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Collagen cross-linkers on dentin bonding: Stability of the adhesive interfaces, degree of conversion of the adhesive, cytotoxicity and in situ MMP inhibition

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ABSTRACT

Objective. To investigate the effect of collagen cross-links on the stability of adhesive properties, the degree of conversion within the hybrid layer, cytotoxicity and the inhibition potential of the MMPs' activity.

Methods. The dentin surfaces of human molars were acid-etched and treated with primers containing: 6.5 wt% proanthocyanidin, UVA-activated 0.1 wt% riboflavin, 5 wt% glutaraldehyde and distilled water for 60 s. Following, dentin was bonded with Adper Single Bond Plus and Tetric N-Bond; and restored with resin composite. The samples were sectioned into resin-dentin "sticks" and tested for microtensile bond strength (μ TBS) after immediate (IM) and 18-month (18M) periods. Bonded sticks at each period were used to evaluate nanoleakage and the degree of conversion (DC) under micro-Raman spectroscopy. The enzymatic activity (P1L10 cross-linkers, P1L22 MMPs' activities) in the hybrid layer was evaluated under confocal microscopy. The culture cell (NIH 3T3 fibroblast cell line) and MTT assay were performed to transdental cytotoxicity evaluation. Data from all tests were submitted to appropriate statistical analysis ($\alpha = 0.05$).

Results. All cross-linking primers reduced the degradation of μ TBS compared with the control group after 18M ($p > 0.05$). The DC was not affected ($p > 0.213$). The NL increased after 18M for all experimental groups, except for proanthocyanidin with Single Bond Plus ($p > 0.05$). All

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of the cross-link agents reduced the MMPs' activity, although this inhibition was more pronounced by PA. The cytotoxicity assay revealed reduced cell viability only for glutaraldehyde ($p < 0.001$).

Significance. Cross-linking primers used in clinically relevant minimized the time degradation of the μ TBS without jeopardizing the adhesive polymerization, as well as reduced the collagenolytic activity of MMPs. Glutaraldehyde reduced cell viability significantly and should be avoided for clinical use.

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1. Introduction

The longevity of hybrid layers depends upon the stability of their components, such as collagen fibrils and polymeric chains [1]. However, collagen fibrils are not completely infiltrated by resin monomers when exposed by acid etching [2,3], thereby impeding optimal protection against denaturation challenges. Unprotected collagen is more prone to creep [4] and cyclic fatigue rupture [5] after prolonged function. Additionally, these resin-sparse collagen fibrils are also filled with and surrounded by water, which participates in the hydrolysis of resin matrices by esterases and collagen by collagenolytic enzymes [6].

Increasing the collagen's resistance against the degradation process may improve the stability of the resin-dentin bonded interface; this was the main purpose of incorporating collagen cross-linkers into the bonding process [7–9]. Collagen cross-linkers are effective in protecting collagen fibrils from degradation through enhancing the collagen's chemical and mechanical properties [10–13]. More recently, their benefits in dentin bonding have been credited to their ability to inhibit the activity of host-derived metalloproteinases [8,14,15]. However, literature showing direct evidence of the activity of endogenous dentin MMPs within the hybrid layer after treatment by cross-linkers agents is still scarce.

On the other hand, collagen degradation is only one part of the biodegradation process, and it is not clear how collagen cross-linkers can affect the adhesive properties of the hybrid layer. Collagen cross-linkers showed an inhibitory polymerization effect on dimethacrylates [16] that may impair the achievement of an adequate degree of conversion inside the hybrid layer and jeopardize the bonding effectiveness [4]. Incomplete polymerization of the adhesive monomers has been suggested as one reason for nanoleakage [17–19] due to the formation of a porous hybridoid structure with reduced sealing ability [20–22]. All of these factors can also affect the cytotoxicity of an adhesive interface [23,24].

Although several collagen cross-linkers, such as glutaraldehyde and proanthocyanidins, have shown therapeutic effects on dentin collagen [25–28]. This requires their use for prolonged application times (10 min to 4 h), which restricts their clinical applicability. The comparison of different, recently accredited collagen cross-linkers, applied at clinically relevant times, might be useful to select the most effective agent in preventing collagen degradation, while inducing low cytotoxicity, with low polymerization inhibition yet providing stable resin-dentin bond interfaces over time.

