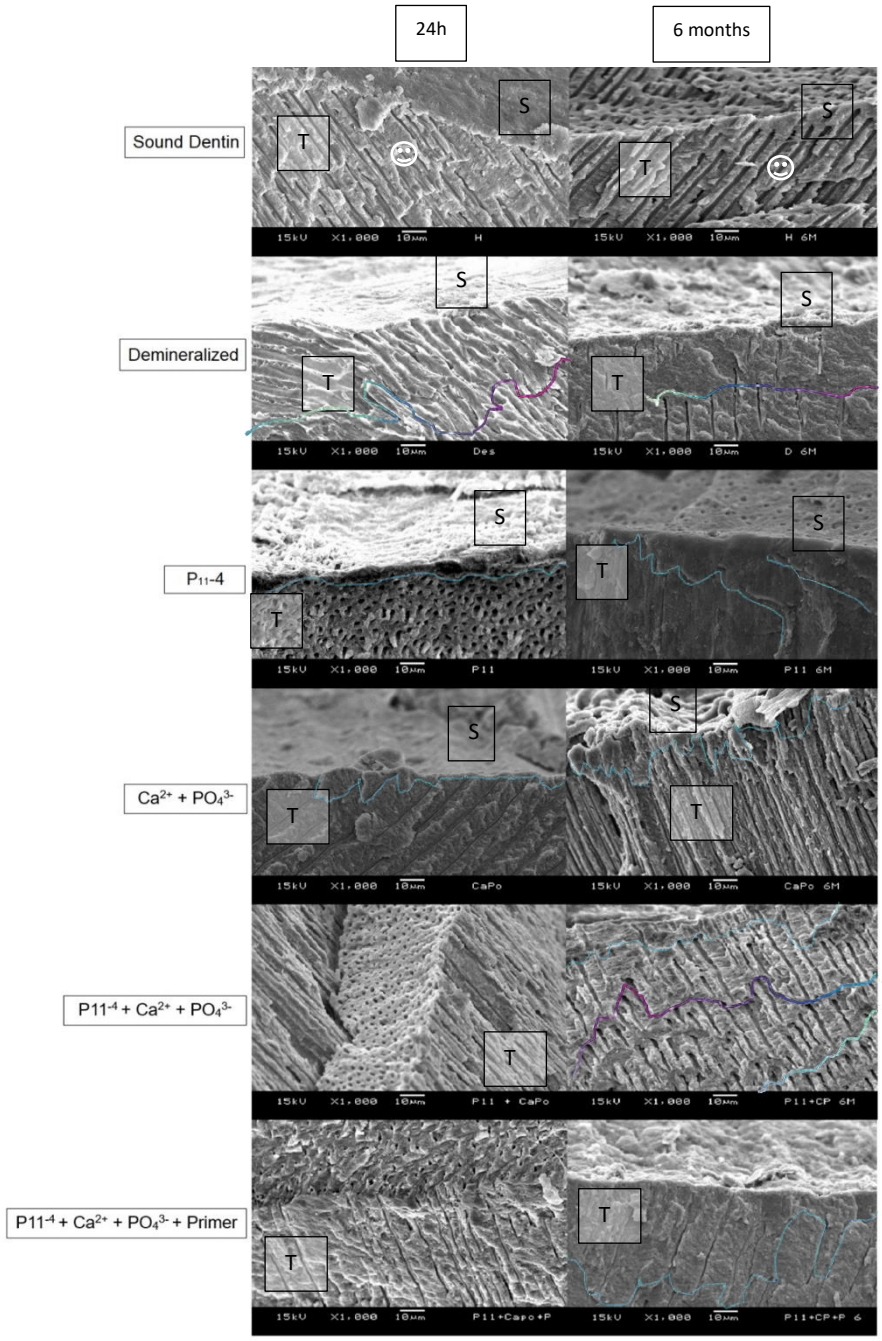


**Figure 8:** Representative SEM micrographs of specimens stored for 24 hours and 6 months in deionized water. Surface (S) and transverse (T) characteristics of the dentin created by cryo-fracturing - Magnification micrograph (×1000). The left-hand column represents micrographs from groups stored in deionized water for 24 hours, and the right-hand column represents micrographs from groups stored in deionized water for 6 months. Smile face represents sound dentin with dentin tubules going straight to a centripetal direction (24h and 6 months). Multicolor lines indicated the depth of demineralized dentin provided by *S mutans* biofilm. There is a degradation of the structure of dentin. Blue line evidenced the mineralization of the dentinal tubules their occlusion.

## DISCUSSION

This study sought to verify the efficacy of a remineralization approach to restore caries-affected dentin, with the goal of enhancing adhesive procedures and increasing the durability of the resin-dentin interface. The first hypotheses tested that pretreatment with P11-4 improves the wettability of dentin affected by caries was accepted. It was found that $P_{11}$-4 indeed improved the wettability of caries-affected dentin, making it more prone to bonding. These results corroborate those from Barbosa-Martins et al., 2018 which also demonstrate that a hydrophobic surface reduces the wettability of the adhesive and hinders its ability to spread across the entire dentin surface, consequently affecting adhesion. When the surface of demineralized dentin was treated with the self-assembling peptide $P_{11}$-4, increase in wettability was observed, indicating the potential of $P_{11}$-4 treatment to improve the surface characteristics of demineralized dentin.

