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Effect of Collagen Cross-Linkers Applied at Different Adhesive Steps on Dentin Microtensile Bond Strength

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Abstract

Aim

The stability of the resin–dentin interface is compromised by collagen exposure and enzymatic degradation, making dentin biomodification with collagen cross-linkers a promising strategy. This study evaluated the effects of proanthocyanidin and theaflavin applied at different adhesive stages on immediate and six-month dentin microtensile bond strength.

Methods

Extracted impacted human third molars were used in this in vitro study(8 experimental groups and 2 control groups, n = 6 teeth per group). Teeth were allocated into groups according to the type of collagen cross-linker and the application step.Proanthocyanidin(PA) and theaflavin(TF)-based cross-linkers were applied at a concentration of 2% for 15 s, either as a dentin pretreatment or incorporated into subsequent adhesive steps.All groups were defined as follows: U, OptiBond Universal;E + U, etching followed by OptiBond Universal;E + PA+U, etching followed by proanthocyanidin pretreatment and OptiBond Universal;E + PAU, etching followed by proanthocyanidin-incorporated OptiBond Universal;PAFL, proanthocyanidin-incorporated OptiBond FL;PA + U, proanthocyanidin pretreatment followed by OptiBond Universal;PAU, proanthocyanidin-incorporated OptiBond Universal;E + TF+U, etching followed by theaflavin pretreatment and OptiBond Universal;E + TFU, etching followed by theaflavin-incorporated OptiBond Universal;TF + U, theaflavin pretreatment followed by OptiBond Universal; and TFU, theaflavin-incorporated OptiBond Universal. Fourier transform infrared spectroscopy(FTIR) was used to evaluate potential chemical interactions following cross-linker application, while scanning electron microscopy(SEM) was performed to assess the resin–dentin interface morphology. Microtensile bond strength(μTBS) was measured immediately and after 6 months of water storage.The significance level set at $p < 0.05$.

Results

Microtensile bond strength significantly decreased after 6 months in the U,E + U, E + PA+U,E + PAU,PAFL,PA + U, and E + TFU groups($p < 0.05$).No significant aging-related reduction was observed in the PAU,E + TF+U,TF + U, and TFU groups($p > 0.05$).Application of proanthocyanidin and theaflavin at specific application steps contributed to better preservation of bond strength after aging.FTIR analysis showed spectral changes in the experimental adhesive liquids after incorporation of proanthocyanidin and theaflavin.SEM revealed protocol-dependent differences in resin–dentin interface morphology.

Conclusions

Early-stage application of proanthocyanidin and theaflavin significantly influenced immediate and 6-month dentin bond strength and durability.

Introduction

Dentin is a heterogeneous and hydrated biological substrate with a high organic content, which inherently complicates adhesive bonding procedures.[1] Approximately 30% of the dentin matrix consists of type I collagen fibrils, which play a critical role in the formation and long-term stability of the hybrid layer during adhesive bonding.[2] Following acid etching, exposure of the collagen network without adequate stabilization may result in progressive degradation of the resin–dentin interface, ultimately leading to a reduction in bond strength over time.[3] Adhesive systems are designed to establish micromechanical and chemical bonding to dentin in order to enhance the retention and longevity of restorative materials.[4] Contemporary adhesive strategies primarily include etch-and-rinse and self-etch systems, both of which depend on sufficient resin monomer infiltration into the demineralized dentin substrate.[5] However, incomplete resin infiltration may leave collagen fibrils partially exposed within the hybrid layer, creating structurally weak regions that are highly susceptible to hydrolytic and enzymatic degradation.[6] The hybrid layer represents a resin–collagen interdiffusion zone and is considered the most vulnerable component of bonded restorations.[7] Its quality is influenced by multiple factors, including dentin moisture conditions, adhesive composition, application technique, and long-term aging processes.[8,9] Moreover, endogenous proteolytic enzymes, particularly matrix metalloproteinases (MMPs) and cysteine cathepsins, become activated following dentin demineralization and contribute to the degradation of exposed collagen fibrils. This enzymatic activity has been identified as a major factor compromising the durability of resin–dentin bonds.[10] To counteract collagen degradation, dentin biomodification strategies involving collagen cross-linking agents have been proposed.[11] These agents induce additional inter- and intrafibrillar cross-links within the collagen matrix, thereby enhancing its mechanical properties and resistance to enzymatic breakdown. Several studies have reported improved immediate and aged microtensile bond strength when collagen cross-linkers are applied as a dentin pretreatment; however, their effectiveness appears to be influenced by both the chemical nature of the agent and the application protocol used.[12,13]