Thus, the aim of this study was to evaluate the transdental cytotoxicity, the stability of the resin-dentin interfaces by microtensile and nanoleakage tests, the degree of conversion for the adhesive by in situ micro-Raman spectroscopy and the collagenolytic activity of the adhesive interface using in situ zymography, both with and without the incorporation of cross-linkers into the dentin bonding protocol.

2. Materials and methods

2.1. Tooth preparation and experimental design

Seventy-six extracted, caries-free human third molars were used after approval of the Institutional Ethics Committee from the State University of Ponta Grossa, Paraná, Brazil (protocol 314.563). The teeth were stored in 0.5% chloramine solution and used within two months after extraction. A flat dentin surface was exposed after wet-grinding the occlusal enamel using 180-grit SiC paper and 600-grit SiC paper for 60 s.

The dentin of forty teeth was etched for 15 s with 35% phosphoric acid gel (Scotchbond etchant, 3M ESPE, St. Paul, USA, batch number N261433), rinsed with water (30 s), air-dried (5 s) and kept slightly moist. The specimens were then randomly allocated to eight groups according to the combination of the main factors: (1) collagen cross-linking treatment (6.5 wt% proanthocyanidin, ultra-violet activated-0.1 wt% riboflavin, 5 wt% glutaraldehyde and distilled water [control group]) and (2) two etch-and-rinse adhesive systems (Adper Single Bond Plus [SB] and Tetric N-Bond [TN], as detailed in Table 1). A total of five teeth were employed per experimental group.

The acid-etched dentin surfaces were primed according to the experimental groups (Table 1). For the riboflavin (RB), the dentin surfaces were further exposed to ultraviolet light for 2 min with a UV lamp (Philips, Hamburg, Germany; $\lambda = 370$ nm at 3 mW/cm^2) before air-drying [8]. The light-curing steps were performed using an LED (Radii Cal, SDI, Bayswater, Victoria, Australia; 1200 mW/cm^2). Resin composite build-ups (Z250, 3M ESPE, Shade A3, batch number N549511) were incrementally constructed, and each portion was light-cured (40 s). The bonded teeth were then stored for 24 h in distilled water at 37°C .

Then, the specimens were longitudinally sectioned in both the mesiodistal and buccolingual directions across the bonded interface in a cutting machine (Buehler, Lake Bluff, USA), to obtain resin-dentin sticks (1 mm^2). The number of premature failures per tooth during specimen preparation was recorded. The cross-sectional area of each stick was measured with a

Table 1 – Description of all experimental groups, composition and application mode.

Product (company)	Composition	Application mode
Proanthocyanidin (PA) primer (Mega Natural Gold, Madera, USA) Batch number 05592502-01	Proanthocyanidin-grape seed extract 6.5% weight, distilled water	After acid etching step, application for 60 s with gentle agitation using a fully saturated applicator. Gently air-drier for 5 s and kept slightly moist the surface
Riboflavin (RB) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 070046	Riboflavin 0.1% weight, distilled water	After acid etching step, application for 60 s with gentle agitation using a fully saturated applicator. After that, irradiation using UVA-light for 2 min. Gently air-drier for 5 s and kept slightly moist the surface
Glutaraldehyde (GA) primer (Fisher Scientific GmbH, Schwerte, Germany) Batch number 186852	Glutaraldehyde 5% weight, distilled water	After acid etching step, application for 60 s with gentle agitation using a fully saturated applicator. Gently air-drier for 5 s and kept slightly moist the surface
Control Group (CT)	Distilled water	After acid etching step, application for 60 s with gentle agitation using a fully saturated applicator. Gently air-drier for 5 s and kept slightly moist the surface
Single Bond Plus (SB) (3M ESPE, St. Paul, USA) Batch number N531785	Ethyl alcohol, BisGMA, silane treated silica (nanofiller), HEMA, copolymer of acrylic and itaconic acids, glycerol 1,3-dimethacrylate, water, UDMA, diphenyliodonium hexafluorophosphate, EDMAB	After treatment according the experimental groups, application 2 consecutive coats of adhesive for 15 s with gentle agitation using a fully saturated applicator. Gently air thin for 5 s to evaporate solvent. Light-cure for 10 s
Tetric N-Bond (TN) (Ivoclar Vivadent AG, Schaan, Liechtenstein) Batch number L50568	Phosphonic acid acrylate, HEMA, BisGMA, UDMA, ethanol, nanofiller, catalysts and stabilizer	After treatment according the experimental groups, application 2 consecutive coats of adhesive for 15 s with gentle agitation using a fully saturated applicator. Gently air thin for 5 s to evaporate solvent. Light-cure for 10 s
BisGMA: bisphenol a diglycidyl ether dimethacrylate; HEMA: 2-hydroxyethyl methacrylate; UDMA: diurethane dimethacrylate; EDMAB: ethyl 4-dimethyl aminobenzoate.		

digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm.

2.2. Resin–dentin microtensile bond strength (μ TBS)

For this test, 40 teeth ($n = 5$ teeth per group) previously restored were used. Each bonded stick was attached to a jig for microtensile testing with cyanoacrylate resin (Super Bonder Gel, Loctite, São Paulo, Brazil) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure modes were evaluated under stereomicroscopy at 40 $\times$ magnification and classified as cohesive adhesive or adhesive/mixed.

2.3. Nanoleakage evaluation (NL)

Two resin-bonded sticks from each tooth at each storage period (not tested in μ TBS) were randomly selected. The specimens were immersed in ammoniacal 50 wt% silver nitrate solution [22] in darkness for 24 h. Then, they were rinsed thoroughly in distilled water and photo-developed (8 h) under fluorescent light to reduce the silver ions into metallic silver grains. The specimens were polished down until 2500-grit SiC paper and 1 and 0.25 μ m diamond paste (Buehler Ltd., Lake Bluff, IL, USA). They were ultrasonically cleaned, air-dried, mounted on stubs and coated with carbon (MED 010, Balzers Union, Balzers, Liechtenstein). The interfaces were observed in a scanning electron microscope (SEM), in the backscattered mode at 12 kV (VEGA 3 TESCAM, Shimadzu, Tokyo, Japan). Three images were taken of each specimen: the first image

was in the center of the stick, while the next two were obtained 0.3 mm left and right from the first picture, respectively [29].

A total of six images were obtained per tooth at each period (3 images $\times$ 2 bonded sticks). A total of 30 images were obtained per group (6 images $\times$ 5 teeth) by a blinded author. We measured the relative percentages of NL within the adhesive and hybrid layers with the ImageTool 3.0 software (University of Texas Health Science Center, San Antonio, USA), as described earlier [19,30].

2.4. In situ degree of conversion (DC) within adhesive/hybrid layers

Two resin–dentin sticks were randomly selected from the immediate period and used to evaluate the DC immediately after sectioning. The sticks were wet polished using 1500- and 2000-grit SiC paper. The specimens were ultrasonically cleaned for 10 min and positioned into micro-Raman equipment (Senterra spectrophotometer Bruker, Ettlingen, Baden-Württemberg, Germany), which was first calibrated for zero and then for coefficient values using a silicon sample. The samples were analyzed using a 20 mW Neon laser with 532 nm wavelength, spatial resolution of 3 μ m, spectral resolution 5 cm^{-1} , accumulation time of 30 s with 5 co-additions and 100 $\times$ magnification (Olympus microscope, London, UK) with a 1- μ m beam diameter. Spectra were obtained at the dentin–adhesive interface, at three random sites (per bonded stick) within intertubular-infiltrated dentin. Spectra of uncured adhesives were taken as references. Post-processing of the spectra was performed using the Opus Spectroscopy Software version 6.5. The ratio of double-bond content of monomer to

polymer in the adhesive was calculated according to the following formula: $DC (\%) = (1 - [R_{\text{cured}}/R_{\text{uncured}}]) \times 100$, where 'R' is the ratio of aliphatic and aromatic peak intensities at 1639 cm^{-1} and 1609 cm^{-1} in cured and uncured adhesives.

