Brazilian Dental Science

UNESP UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Instituto de Ciência e Tecnologia Campus de São José dos Campos

Original Article DOI: https://doi.org/10.4322/bds.2026.e4948

Longitudinal dentin bond strength evaluation using different application modes of a metalloproteinase inhibitor

Avaliação longitudinal da resistência de união da dentina utilizando diferentes métodos de aplicação de um inibidor de metaloproteinases

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How to cite: Kury M, Totti M, Feldens TT, Goulart M, Cavalli V, Coelho-de-Souza FH, et al. Longitudinal dentin bond strength evaluation using different application modes of a metalloproteinase inhibitor. Braz. Dent. Sci. 2026;29:e4948. https://doi.org/10.4322/bds.2026.e4948

ABSTRACT

Objective: This study evaluated the influence of diluted chlorhexidine (CHX, wt %) in the dental unit waterline (DUWL) on dentin bond strength. **Material and Methods:** Eighty human molars were coronally sectioned and randomly assigned to five groups (n=10), as follows: (G1) 37% phosphoric acid etching (H_3PO_4) rinsed with distilled water (DW); (G2) H_3PO_4 rinsed with 2% CHX; (G3) H_3PO_4 rinsed with 5% CHX; (G4) H_3PO_4 rinsed with DW followed by 2% CHX application; and (G5) H_3PO_4 rinsed with DW followed by 5% CHX application. The adhesive system (Adper Scotchbond Multi-Purpose Plus) was applied, and composite resin blocks (Filtek Z350 XT) were incrementally built up. Teeth were sectioned to obtain microtensile specimens, which were tested for bond strength after 24 h or 12 months of storage. Fracture patterns were examined under a stereomicroscope (40×) and hybrid layer morphology was evaluated using scanning electron microscopy (SEM). Data were analyzed using two-way ANOVA and Tukey tests (α=0.05). **Results:** CHX in the DUWL did not reduce bond strength compared to the control group, regardless of concentration or storage time. Application of 5% CHX as a primer decreased immediate bond strength, although this effect was reversed after water-aging. No significant differences were observed among groups in the longitudinal evaluation. Failures were predominantly adhesive, and SEM analysis showed homogeneous hybrid layer formation without gaps or porosity. **Conclusion:** Diluted CHX in the DUWL does not adversely affect resin-dentin bond strength. Although a temporary reduction in bond strength was observed with 5% CHX primer application, this effect was mitigated over time.

KEYWORDS

Adhesive system; Bond strength; Chlorhexidine; Dentin; Metalloproteinase.

RESUMO

Objetivo: Avaliar a influência da clorexidina (CHX) diluída na linha de água da unidade odontológica (DUWL) na resistência de união à dentina. **Material e Métodos:** Oitenta molares humanos foram seccionados e divididos aleatoriamente em cinco grupos (n=10), conforme os tratamentos: (G1) condicionamento com ácido fosfórico a 37% (H_3PO_4) e enxágue com água destilada (DW); (G2) H_3PO_4 enxaguado com CHX a 2%; (G3) H_3PO_4 enxaguado com CHX a 5%; (G4) H_3PO_4 enxaguado com DW seguido de aplicação de CHX a 2%; (G5) H_3PO_4 enxaguado com DW seguido de CHX a 5%. Foi aplicado o sistema adesivo (Adper Scotchbond Multi-Purpose Plus) e realizada restauração em resina composta (Filtek Z350 XT). Após 24 horas ou 12 meses de armazenamento, espécimes foram testados em microtração. Fraturas foram analisadas em estereomicroscópio (40×) e a camada híbrida por microscopia eletrônica de varredura (MEV). Os dados foram analisados por ANOVA dois fatores e teste de Tukey (α=0,05). **Resultados:** O uso de CHX na DUWL não reduziu a resistência de união comparado ao controle, independentemente da concentração ou tempo. A aplicação de CHX a 5% como primer reduziu a resistência imediata, mas esse efeito foi revertido após envelhecimento em água. As falhas foram majoritariamente adesivas e a MEV revelou camada híbrida homogênea, sem porosidades. **Conclusão:** A CHX diluída na DUWL não afeta negativamente a união resina-dentina. A redução temporária com CHX a 5% como primer foi minimizada ao longo do tempo.