Proanthocyanidin(PA) is a naturally occurring polyphenolic compound commonly derived from grape seed extract and other plant sources. Due to its strong affinity for collagen and its ability to form hydrogen bonds with collagen fibrils, proanthocyanidin has been extensively investigated as a dentin collagen cross-linker.[14] Previous studies have demonstrated that proanthocyanidin can increase dentin stiffness, enhance resistance to enzymatic degradation, and improve microtensile bond strength, particularly when applied prior to adhesive procedures.[15,16] Nevertheless, the influence of applying proanthocyanidin at different stages of the adhesive protocol on bond durability remains unclear. Theaflavin, a polyphenolic compound derived from black tea, has recently attracted interest as a potential collagen cross-linking agent. Its antioxidant properties and reported ability to stabilize collagen

and inhibit collagen-degrading enzymes suggest a possible role in improving dentin bond durability.[17] Although preliminary findings indicate that theaflavin may enhance resistance of dentin collagen to degradation and influence bonding performance, evidence regarding its application at different adhesive steps and its long-term effects on dentin bond strength is still limited.[18] Despite growing interest in natural collagen cross-linkers, most existing studies have focused primarily on their use as dentin pretreatment agents. Comparative data evaluating the impact of applying these agents at different stages of the adhesive protocol, particularly under both self-etch and etch-and-rinse strategies, remain scarce. This represents a significant gap in the literature, as the timing of cross-linker application may play a crucial role in determining the integrity and durability of the resin–dentin interface. Therefore, the aim of this study was to evaluate the effect of collagen cross-linkers, specifically proanthocyanidin and theaflavin, applied at different stages of the adhesive protocol on immediate and 6-month dentin microtensile bond strength. Both self-etch and etch-and-rinse adhesive modes were included as control conditions to allow comprehensive assessment of application timing. The findings of this study aim to clarify the influence of cross-linker application stage on bond durability and to contribute to the development of optimized adhesive strategies. Additionally null hypothesis of this study was that the application of proanthocyanidin (PA) and theaflavin (TF), regardless of the mode of application (as a separate primer or incorporated into the adhesive system), would have no significant effect on immediate or six-month dentin microtensile bond strength, nor on the interfacial morphology of the resin–dentin interface.

Methods

Sample preparation

This study was approved by the Ethics Committee of *** University, Faculty of Dentistry (Approval No:12.06.2023–1775084). All procedures were conducted in accordance with the Declaration of Helsinki. The wisdom teeth extracted from patients after obtaining informed consent forms were collected. Extracted human third molars stored in 0.1% thymol solution at 4°C until use were included in this in vitro study. The teeth were embedded in chemically cured acrylic resin and sectioned perpendicular to the long axis using a low-speed precision cutting device (Isomet 1000 Precision Saw, Buehler, Düsseldorf, Germany) under continuous water cooling. Occlusal enamel was removed approximately 3 mm below the cusp tips using a diamond cutting disc to expose mid-coronal dentin surfaces. The exposed dentin surfaces were polished with 600-grit silicon carbide abrasive paper (Buehler, Düsseldorf, Germany) under water cooling for 60 s to obtain a standardized smear layer. A total of ten groups were established, including eight experimental groups and two control groups (n = 6 teeth per group). Specimens were randomly allocated to the groups according to the type of collagen cross-linker and the application stage within the adhesive protocol.