2.5. In situ zymography

A dye-quenched MMP probe based on gelatin was prepared by means of a fluorescein isothiocyanate (FITC) hypersaturated gelatin. 5 mg of FITC was dissolved in 2 ml 0.1M sodium carbonate/bicarbonate buffer (pH 9.0, Sigma Aldrich, Milwaukee, USA). This reactant was added dropwise to a 1 mg/ml gelatin solution in the dark and was incubated at room temperature for 2 h. The reacted FITC–gelatin conjugate was isolated from unbound FITC by means of a G-25M Sephadex column. The fluorescein-to-protein ratio of >15 was confirmed from absorbance readings at 495 nm and 280 nm, respectively. This MMP-substrate confocal dye was dissolved (0.3 wt%) in distilled water and actively applied for 60 s onto the phosphoric acid-etched dentin before the bonding agents and cross-linking primers were applied.

Three teeth per group ($n=3$) were bonded as previously described and cut into resin–dentin slabs; their interfaces were observed by confocal laser scanning microscopy (CLSM), similarly to a previous study [31]. The specimens were examined using a CLSM (Leica SP5 CLSM, Heidelberg, Germany) equipped with a $63\times/1.4$ NA oil immersion lens using 468-nm laser illumination. The z-stack scans (one at each micrometer up to $20\text{ }\mu\text{m}$ below the surface) were compiled into single projections. Each resin–dentin interface was entirely characterized, and images representing the MMP-activity observed along the bonded interfaces were captured.

2.6. Cytotoxicity evaluation: cell culture and MTT assay

Twenty-four teeth were used in this test. Dentin disks with a thickness of 0.6 mm were obtained from the mid-coronal dentin of each tooth using a cutting machine (Isomet 1000, Buehler, Lake Bluff, USA). The disks were carefully examined with a stereomicroscope at $40\times$ to confirm the absence of enamel and defects resulting from pulp horn projections. Then, the occlusal sides of the disks were manually finished with wet 320-grit silicon carbide paper to reach a final thickness of 0.5 mm (Mitutoyo South Americana Ltd., Suzano, SP, Brazil). Afterwards, a smear layer was produced with 600-grit SiC on both sides of the disks and immediately removed by 0.5 M EDTA (pH 7.4) for 60 s. After abundant rinsing with deionized water, the dentin's permeability was measured through a hydraulic conductance device [32] to permit a homogeneous distribution of the dentin disks among the experimental groups ($n=6$ disks per group). The dentin disks were positioned in metallic devices and autoclaved ($20\text{ min}/121^\circ\text{C}$). The occlusal surfaces of the dentin disks were etched with 35% phosphoric acid (15 s), carefully rinsed with deionized water (10 s) and blot dried with sterile cotton pellets.

The NIH/3T3 fibroblast cell line was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin ($10,000\text{ U}/100\text{ }\mu\text{g}/\text{ml}$) at 37°C with 5% CO_2 in a humidified atmosphere. Briefly, 4×10^4

cells were added to each well of a 24-well plate. After 24 h, the medium was removed; the cells were washed twice with phosphate buffer solution (PBS). DMEM containing 1% fetal bovine serum and trans-well chambers were added into each well. The dentin disks were carefully added, and $10\text{ }\mu\text{l}$ of each cross-linking primer was applied onto the occlusal surface. For the riboflavin group, the UVA-light was irradiated by a lamp, as previously described, using a 10.4 mm tip that completely covered the culture well (24-well format). This protocol followed Bouillaguet et al. [33] to prevent modifications to the cell mitochondrial activity.

The trans-well was removed and the cells were washed with phosphate-buffered saline (PBS) 24 h later. MTT cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, USA) according to the method of Tada et al. [34] with some modifications. MTT solution was added ($1.0\text{ mg}/\text{ml}$), and cell viability was then assessed in a colorimetric assay using mitochondrial dehydrogenase activity in active mitochondria to form purple formazan. The absorbance of each well was read at 570 nm using a plate reader (EL808B, BioTech Instruments Inc., Winooski, VT, USA). Cell viability was expressed as the percentage of optical values in the treated samples versus the concurrent control (no treatment), considered as 100%. All of the experiments were performed in triplicate.

2.7. Statistical analysis

The μTBS (MPa) and NL (%) from the same experimental unit were averaged for statistical purposes at each storage time. The bonded sticks with premature and cohesive failures were not included in the tooth mean, due to their low frequency in the experiment.