PALAVRAS-CHAVE

Sistema adesivo; Resistência de união; Clorexidina; Dentina; Metaloproteinase.

INTRODUCTION

The hybrid layer, formed by the infiltration of resin monomers into exposed collagen fibrils within demineralized dentin, plays a critical role in the long-term bond strength of adhesive restorations [1,2]. One of the key factors that assures the stability of this layer is the proper impregnation and polymerization of monomers within the dental substrate, which provides adequate dentin sealing and retention of restorative materials [3]. However, discrepancies between the depth of demineralization and the extent of resin infiltration may leave interfibrillar spaces containing exposed collagen. Such spaces often retain water, which favors the action of endogenous enzymes (particularly matrix metalloproteinases – MMP-2, MMP-8 and MMP-9 and cysteine cathepsins)—which degrade unprotected collagen over time [4,5].

Recent research in Adhesive Dentistry is focused on strategies that may efficiently inhibit the metaloproteinases and preserve the integrity of the hybrid layer. MMPs are zinc-dependent endopeptidases that function in aqueous environments and require zinc (Zn^{2+}) and calcium ions, as well as interactions with collagen-binding proteins, to remain active [6]. Therefore, their inhibition is achieved by agents capable to bind to their active sites [6,7]. Chlorhexidine (CHX) appears as a MMP inhibitor possibly due to its capability to chelate zinc and calcium and act as a cross-linking agent [8,9]. CHX has been demonstrated inhibitory effects on MMP's even at low concentrations, ranging between 0.5 and 5 wt%, while concentrations below 0.1% are usually ineffective [10,11].

The use of CHX in the dentin treatment after etching and prior to the bonding application allows the impregnation of exposed collagen fibrils after demineralization [9]. CHX demonstrated more affinity for demineralized dentin, and its binding is not disrupted by subsequent resin infiltration. Hence, this might be the reason why the chlorhexidine acts inhibiting MMPs over time [6]. Several studies reported that the use of CHX application as a primer does not significantly affect immediate bond strength or hybrid layer morphology [12-14]. However, a recent systematic review confirmed that, up until this moment, the evidence remains insufficient to neither recommend nor discourage the application of CHX as therapeutic primer in composite restorations [10], highlighting the importance of well-designed studies to answer if the application of CHX would slow down the degradation of hybrid layer.

Despite its potential benefits, the use of CHX introduces an additional clinical step. As multi-step bonding procedures are complex and technically sensitive, the addition of another clinical step may face resistance by clinicians [10]. Alternative approaches—such as incorporating CHX directly into etchants or adhesives—have shown mixed results regarding bond strength and hybrid layer formation [15-17]. Another possible strategy would be to dilute CHX into the dental unit waterline (DUWL) to rinse the etchant. However, the effects of this approach on resin–dentin bonding remain unknown.

In view of these facts, the aim of the present study was to evaluate whether rinsing phosphoric acid with diluted CHX via DUWL or applying CHX in different concentrations affects longitudinal dentin bond strength, failure mode and hybrid layer morphology. The null hypothesis tested was that the use of different concentrations of CHX would not reduce immediate or longitudinal bond strength.

MATERIAL AND METHODS

Specimen restoration

The experimental protocol was reviewed and approved by the Institutional Ethics Committee (Protocol No. 34145214.6.0000.5347), in accordance with the ethical standards of the institution and national research guidelines. Eighty freshly extracted human molars, extracted for clinical reasons unrelated to this research, were stored in a 0.1% thymol solution. The occlusal enamel of each tooth was sectioned perpendicularly to the dental axis using a double-faced diamond disc. The exposed occlusal dentin was sequentially polished with silicon carbide sandpapers of decreasing grit sizes (#150, #320, #400, and #600; Saint Gobain Adesivos Ltda., Jundiaí, SP, Brazil), mounted on a mechanical polisher (APL94, AROTEC S.A., Cotia, SP, Brazil) under constant water irrigation. Teeth were then stored in distilled water until use.