Treatment

Proanthocyanidin (PA) and theaflavin (TF) were dissolved in ethanol to obtain 2% (w/v) solutions and used either as dentin pretreatment primers or incorporated into the adhesive system. All solutions were freshly prepared and used at room temperature. A total of ten groups were established, including two control groups and eight experimental groups (n = 6 teeth per group). In the control groups, OptiBond Universal adhesive (Kerr Corporation, Orange, CA, USA) was applied either in self-etch mode on sound dentin or following phosphoric acid etching (37.5%, 15 s) in etch-and-rinse mode. In the experimental groups, PA or TF was applied for 30 s as a dentin pretreatment or delivered via PA- or TF-incorporated OptiBond Universal formulations, depending on group allocation. All groups were defined as follows:

U, OptiBond Universal,

E + U, etching followed by OptiBond Universal,

E + PA+U, etching followed by proanthocyanidin pretreatment and OptiBond Universal,

E + PAU, etching followed by proanthocyanidin-incorporated OptiBond Universal,

PAFL, proanthocyanidin-incorporated OptiBond FL,

PA + U, proanthocyanidin pretreatment followed by OptiBond Universal,

PAU, proanthocyanidin-incorporated OptiBond Universal,

E + TF+U, etching followed by theaflavin pretreatment;OptiBond Universal,

E + TFU, etching followed by theaflavin-incorporated OptiBond Universal,

TF + U, theaflavin pretreatment followed by OptiBond Universal,

TFU, theaflavin-incorporated OptiBond Universal.

After application, excess solvent was gently air-dried, and the adhesive was light-cured for 15 s using an LED curing unit (Elipar™ DeepCure-S, 3M ESPE, St Paul, MN, USA). Following adhesive procedures, a microhybrid resin composite (Herculite Classic, Kerr, Orange, CA, USA) was incrementally built up on the dentin surfaces and light-cured according to the manufacturer's instructions.

Microtensile Bond Strength Testing

After completion of the composite build-ups, the specimens were stored in distilled water at 37°C for 24 h. For aging evaluation, half of the specimens were further stored in distilled water at 37°C for 6 months, with the storage medium renewed weekly. Following the storage periods, the specimens were sectioned longitudinally in both mesiodistal and buccolingual directions using a low-speed precision cutting device (Isomet 1000 Precision Saw, Buehler, Düsseldorf, Germany) under water cooling to obtain resin–dentin beams with a cross-sectional area of approximately 1.0 mm^2. Each beam was examined under a

stereomicroscope to exclude specimens with interfacial defects or irregular geometry. The cross-sectional area of each beam was measured prior to testing. The beams were attached to a microtensile testing jig using cyanoacrylate adhesive and subjected to tensile loading using a microtensile testing device (Microtensile Tester, Bisco Inc., Schaumburg, IL, USA) at a crosshead speed of 1 mm/min until failure. Microtensile bond strength values were calculated in megapascals (MPa) by dividing the maximum load at failure (N) by the bonded cross-sectional area (mm^2). The mean μTBS value for each tooth was calculated.

Scanning Electron Microscope (SEM) Analysis

SEM analysis was performed at the Istanbul University Faculty of Science, Nano and Optoelectronics Research Laboratory, using a Versa 3D FIB-SEM (Dual-Beam™) system (FEI Company, Hillsboro, OR, USA). The fracture surfaces of resin–dentin beams obtained after microtensile bond strength testing were prepared for scanning electron microscopy (SEM) evaluation. Representative dentin beams from each group were fixed in 2.5% glutaraldehyde solution for 24 h at room temperature, followed by rinsing with distilled water. The specimens were subsequently dehydrated through an ascending ethanol series (50%, 70%, 80%, 90%, and 100%), with each step performed for 10 min. After dehydration, the specimens were embedded in epoxy resin to stabilize the resin–dentin interface and to allow precise sectioning. Once polymerization was completed, each dentin beam was individually sectioned from the epoxy block to expose the fracture surface. The specimens were then mounted on aluminum SEM stubs using conductive carbon adhesive tabs, ensuring that the fracture surface was oriented upward for imaging. Prior to SEM observation, all specimens were sputter-coated with a thin layer of gold to provide electrical conductivity. Gold coating was performed under vacuum conditions for approximately 60 s using a sputter coater. SEM examination of the fracture surfaces was carried out at different magnifications (×1,000, ×5,000, and ×10,000) to evaluate interfacial morphology and failure characteristics.