The Kolmogorov–Smirnov test was employed to assess whether the data from each test (μTBS , NL, DC and cytotoxicity) followed a normal distribution. Barlett's test was performed to determine if the assumption of equal variances was valid. After observing the data's normality and equality of the variances, the data from the μTBS (MPa) and NL (%) of each adhesive were subjected to a two-way repeated measure ANOVA (on solutions and storage time). The data from the DC (%) and cytotoxicity (%) of each adhesive were subjected to a one-way ANOVA (on solutions). For all of the test Tukey's test was used for pairwise comparisons ($\alpha=0.05$).

3. Results

3.1. Microtensile bond strength

Approximately 29–37 bonded sticks could be obtained per tooth, including the pre-test failures. The mean cross-sectional area was $0.9 \pm 0.13\text{ mm}^2$, and no differences among the groups were detected (data not shown; $p>0.05$). Most of the failures were mixed (data not shown). None of the cross-linker primers affected the immediate μTBS of either adhesive (Table 2). For SB, all of the primers except the control group produced stable μTBS after 18 months of storage. A similar trend was observed for TN, except that degradation was also observed in the glutaraldehyde group (Table 2).

Table 2 – Means and standard deviations of μ TBS (MPa) for all experimental groups.

Groups	SB		TN	
	Immediate	18-month	Immediate	18-month
Control	39.5 (7.9) a	13.9 (1.8) b	36.8 (4.7) A	13.9 (1.8) D
Proanthocyanidin	36.2 (5.5) a	31.9 (4.3) a	29.2 (1.2) ABC	27.6 (6.3) BC
UVA-riboflavin	37.1 (9.7) a	31.6 (3.5) a	31.5 (6.9) AB	25.1 (1.3) BC
Glutaraldehyde	38.5 (2.4) a	29.7 (2.6) a	35.7 (1.9) AB	24.2 (1.4) C

Comparisons are only valid within the same adhesive system. Means identified with the same letter are statistically similar (Tukey's test; $p > 0.05$).

Table 3 – Means and standard deviations of nanoleakage (%) for all experimental groups.

Groups	SB		TN	
	Immediate	18-month	Immediate	18-month
Control	7.2 (4.5) Aa	20.3 (4.6) Bb	19.5 (3.8) Ff	25.9 (3.5) Eg
Proanthocyanidin	4.6 (2.1) Aa	8.0 (0.9) Aa	13.2 (4.8) Df	15.7 (1.9) Cg
UVA-riboflavin	4.5 (2.6) Aa	11.6 (1.6) Ab	13.9 (1.9) Df	20.0 (2.1) Dg
Glutaraldehyde	6.1 (2.3) Aa	14.7 (2.4) Bb	17.6 (7.2) Ef	24.2 (2.3) Eg

Comparisons are only valid within the same adhesive system. In each column, means identified with the same uppercase letter are statistically similar. In each row (for each adhesive), means identified with the same lowercase letters are statistically similar (Tukey's test; $p > 0.05$).

Table 4 – Means and standard deviations of the degree of conversion (%) for all experimental groups.

Groups	SB	TN
Control	79.4 (7.2) A	77.6 (6.8) a
Proanthocyanidin	79.7 (6.0) A	77.7 (7.8) a
UVA-riboflavin	73.7 (5.8) A	71.9 (7.4) a
Glutaraldehyde	77.5 (9.0) A	72.7 (8.8) a

Comparisons are only valid within the same adhesive system. No significant difference was observed among groups (Tukey's test; $p > 0.05$).

3.2. Nanoleakage

For SB, reduced nanoleakage was observed at the immediate period for all of the primers (Table 3). This increased significantly after 18 months for all of the primers ($p < 0.001$), except proanthocyanidin. For TN, a more pronounced nanoleakage was observed at the immediate period for the control and glutaraldehyde groups ($p < 0.001$). This increased significantly after 18 months of water storage for all of the groups ($p < 0.001$) (Table 3). Representative SEM images of the resin-dentin interfaces for the different experimental groups are depicted in Fig. 1.

3.3. Degree of conversion

No statistically significant differences ($p > 0.213$) were observed among the experimental groups for both of the adhesive systems (Table 4).