Samples were randomly distributed into five groups, according to dentin surface treatment protocols:

1) DW: Etching with 37% phosphoric acid (Scotchbond™ Universal Etching Gel, 3M Oral Care, St. Paul, Minessota, USA - H_3PO_4) for 15 s, followed by rinsing with distilled water for 30 s. Dentin surface was gently dried with an air blast for 5 s at a distance of 10 cm to remove excess surface water, maintaining the substrate visibly moist.

2) 2%CHX_rinse: Etching with H_3PO_4 for 15 s, followed by rinsing with 2% chlorhexidine from DUWL for 30 s (50 ml of the solution). Dentin surface was gently dried with an air blast, for 5 s at a distance of 10 cm to remove excess surface water, maintaining the substrate visibly moist.

3) 5%CHX_rinse: Etching with H_3PO_4 for 15 s, followed by rinsing with 5% chlorhexidine from DUWL for 30 s (50 ml of the solution). Dentin surface was gently dried with an air blast, for 5 s at a distance of 10 cm to remove excess surface water, maintaining the substrate visibly moist.

4) 2%CHX_primer: Etching with H_3PO_4 for 15 s and rinsing with distilled water for 30 s, followed by the application of 2% chlorhexidine for 120 s using a microbrush (20 μL volume). Dentin surface was gently dried with an air blast, for 5 s at a distance of 10 cm to remove excess surface water, maintaining the substrate visibly moist.

5) 5%CHX_primer: Etching with H_3PO_4 for 15 s and rinsing with distilled water for 30 s, followed by the application of 5% chlorhexidine for 120 s using a microbrush (20 μL volume). Dentin surface was gently dried with an air blast, for 5 s at a distance of 10 cm to remove excess surface water, maintaining the substrate visibly moist.

An etch-and-rinse adhesive system (Adper Scotchbond Multi-Purpose Plus, 3M Oral Care, St. Paul, Minessota, USA) was applied in two consecutive coats with microbrush agitation for 15 s, followed by gentle air-stream evaporation for 5 s and photoactivation for 10 s at 1200 mW/cm^2 using a LED curing unit (Radii Plus, SDI Ltd., Bayswater, Australia), with the light tip held approximately 1 mm from the surface. Nanofilled composite resin blocks (Filtek Z350 XT, 3M Oral Care, St. Paul, Minessota, USA) were incrementally built up in three 2mm-layers and individually light cured for 40 s. Immediately afterward, the roots were sectioned and the pulp chambers were restored with Filtek Z350XT composite resin and Adper Single Bond 2 adhesive resin (3M Oral Care, St. Paul, Minessota, USA).

Microtensile test

Seven restored teeth per group were sectioned into sticks with a cross-sectional area of 1 mm^2 using a low-speed diamond saw (EXTEC Labcut 1010, Enfield, CT, USA). Forty sticks were selected from the central portion of the restored teeth. Half of the sticks were stored in distilled water at 37°C (± 2°C) for 24 h while the remaining half were stored for 12 months, with weekly water changes [18]. After each storage period, the specimens were fixed to a microtensile testing device (DL-2000, EMIC, São José dos Pinhais, PR, Brazil) with cyanoacrylate-based adhesive and subjected to tensile loading until failure in a universal testing machine at a crosshead speed of 0.5 mm/min.

Scanning Electron Microscopy (SEM)

One additional tooth from each group was sectioned into 1mm-thick slices. The slices were manually polished with #1000 and #1500 silicon carbide sandpapers (Saint Gobain Adesivos Ltda., Jundiaí, SP, Brazil). After obtaining flat, polished surfaces, specimens were etched with 37% phosphoric acid for 15 s and then with 1% sodium hypochlorite for 120 s (FANEM 315 SE, FANEM, São Paulo, Brazil), rinsed with distilled water for 15 s and gently dried with absorbent paper. The specimens were dried overnight in an oven then sputter-coated with gold (MED 010, Baltec, Balzers, Liechtenstein). SEM micrographs were obtained using a scanning electron microscope (Jeol Inc., Peabody, MA, USA) and representative areas of the hybrid layer were photographed at 1500x and 3000x magnifications.