Fourier Transform Infrared spectroscopy (FTIR) analysis

Four experimental solutions were analyzed: PA, Bond + PA, TF, and Bond + TF. Fourier Transform Infrared (FTIR) spectroscopy was performed to evaluate the chemical composition of each solution. Spectra were recorded within the range of 4000–400 cm^{-1} using an FTIR spectrometer under standardized conditions. For all groups, the cross-linking agents (PA and TF) were first dissolved in ethanol to obtain homogeneous solutions. Subsequently, for the Bond + PA and Bond + TF groups, the ethanol-based cross-linker solutions were mixed with the bonding agent immediately prior to FTIR analysis to ensure uniform distribution within the adhesive matrix. All samples were measured at room temperature, and background spectra were collected before each measurement. The obtained spectra were plotted as absorbance versus wavenumber (cm^{-1}), and the characteristic functional group regions were recorded for comparative analysis among the groups.

Statistical Analysis

Paired samples t-test or Wilcoxon signed-rank test was used for intra-group comparisons, and failure mode distributions were analyzed using the Monte Carlo–corrected Fisher's exact test ($p < 0.05$).

Results

Within-group analysis showed that aging significantly influenced microtensile bond strength in a protocol-dependent manner (Table 1). Conventional adhesive protocols and several proanthocyanidin-containing groups exhibited a significant reduction in bond strength after 6 months ($p < 0.05$). In contrast, the proanthocyanidin pre-treatment followed by universal adhesive showed a significant increase in bond strength after aging, while most theaflavin-containing groups maintained stable bond strength values over time.

Microtensile Bond Strength

Intra-group comparisons of immediate and 6-month microtensile bond strength (μTBS) values are presented in Table 2. Among all groups, the highest immediate μTBS value was recorded in the E + U group (41.32 ± 15.67 MPa), followed by the E + PA+U group (34.84 ± 13.45 MPa). The lowest immediate μTBS values were observed in the PAU (11.30 ± 4.68 MPa) and PA + U (11.85 ± 4.46 MPa) groups. After 6 months, the highest μTBS values were detected in the E + U group (28.94 ± 9.35 MPa) and the U group (27.61 ± 9.65 MPa). The lowest 6-month μTBS values were observed in the TFU (11.63 ± 4.50 MPa) and PAU (12.22 ± 3.03 MPa) groups. Statistically significant differences between immediate and 6-month μTBS values were observed in the U ($p = 0.048$), E + U ($p < 0.001$), E + PA+U ($p < 0.001$), E + PAU ($p = 0.025$), PAFL ($p = 0.004$), PA + U ($p = 0.001$), and E + TFU ($p = 0.003$) groups. No statistically significant differences were found between immediate and 6-month μTBS values in the PAU ($p = 0.253$), E + TF+U ($p = 0.597$), TF + U ($p = 0.961$), and TFU ($p = 0.561$) groups.

SEM

SEM evaluation revealed distinct morphological differences among the experimental groups (Table 3). The U group exhibited a non-etched dentin surface characterized by the presence of a smear layer partially occluding dentinal tubules. Tubule orifices were not fully open, and the surface displayed a relatively smooth and compact morphology without signs of demineralization. In contrast, the E + U group demonstrated a demineralized dentin surface with clearly exposed and widened dentinal tubules. The smear layer was removed, and tubule orifices were patent and well defined. The intertubular dentin exhibited a rough and porous morphology. The E + PA+U group showed reduced visibility of open dentinal tubules compared to E + U. Tubule orifices appeared partially covered, and the surface presented a heterogeneous morphology with localized deposits and a thin surface layer. The E + PAU group exhibited identifiable dentinal tubules with partial narrowing or coverage of some tubule orifices. The intertubular dentin appeared relatively more homogeneous and less porous compared to E + U, and no extensive surface precipitate accumulation was observed.The E + TF+U group demonstrated clearly

exposed dentinal tubules with distinct resin tag formation extending into multiple tubule lumens. In the E + TFU group, dentinal tubules were widely exposed following acid etching; however, well-defined and elongated resin tags were not distinctly evident. Limited intratubular material was observed in some tubules. Non-etched groups (TF + U, PA + U, and TFU) exhibited similar SEM characteristics, with dentin surfaces covered by a smear layer. Dentinal tubules were largely obscured, and tubule orifices were not clearly patent. No pronounced resin tag formation was observed in these groups.