3.4. In situ zymography

In the control groups of both of the adhesive systems, the in situ zymography revealed an intense green fluorescence at the hybrid layer, indicating strong activity of MMPs (Fig. 2). The MMP activity for both adhesives was completely eliminated

with the preliminary application of the proanthocyanidin primer. In the UVA-riboflavin and glutaraldehyde groups, an expressive reduction of MMP activity was also observed for both adhesives, although not to the same extent as that achieved with proanthocyanidin (Fig. 2).

3.5. Cell viability

The treatment with glutaraldehyde reduced $81.1 \pm 4\%$ of cell viability in which was statistically different than the other groups ($p < 0.001$). UVA-riboflavin (0.1%) and proanthocyanidin (6.5%) did not alter the cell viability, compared with the control group (Fig. 3).

4. Discussion

This study evaluated the use of aqueous primers containing different collagen cross-links in the bonding protocol applied on demineralized dentin under clinically relevant time periods. Although some studies have had the same aim [8,12,35], they did not compare the most effective agents in a single experimental design. We observed that all of the cross-linking agents employed produced stable bond strength after 18 months of water storage for both adhesives, which is in agreement with previous studies [8,27].

The chemical and structural characteristics of each cross-linking agent determine its ability to interact and modify the dentin matrix and the consequent impact on the stability of the collagen, the vulnerability of the adhesive interfaces to degradation and its cytotoxic potential [36,37]. For instance, glutaraldehyde has a five-carbon aliphatic molecule with an aldehyde at each end of the chain, rendering it bifunctional. The aldehyde group is able to interact chemically with the amino groups of collagen [38], thus increasing the strength of the collagen [39] and minimizing the degradation of the adhesive interface [28]. The disadvantage of this agent is that it