Statistical analysis

Data were first subjected to exploratory analysis to confirm assumptions of normality (Shapiro-Wilk test, $p > 0.05$) and homoscedasticity (Levene´s test, $p > 0.05$). Subsequently, a two-way ANOVA followed by Tukey's post-hoc test was performed, with the significance level set at $\alpha = 0.05$.

Failure modes were qualitatively assessed under a stereomicroscope (Olympus CX21, Olympus, Tokyo, Japan) at 40× magnification. Failures were classified as adhesive, cohesive in composite, cohesive in dentine or mixed (i.e., up to 50% of the dentin surface covered with adhesive or resin composite).

RESULTS

Mean μTBS values obtained according to the factors dentin treatment protocols and evaluation times are described in Table I. Pre-test failures were not found in any experimental group. Although no interaction was detected between the study factors, statistical differences were observed for evaluation time (p=0.043) and dentin treatment (p=0.002). At the immediate evaluation, the control group (DW) exhibited the highest mean bond strength (38.0 ± 16.4 MPa), which was statistically similar to 2%CHX_primer (37.0 ± 5.5 MPa) but significantly higher than 5%CHX_primer (26.4 ± 7.1 MPa). Both CHX rinse groups (2%CHX_rinse and 5%CHX_rinse) presented intermediate values (30.2 ± 5.8 MPa and 29.6 ± 7.6 MPa, respectively), which did not differ significantly from either the DW or primer groups. Notably, 5%CHX_primer showed the lowest immediate bond strength, differing statistically from DW and showing a trend toward lower values than the other groups. After 12 months of water storage, no statistically significant differences were detected either within or between groups. 5%CHX_primer remained the group with the lowest average value (25.0 ± 6.3 MPa), although without significant degradation compared to its baseline. No significant differences were detected among groups at the 12-month evaluation.

Table I - Bond strength values at immediate and 12-month evaluation*

Surface Treatment	Bond Strength (μTBS)	
	Immediate	12 months
DW	38.0 (16.4) Aa	33.7 (4.9) Aa
2%CHX_rinse	30.2 (5.8) Aab	27.3 (7.1) Aa
5%CHX_rinse	29.6 (7.6) Aab	27.8 (7.4) Aa
2%CHX_primer	37.0 (5.5) Aab	30.6 (6.3) Aa
5%CHX_primer	26.4 (7.1) Ab	25.0 (6.3) Aa

*Means followed by different letters differ statistically at 5% (uppercase letters compare evaluation times in lines and lowercase letters compare treatment protocols in columns).

The examination of failure mode is shown in Figure 1. Immediately after bonding, all experimental groups exhibited a predominance of adhesive failures in dentin (ADH). This mode was particularly frequent in the 5%CHX_primer and 2%CHX_primer groups, where ADH accounted for more than 50% of the failure types. The 2%CHX_rinse group showed a more balanced distribution, with ADH being slightly predominant but accompanied by a higher incidence of cohesive failures in resin (COR) and mixed failures (MIX) compared to the other CHX-treated groups. Notably, cohesive in dentin failures (COD) were consistently the least frequent in all groups at the immediate time point.

After 12 months of water storage, the distribution of failure modes remained generally similar across groups, with ADH continuing to be the dominant failure type. A slight increase in the proportion of ADH was observed in nearly all groups, particularly in 5%CHX_rinse and 2%CHX_primer. Consequently, MIX failure rates decreased in these same groups. Conversely, the incidence of COR and COD either remained stable or slightly decreased. The DW group maintained the failure mode distribution over time, which was not observed in the CHX-based groups.

SEM micrographs (Figure 2) revealed slight differences in hybrid layer morphology among the experimental groups and over time. At the immediate evaluation, the DW group exhibited