The $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ + Primer group also exhibited improved wettability, which can be explained by the synergistic effect of $P_{11}$-4, with the $Ca^{2+}$ + $PO_4^{3-}$ solution and the primer of the Clearfil SE Bond adhesive system on demineralized dentin. When P11-4 was triggered by the acid present in the primer of the Clearfil adhesive system, it underwent hierarchical self-assembly in scaffolding, forming ribbons that will give rise to collagen fibrils (21). These scaffolds bind to calcium and phosphate ions available on the dentin surface, giving rise to mineralized tissue (10). The use of the $Ca^{2+}$ + $PO4^{3-}$ solution was intentional to reproduce the interference of the dentinal fluid in the degradation of the restoration, as well as the remineralization process (10), since the liquid selected for the storage of the samples was deionized water.

The second hypothesis that pretreatment with $P_{11}$-4 improves resin/dentin bond strength was not accepted. Despite the promising improvement in the wettability of demineralized dentin observed with $P_{11}$-4, the microtensile bond strength test results at both storage times in deionized water show no statistically significant difference between the dentin treated with $P_{11}$-4 and the demineralized dentin alone. While helpful to keep the peptide in the hydrogel form during application, the pH from Clearfill was likely not completely neutralized upon contact with the dentin substrate, which would then have led to self-assembly of the peptide in fibrils, as shown by others when using buffered solutions (10). In addition, even if the pH had been neutralized, the time

elapsed between acidic primer application and polymerization was likely not sufficient to allow for proper assembly. Indeed, in previous studies that demonstrated the mechanism of hierarchical organization of this peptide, the change in conformation upon pH triggering was greater than 5 min (12). It is also possible that some other component in the adhesive formulation destabilized the fibril formation, such as organic solvents like water, which potentially denatured the supramolecular interactions between peptide units (11). Both of those possibilities need to be further investigated in a systematic manner using protein morphology techniques and other methods.

The third hypothesis tested that pretreatment with $P_{11}$-4 improves the stability of the hybrid layer and consequently improves the longevity of the bond was also not accepted. After 24 h storage, the sound dentin group did not have appreciable infiltration of silver nitrate, while the demineralized group had a much more pronounced infiltration, with visibly thicker layers of silver nitrate forming. All the other groups showed intermediary results, with some areas of greater infiltration, and other areas of intact bonding. However, at 6 months of storage, all groups showed breakpoints in the hybrid layer, with silver nitrate deposition, indicating potential degradation over time. The $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ group were not able to prevent this from happening and showed similar behavior than the group with caries-affected dentin alone.

EDS quantification demonstrated a lower percentage of silver nitrate for the $P_{11}$-4 + $Ca^{2+}$ + $PO_4^{3-}$ group stored in deionized water for 24 hours. These results can be attributed to the deposition of Ca and PO4 on the demineralized dentin in an organized way, hinder the silver nitrate deposition. Some of the groups that were stored for 6 months showed a slightly decrease in the deposition of silver nitrate, showing a more homogeneous hybrid layer. Those results are contrary to that observed in the study by Sousa et al, 2019, which demonstrated that $P_{11}$-4 was able to form a homogeneous and less porous hybrid layer. The difference between both studies is on adhesive system used. Sousa et al, 2019 and Moreira et al 2022 showed a decrease on the silver nitrate deposited on hybrid layer using a etch & rinse adhesive system. When that adhesive is used, phosphoric acid is removed from the dentin surface by water rinsing, then, the low pH is increased and P11-4 can assembly in Beta-sheets, organizing the deposition of $Ca^{2+}$ and $PO4^{3-}$ and make easier the hidroxyapaptite formation. The opposite occurs when a self-etching system is used, pH remains acidic for long time, besides the dentin buffer ability (3). So, P11-4 can change its form from

a monomeric to polymeric phase, disturbing the mineral stability and opening porous on the hybrid layer. Then, instead of P11-4 provide a deep modification on the dentin, our study supports the hypothesis that P11-4 effectiveness is affected by the pH of the adhesive system selected for the development of the study.

Cryofracture was performed on the dentin disks in order not to damage them with a diamond cutting disk, and to evaluate the surface morphology without any interference after the applied treatments. No alteration or deposition of any material was observed on the surface and side of the dentin slices, and the decrease of smear layer was due to the cleaning in the ultrasonic bath for 30 minutes after polishing, in the interval of each grain of sandpaper used.