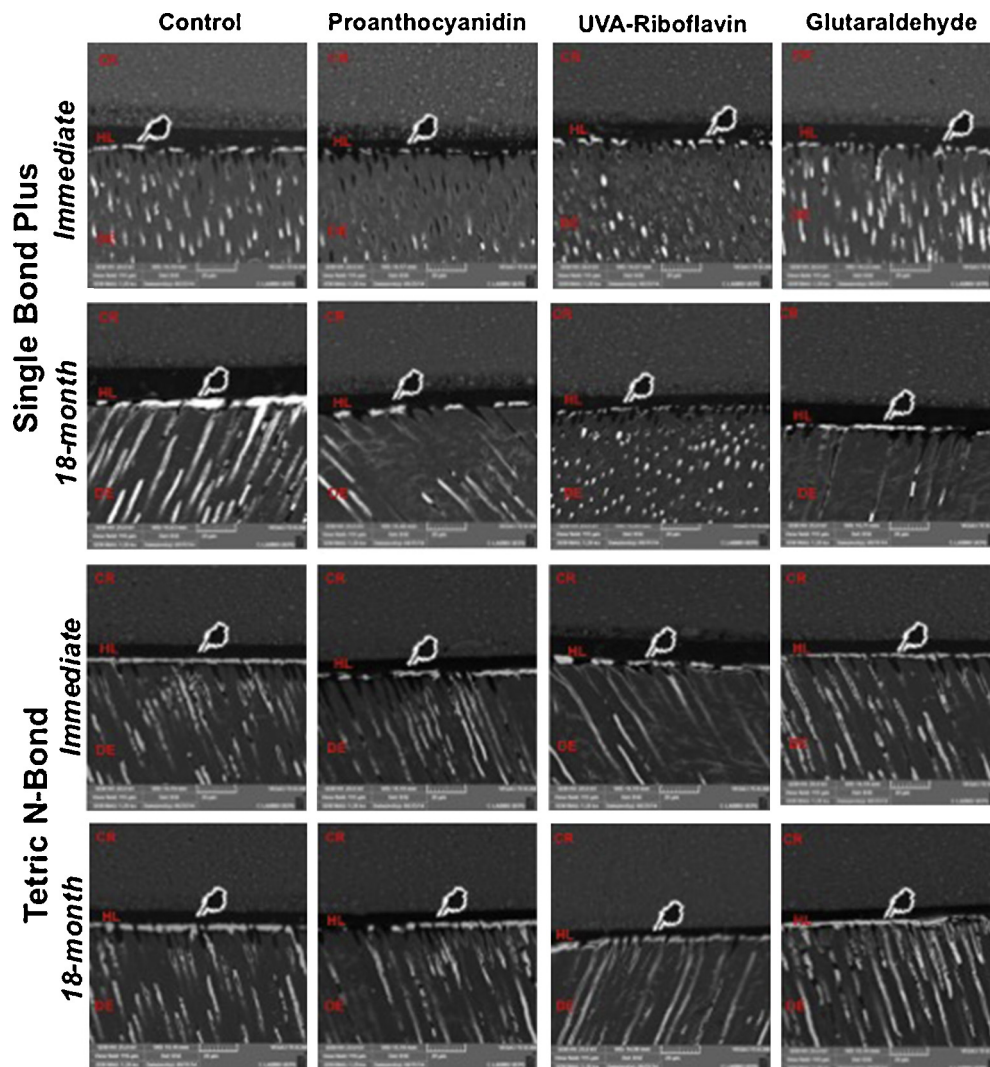


Fig. 1 – Representative backscattered SEM images of the resin–dentin interfaces bonded for different experimental groups. Silver nitrate uptake (white hands) occurred practically within the hybrid layer in all groups. When control groups was performed significantly increase of silver nitrate deposition occurred within the HL, as well as, adhesive layer and inside the dentinal tubules in comparison with the different MMP-inhibitors applied, mainly after 18-month of water storage. CR: composite-resin; HL: hybrid layer; DE: dentin.

promotes rapid surface cross-linking of the tissue, generating a barrier that impedes its further diffusion into the tissue bulk, jeopardizing the fixation of the tissue as the depth of the tissue increases [37,40]. This might be one of the reasons why this agent was not capable of completely inhibiting the activity of MMPs in the deeper region of the hybrid layer (Fig. 2).

Riboflavin (vitamin B2) was also shown to reduce the degradation of the adhesive interface in both the present and earlier studies [8,35]. The high energy of UVA-light (365 nm) breaks down weak and intrinsic cross-links among collagen fibrils and generates free oxygen radicals. The reactive oxygen species can induce the formation of new covalent and strong cross-links within collagen [41]. This occurs through the binding of the functional hydroxyl groups in riboflavin to proline and/or lysine in the collagen [42].

Proanthocyanidins are natural polyphenolic compounds that are widely used as food supplements. Due to their

free-radical scavenging capacity, high affinity to protein and antioxidant potential, these materials have been extensively studied for enhancing dentin bonds [36,43,44]. The stabilization of the dentin bonding after water storage can be explained by four interaction mechanisms between the proanthocyanidin and proteins, including covalent, ionic and hydrogen bonding, as well as hydrophobic interactions [36,44,45]. Proline-rich proteins like collagen have an extremely high affinity to proanthocyanidin [46], yielding strong bonds. This interaction primarily occurs through hydrogen bonding between the protein amide carbonyl group of the collagen and the phenolic hydroxyl group of the cross-linking agent [47].

Riboflavin and proanthocyanidin are very safe agents, as they did not reduce cell viability in the cytotoxicity test, thus overcoming some of the drawbacks that are typically encountered with synthetic cross-linking agents such as