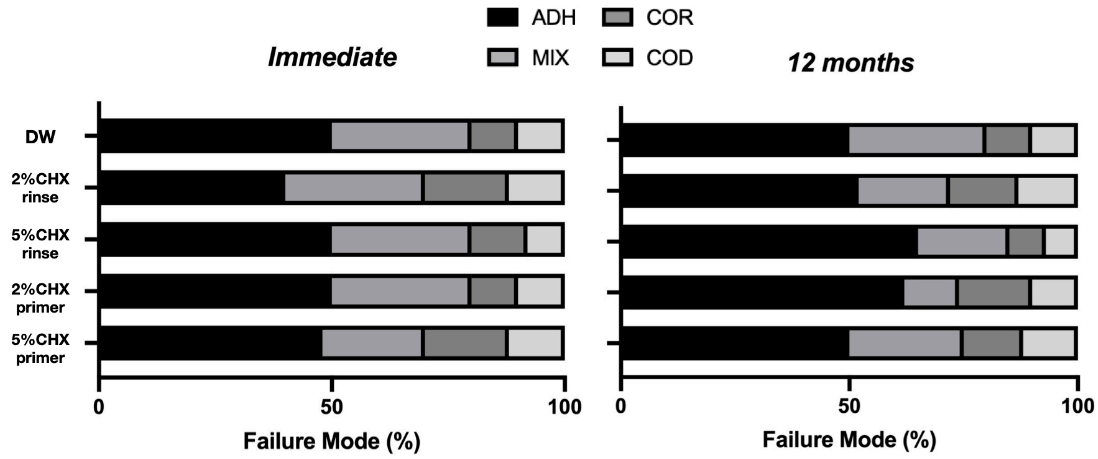

Figure 1 - Graph with the failure mode pattern of the tested specimens immediately and 12 months after the restorative procedures.

a moderately thick hybrid layer with visible resin tags, indicating adequate resin infiltration. The CHX rinse groups (2% and 5%) showed defined hybrid layers, with reduced resin tag formation observed in the 5% CHX rinse group. In contrast, both CHX primer groups presented more homogeneous and thicker hybrid layers, with better resin tag penetration, especially in the 2%CHX_primer group. After 12 months of storage, all groups exhibited some degree of degradation. The DW and CHX rinse groups showed reduced hybrid layer definition and fewer resin tags.

DISCUSSION

Mean μTBS values demonstrated that the use of CHX diluted in DUWL did not compromise bond strength compared to the control group (DW). However, the null hypothesis was partially accepted, since the 5% CHX primer group showed lower immediate bond strength (24 h) compared with the control group. Although applying 5% CHX as a primer resulted in lower bond strength at 24 hours, this decrease was no longer observed after 12 months. Moreover, CHX-treated groups showed no statistical differences from DW at longitudinal evaluation. These results suggest that CHX, regardless of concentration or application method, does not undermine resin–dentin bond strength in a 12-month period, corroborating previous findings [11,19] of MMP-inhibitory effects.

Notably, the 2% CHX primer group yielded immediate bond strength comparable to the control and maintained its values after aging, which is consistent with studies identifying 2% CHX as the most clinically effective concentration for dentin pretreatment [8]. In contrast, 5% CHX primer produced the lowest mean values initially ($p<0.05$) with a trend toward interference in the adhesive interface, although this deviation leveled off over time. Collares et al. [20] also reported no linear dose–response relationship between CHX concentration and bond strength, highlighting the importance of application mode, vehicle, and contact time.

The application of 5% CHX as a surface treatment has been poorly investigated [8]. In our study, while this protocol reduced immediate bond strength, it also exhibited minimal μTBS reduction over time, as confirmed by microscopies. SEM images showed that CHX application—including the novel DUWL method—did not alter hybrid layer morphology over time. Mobarack [21] reported that 5% CHX effectively slowed bond strength loss in caries-affected dentin over two years, whereas the effect of 2% CHX diminished, possibly due to better substantivity in porous substrates [22]. However, because CHX is water-soluble, it may leach from the hybrid layer over time [23]. Importantly, the present study was focused in sound dentin, which is less porous than caries-affected tissue [22].