FTIR Analysis

Four experimental solutions were analyzed: PA, Bond + PA, TF, and Bond + TF. Fourier Transform Infrared (FTIR) spectroscopy was performed to evaluate the chemical composition of each solution(Table 4). Spectra were recorded within the range of 4000–400 cm^{-1} using an FTIR spectrometer under standardized conditions. The obtained spectra were plotted as absorbance versus wavenumber (cm^{-1}), and the characteristic functional group regions were recorded for comparative analysis among the groups.(Table 4) It is observed from the altered wavelengths that the PA and TF solutions have homogenously distributed in the combined groups (Bond + PA and Bond + TF). All samples were measured at room temperature, and background spectra were collected before each measurement.

Discussion

Naturally derived collagen cross-linkers such as proanthocyanidin (PA) and theaflavin (TF) have attracted increasing attention as dentin biomodification agents due to their potential to enhance the long-term stability of the hybrid layer by improving collagen stiffness and resistance to enzymatic degradation.[19–21] Degradation of exposed collagen fibrils within incompletely infiltrated hybrid layers is widely recognized as a key mechanism underlying the deterioration of resin–dentin bonds over time, primarily mediated by matrix metalloproteinases (MMPs) and cysteine cathepsins activated during dentin demineralization.[20,22] Therefore, strategies that combine collagen cross-linking with suppression of endogenous proteolytic activity are considered clinically promising approaches to improve adhesive durability.[12,21]

Significant differences between immediate and six-month μTBS values were observed in the U, E + U, E + PA+U, E + PAU, PAFL, PA + U, and E + TFU groups. In contrast, no statistically significant aging-related differences were detected in the PAU, E + TF+U, TF + U, and TFU groups. These findings indicate that the effect of collagen cross-linkers on bond durability was strongly dependent on the application protocol. Besides, with the support of the SEM analysis to the mechanical findings, the hypothesis of the current study is rejected.

In the present study, microtensile bond strength (μTBS) testing was selected as the primary outcome measure due to its sensitivity to interfacial changes and its suitability for evaluating both immediate bond strength and long-term durability following aging.[23] Scanning electron microscopy (SEM) was used to qualitatively assess resin–dentin interfacial morphology, including hybrid layer continuity, resin tag

formation, and interfacial gaps, providing structural support for the mechanical findings.[23,24] FTIR analysis was included to verify potential chemical interactions between proanthocyanidin or theaflavin and the adhesive formulations and to confirm that the incorporation of these cross-linkers did not alter the fundamental chemical structure of the adhesives.[25,26]

A concentration of 2% was selected for both proanthocyanidin and theaflavin based on previous studies reporting that low-concentration polyphenolic cross-linkers are sufficient to enhance collagen stability and inhibit enzymatic degradation without adversely affecting resin infiltration or adhesive polymerization.[13,18,21,25] Higher concentrations have been associated with increased solution viscosity and potential interference with adhesive penetration and degree of conversion.[41] Therefore, 2% was considered a clinically relevant and biologically effective concentration for collagen biomodification.

In previous studies, proanthocyanidin and theaflavin have been dissolved not only in ethanol but also in water, ethanol–water mixtures, acetone, and dimethyl sulfoxide (DMSO), all of which have been reported to provide adequate solubility and biological activity of polyphenolic cross-linkers.[13,17,18,30] DMSO, despite its high solvating capacity, has been associated with cytotoxicity concerns and potential interference with adhesive polymerization. In contrast, ethanol offers balanced solvating ability, good compatibility with adhesive systems, and favorable volatility, making it a clinically suitable carrier for natural cross-linkers.[13,17,18,28] In the current study, FTIR analysis was performed in terms of evaluating the proper dissolution of the cross-linkers in the adhesive bondings (Bond + PA, and Bond + TF). The spectra of PA and TF showed characteristic polyphenolic absorption bands, including broad O–H stretching vibrations (≈ 3200–3500 cm^{-1}), aromatic C = C stretching (≈ 1600 cm^{-1}), and C–O stretching bands within the fingerprint region (≈ 1200–1000 cm^{-1}). The adhesive-containing groups (Bond + PA and Bond + TF) exhibited the characteristic methacrylate-related peaks of the bonding agent, particularly the C = O stretching band (≈ 1720 cm^{-1}) and resin-associated bands in the fingerprint region. No additional crystalline-, precipitation-, or aggregation-related peaks were observed in any group, indicating that PA and TF were homogeneously dissolved in the ethanol-based medium and remained chemically compatible when incorporated into the adhesive system.