Studies have shown that the application of $P_{11}$-4 to caries-affected dentin in conjunction with a conventional adhesive system was able to improve wettability and bond strength (10,11,12), but when used with a self-etching adhesive system, its effectiveness was lost precisely by the presence of an acidic pH of 2.0, causing instability in self-assembly. These findings shed light on the importance of considering the chemical compatibility of materials when attempting to enhance adhesive procedures. Indeed, the study's results suggest that further exploration is necessary to identify more suitable combinations of materials or modifications to ensure optimal bond strength and longevity in restorative dentistry. Additionally, future studies could investigate alternative self-etching adhesive systems or adjust the pH of the primer to assess their compatibility with $P_{11}$-4 and their potential impact on bond strength outcomes.

## CONCLUSION

$P_{11}$-4 improved the wettability of demineralized dentin, making its surface hydrophilic, producing a more regular hybrid layer, with less silver infiltration, but it did not have a significant effect on improving bond strength in long time due to its instability in the presence of the acidic pH of the self-etching adhesive system. Therefore, P11-4 does not improve the longevity of the resin/dentin bond strength using self-etching adhesive system.

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## 3. CONCLUSÃO

O $P_{11}$-4 melhorou a molhabilidade da dentina desmineralizada, tornando sua superfície hidrofílica, produzindo uma camada híbrida mais regular, com menor infiltração de prata, mas não apresentou efeito significativo na melhora da resistência de união devido a sua instabilidade diante do pH ácido do sistema. adesivo autocondicionante, consequentemente não alcançou a longevidade da união resina/dentina.

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**ANEXO 1:** Comitê de ética

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Continuação do Parecer: 5.079.594

**Conclusões ou Pendências e Lista de Inadequações:**

Não há mais pendências por resolver (vide texto acima).

**Considerações Finais a critério do CEP:**

Parecer de aprovação de Protocolo emitido "ad referendum" conforme autorização do Colegiado na reunião de 03/02/2021. O parecer será submetido para homologação na reunião de 15/12/2021.

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

| Tipo Documento | Arquivo | Postagem | Autor | Situação |
|---|---|---|---|---|
| Informações Básicas do Projeto | PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1836709.pdf | 04/11/2021 01:10:35 | | Aceito |
| Projeto Detalhado / Brochura Investigador | Projeto_corrigido.pdf | 04/11/2021 01:10:15 | LARISSA MARCELINO | Aceito |
| Outros | Termo_doacao_corrigido.pdf | 26/10/2021 23:49:02 | LARISSA MARCELINO | Aceito |
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**UF:** SP **Município:** PIRACICABA
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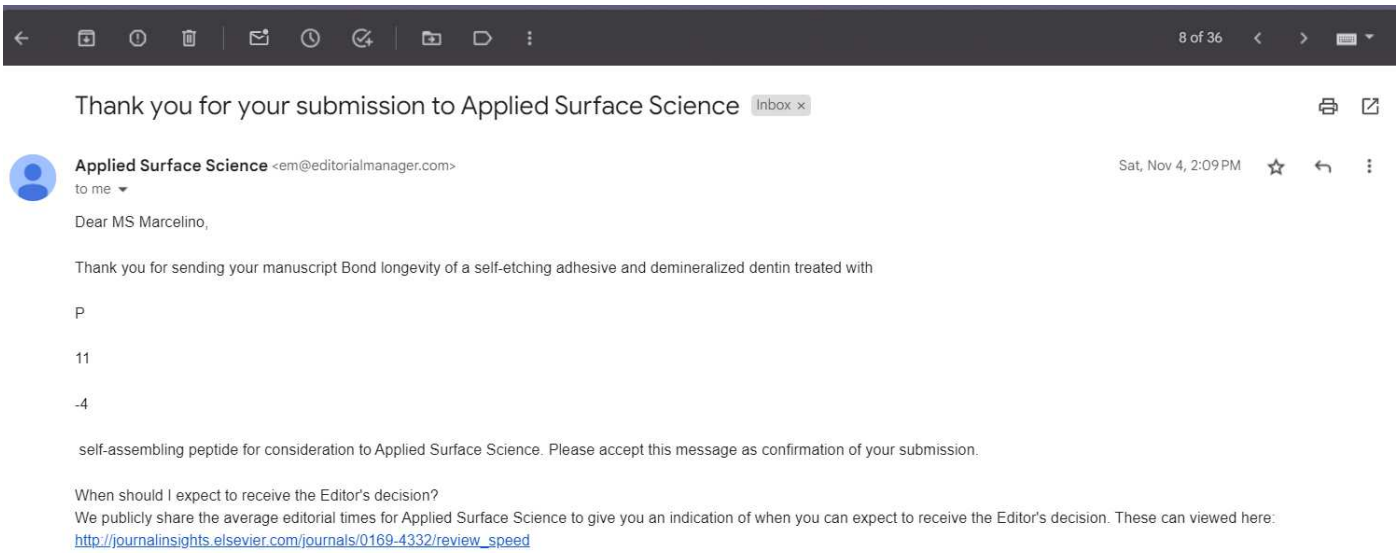
**ANEXO 2:** Comprovante de submissão do artigo



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