SEM analysis corroborated quantitative data by revealing well-preserved hybrid layers in CHX-treated specimens, especially those with 2% CHX (both rinse and primer). These groups maintained a continuous hybrid layer with visible resin tags after 12 months, indicating stable resin infiltration and minimal collagen degradation —effects aligned with prior work showing CHX's inhibition of collagenolytic activity and hybrid

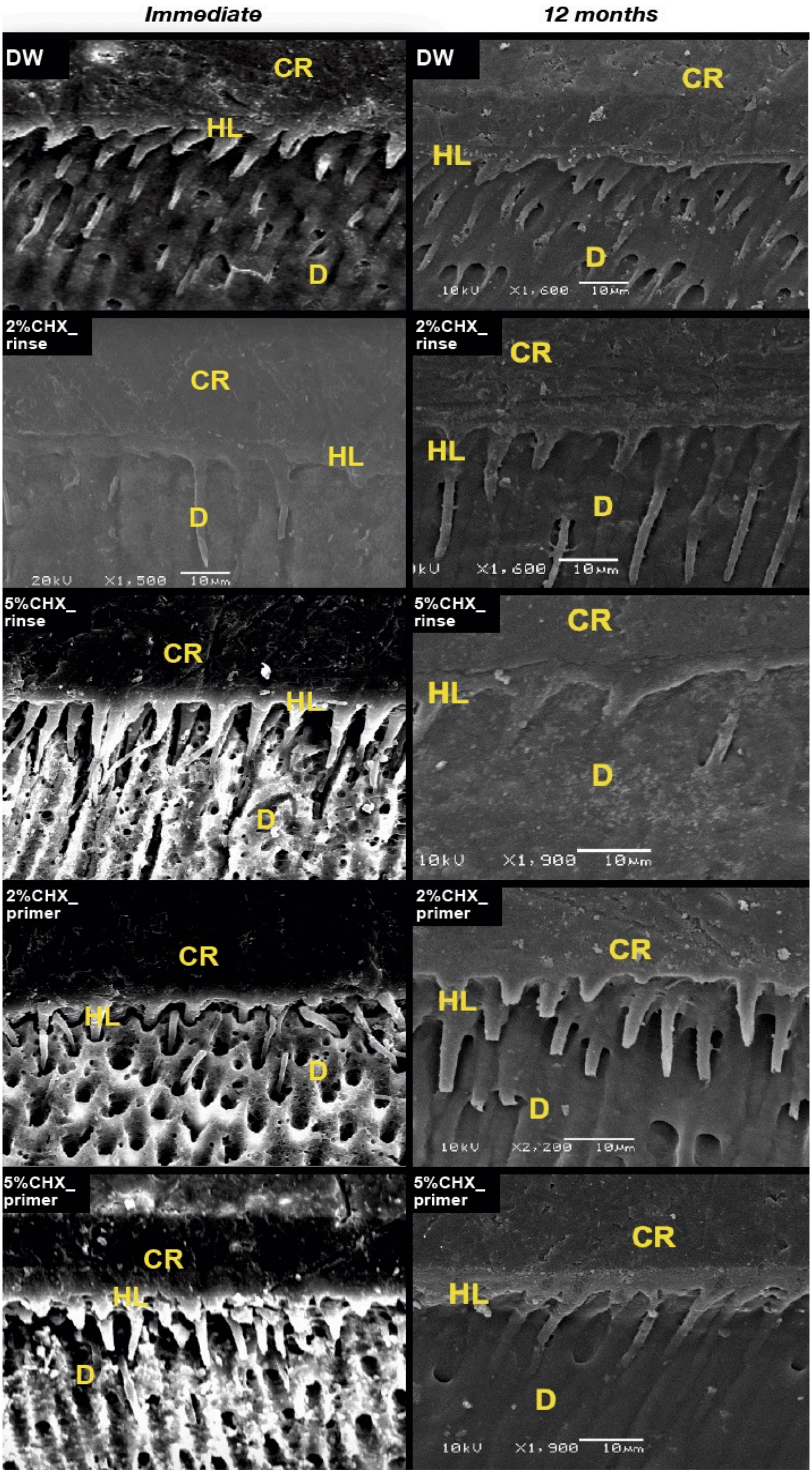

Figure 2 - Representative scanning electron microscopy (SEM) images of the adhesive interface in different experimental groups, evaluated immediately (left column) and after 12 months of water storage (right column). In the DW and CHX-treated groups, variations in hybrid layer thickness, continuity, and resin tag penetration can be observed. CHX used as a primer (2% and 5%) resulted in more uniform and well-defined hybrid layers, particularly evident in the 2%CHX_primer group, which maintained its interfacial structure even after aging. HL = hybrid layer, D = dentin, CR = composite resin, CR = composite resin.

layer breakdown over time [24,25]. In contrast, control and 5% CHX groups exhibited more notable hybrid layer degradation, with fewer resin tags and disrupted interfaces. While these are qualitative observations, they underscore the potential of 2% CHX protocols, though further validation is needed for higher concentrations. Morphological degradation observed under SEM may not necessarily translate to significant loss of mechanical retention, possibly due to adhesive infiltration depth and polymerization quality.