Among the evaluated groups, the highest immediate μTBS values were observed in the E + U control group, while the most favorable performance among the cross-linker-treated groups was achieved when PA was applied as a dentin pretreatment after acid etching (E + PA+U).[25] This finding supports the concept that acid etching exposes a demineralized collagen scaffold that may benefit from biomodification prior to adhesive infiltration, thereby enhancing collagen stability and facilitating more effective hybrid layer formation.[12,19,21] Although the E + PA+U group demonstrated the highest immediate μTBS values, its SEM images did not reveal prominent or well-organized resin tag formation and showed a relatively irregular surface morphology. This apparent discrepancy can be explained by the fact that bond strength is not primarily determined by resin tag length or density[31], but rather by the quality of hybrid layer formation and collagen stability within the intertubular dentin. PA, when applied after acid etching, has been shown to enhance collagen cross-linking, increase fibrillar stiffness, and

inhibit matrix metalloproteinase activity, thereby improving the mechanical properties and stability of the hybrid layer.[12,32] Therefore, enhanced immediate bond strength may be achieved even in the absence of morphologically prominent resin tags.

In contrast, the E + TF+U group exhibited more distinct resin tag formation and a more organized micromorphological appearance, yet demonstrated lower immediate μTBS values. This finding further supports the notion that resin tag formation alone does not directly correlate with bond strength.[33] However, the preservation of bond strength after six months suggests improved long-term stability. TF may diffuse into the demineralized collagen matrix and promote biomodification, contributing to resistance against enzymatic and hydrolytic degradation over time.[13,34] Thus, while PA may enhance immediate mechanical performance, TF may contribute more significantly to long-term durability. This finding may be explained by another mechanism. Proanthocyanidin is high molecular weight and strong interaction with adhesive components may also interfere with long-term resin infiltration and polymer stability, leading to greater aging-related degradation.[16,29] In contrast, although theaflavin produced lower initial bond strength, its antioxidant activity and inhibitory effect on collagen-degrading enzymes may provide more sustained protection of the hybrid layer over time, resulting in improved bond durability despite lower baseline values.[17,25] These findings indicate that long-term bond stability may depend more on enzymatic inhibition and interfacial protection than on initial bond strength.

In contrast, lower μTBS values were predominantly associated with groups in which PA or TF was applied without prior acid etching or incorporated directly into the adhesive system. These findings suggest that the presence of polyphenolic cross-linkers on smear-layer-covered dentin or within adhesive formulations may hinder resin infiltration or interfere with adhesive polymerization. Changes in adhesive viscosity, solvent balance, or degree of conversion have been proposed as potential mechanisms underlying these adverse effects.[27,28] Similar findings were reported by Epasinghe et al. and Bedran-Russo et al., who demonstrated that proanthocyanidin applied as a dentin pretreatment after acid etching improved bond strength and interfacial integrity, whereas incorporation of polyphenolic cross-linkers into adhesive systems or application on smear-layer–covered dentin resulted in reduced bonding performance due to impaired resin infiltration and altered adhesive properties.[12,13,18] These effects may be mechanistically explained by the ability of polyphenolic compounds to interact with both collagen fibrils and resin monomers through hydrogen bonding and hydrophobic interactions. When applied on smear-layer–covered dentin, limited demineralization restricts exposure of the collagen network, thereby reducing effective cross-linking and impairing resin diffusion into the substrate.[4,31] Moreover, the incorporation of polyphenols directly into adhesive formulations may alter the physicochemical properties of the adhesive, including increased viscosity, modification of solvent evaporation dynamics, and interference with free-radical polymerization reactions.[35,36] Polyphenolic molecules contain multiple hydroxyl groups capable of radical scavenging activity, which may reduce the degree of conversion of methacrylate-based adhesives when present within the adhesive matrix.[36,38] A reduced degree of conversion has been directly associated with compromised mechanical properties, increased nanoleakage, and lower immediate bond strength. [31,37] Mazzoni et al.[16] and Hass et al.[27]