Other MMP inhibitors have been explored in Adhesive Dentistry, such as metallic ions, polyphenols, flavonoids, and antibiotics as alternative protease inhibitors and collagen cross-linkers [8,9]. Cross-linkers, such as EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) and proanthocyanidins, have shown similar MMP-inhibitory effects than 2% CHX in confocal studies [26-29]. However, proanthocyanidins' dark coloration and the unknown impact of EDC on adhesive polymerization raises a drawback for their clinical applications [30]. We acknowledge that the aforementioned studies performed additional analyses, such as in situ zymography, which are essential to confirm the MMP inhibition potential, and this represents a limitation in our research.

Although a randomized clinical trial comparing bonding protocols with and without CHX application showed no differences [31], the current evidence is insufficient to firmly recommend or refute prior CHX surface treatment in adhesive restorations [32]. Meta-analyses underline limitations across clinical studies - lack of blinding, inadequate sample size and follow-up shorter than six months that must be addressed in future RCTs [32]. Further clinical investigation of CHX in DUWL is warranted to collaborate with this debate.

Even if clinical trials validate CHX incorporation into bonding protocols, MMP activity is not the sole determinant to restoration failure [33]. Factors like cavity geometry, dentin condition, [33] and patient's caries risk also influence outcomes. The ultimate goal remains to sustain oral health and monitor restorations over time [34].

Simplified protocols that maintain bonding effectiveness over time are crucial in Operative Dentistry. From a clinical standpoint, incorporating CHX via DUWL may reduce technique sensitivity and simplify protocols compared to conventional CHX application, offering practical advantages. However, the operator should be careful because using CHX via DUWL could potentially interrupt the chairside workflow and cause residual CHX accumulation, whereas a conventional triple-syringe rinse provides easier control and immediate evacuation.

The present results support that 2% CHX in DUWL is a promising strategy to enhance resin–dentin bond longevity without adding extra procedural steps. One might say that the absence of higher bond strength values counter indicates this application, but longitudinal *in vitro* models [35,36], including up to a decade of aging, have demonstrated CHX´s ability to preserve hybrid layer morphology and function [37]. Nonetheless, longer-term follow-up and evaluation of enzymatic activity remains necessary to detect potential future distinctions.

CONCLUSION

Within the limitations of the present study, the following conclusions can be drawn:

- Rinsing phosphoric acid with CHX diluted in the dental unit waterline does not adversely affect dentin bond strength to resin composite, irrespective of the evaluation period.
- Among the protocols evaluated, only 5% CHX applied as a primer after etching led to a significant reduction in bond strength; however, this difference was not sustained over time.
- After one year storage, all CHX-containing groups preserved dentin bond strength, showing no differences compared to the control.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author's Contributions

MCGE, TTF, FHCS: Conceptualization. MT, MG Methodology. MK: Software. MCGE: Validation. FHCS, MK: Formal Analysis. MT, MG: Investigation. MCGE: Resources. MK: Data Curation. MK: Writing – Original Draft Preparation.

MCGE, VC: Writing – Review & Editing. MCGE, TTF, FHCS, VC: Visualization. MCGE: Supervision. MCGE: Project Administration. MCGE: Funding Acquisition.

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

Funding

This study was partially supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Finance Code 001).

Regulatory Statement

This study was conducted in accordance with all the provisions of the local human subjects oversight committee guidelines and policies of the University where it was conducted. The approval code for this study is: 34145214.6.0000.5347.

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Editor-in-chief and Section Editor:
Sergio Eduardo de Paiva Gonçalves

Date submitted: 2025 Sep 01
Accept submission: 2025 Nov 18