found that incorporating bioactive compounds into adhesives might disrupt resin polymerization, modify viscosity and solvent balance, and impede monomer infiltration, thereby adversely impacting hybrid layer formation and bond durability. Additionally, excessive surface precipitation or incomplete solvent displacement may obstruct resin monomer infiltration into the demineralized collagen matrix, further weakening hybrid layer formation.[10,34] Collectively, these mechanisms support the present findings, suggesting that while polyphenolic cross-linkers can enhance bond performance when applied after proper acid etching, their application on smear-layer–covered dentin or incorporation into adhesive systems may adversely affect resin infiltration, polymerization efficiency, and overall interfacial integrity.

Aging significantly influenced bond strength in a protocol-dependent manner. Groups with lower initial interfacial integrity exhibited more pronounced reductions in μTBS after 6 months of water storage, consistent with the susceptibility of inadequately infiltrated collagen to hydrolytic and enzymatic degradation.[20,22] In line with these mechanisms, the TF-incorporated adhesive group (TFU) exhibited lower microtensile bond strength values. Although TF has demonstrated collagen-stabilizing potential, the results pointed out that its cross-linking capacity appears to be lower and more protocol-dependent.[17,25] In particular, Liu et al.[39] reported that TF at certain concentrations exhibited collagen stabilization comparable to PA, and even superior performance at lower concentrations; however, the authors emphasized the strong influence of exposure time and solution parameters. Similarly, Chen et al.[40] demonstrated that TF-induced cross-linking was highly dependent on treatment duration and solvent conditions. In contrast, PA has been consistently reported to enhance dentin mechanical properties and enzymatic resistance with relatively robust outcomes across various protocols.[18] Although theaflavin (TF) has demonstrated collagen-stabilizing potential, its effectiveness appears to be more dependent on application parameters such as concentration, exposure time, and delivery strategy.

The natural origin and reported biocompatibility of PA and TF represent important advantages over synthetic cross-linkers, supporting their potential use in adhesive dentistry.[12,19,25] When applied using optimized protocols, these agents may contribute to improved longevity of adhesive restorations by enhancing hybrid layer stability and reducing collagen degradation. However, the present study has limitations inherent to its in vitro design. Water storage was used as the sole aging method, and additional challenges such as thermal cycling or mechanical loading were not included. Furthermore, only a single concentration and application time for each cross-linker and one adhesive system were evaluated. Variations in concentration, application duration, or adhesive chemistry may influence the balance between beneficial collagen cross-linking and potential interference with resin infiltration. Future studies should address these variables and incorporate additional durability assessments to better simulate clinical conditions.

Conclusion

Within the limitations of this study, the application stage of collagen cross-linkers significantly influenced dentin microtensile bond strength and its stability over time. Higher immediate and 6-month bond

strength values were obtained in groups where the adhesive was applied using an etch-and-rinse strategy without cross-linker incorporation or when proanthocyanidin was applied as a dentin pretreatment. In contrast, lower bond strength values were observed when collagen cross-linkers were applied without prior acid etching or incorporated directly into the adhesive system. Aging affected the microtensile bond strength of several groups, with statistically significant differences between immediate and 6-month measurements depending on the application protocol.

Recommendations

The limitations of this study include its in vitro design, the evaluation of a single concentration and application time of the cross-linking agents, and the use of a limited aging protocol. Therefore, future studies should investigate different concentrations, application durations, adhesive systems, and extended aging conditions, as well as validate these findings through clinically relevant in vivo studies.

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Tables

Tables 1 to 4 are available in the Supplementary Files section.

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