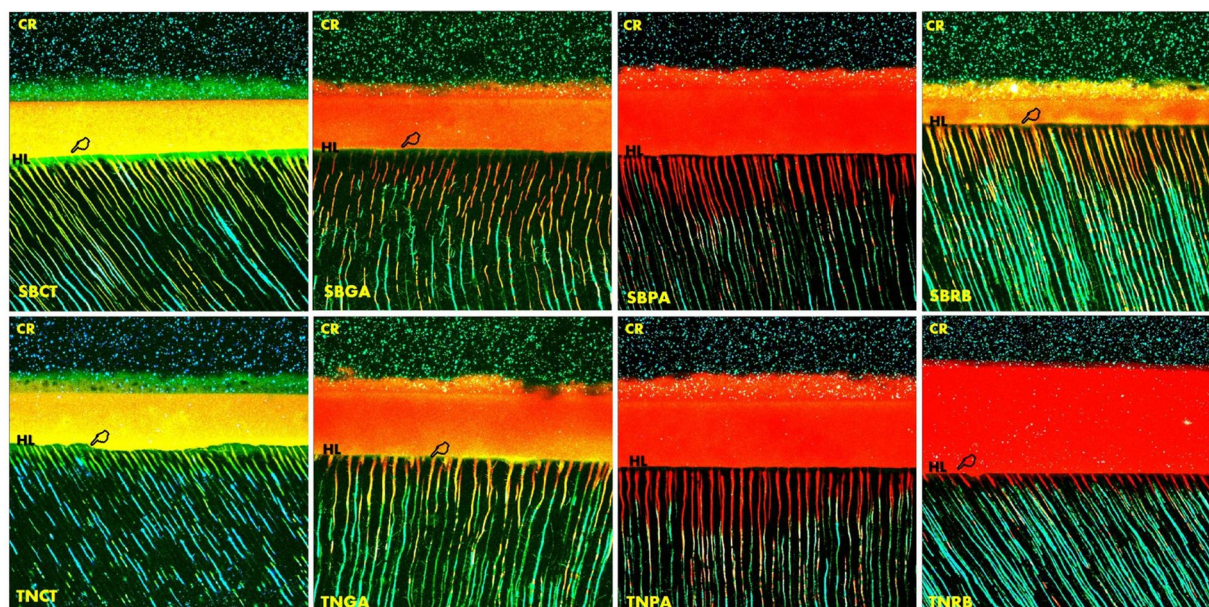


Fig. 2 – The confocal micrographs of the in situ MMP-activity at the dentin-adhesive interface after incubation for 24 h. The figures SBCT and TNCT represent the control groups and show the intense activity of MMPs (green staining) at the hybrid layer (black hand) and beneath it. The figures SBGA and TNGA represent the glutaraldehyde groups and exhibit the considerable reduction of the intensity of the active MMP-binding fluorescent dye. However, there is still moderate green staining (enzymatic activity) at the top of the hybrid layer. In contrast, the cross-link treatment using proanthocyanidin (Figure PA) eliminated almost entirely the MMP-activity represented by the lack of green staining at the thick hybrid layer. In the case of UVA-activated riboflavin the green staining was somewhat between the control group and the treatment with glutaraldehyde. The pre-treatment using riboflavin reduced the MMP activity but not in a greater extent as proanthocyanidin. CR: composite-resin and HL: hybrid layer.

glutaraldehyde. The high cytotoxicity of glutaraldehyde might be because this agent suffers from depolymerization [40,48]. The residues from this depolymerization along with uncured molecules render it very cytotoxic, as reported based on the MTT assay of the present investigation and in previous investigations [36,49,50]. These two disadvantages make glutaraldehyde the least adequate cross-linking agent for incorporation into the bonding protocol.

The literature about the cytotoxic potential of riboflavin is controversial [33,37,42,51]. We used the transdental method in the present study's MTT assay to better simulate the clinical situation. In this case, the product is applied onto the

dentin surface, where it is capable of protecting the underlying cells. The other studies reporting that riboflavin is cytotoxic applied the product directly onto the cell culture [33,37]. On the other hand, proanthocyanidin's lack of cytotoxicity is in agreement with other studies [36,48,52]. The high affinity of proanthocyanidin to the organic matrix of collagen may lead to effective cross-linking without the production of cytotoxic residual molecules, rendering it a safe product for use in dentin bonding.

A high degree of conversion for the adhesive is critical to produce a high cross-linked polymer within the hybrid layer and also yield adhesive interfaces that is less prone to degradation [17–19]. Faithfully, the use of the cross-linking agents did not significantly affect the degree of conversion within the hybrid layer, which suggests that none of the agents jeopardized the polymerization of adhesive system when applied like primers.

Although proanthocyanidins were reported to have the potential to reduce the polymerization efficacy of resins, this seems to depend on the concentration of the agent [9,53] as well as how this cross-linking is incorporated into the bonding process. For instance, the incorporation of proanthocyanidin into a simplified etch-and-rinse adhesive system in concentrations equal to or higher than 2% resulted in inadequate polymerization of the adhesive and the formation of microvoids within the adhesive layer. [53,54] In the present study, the polymerization of the adhesive was not jeopardized, even when applying proanthocyanidin at higher

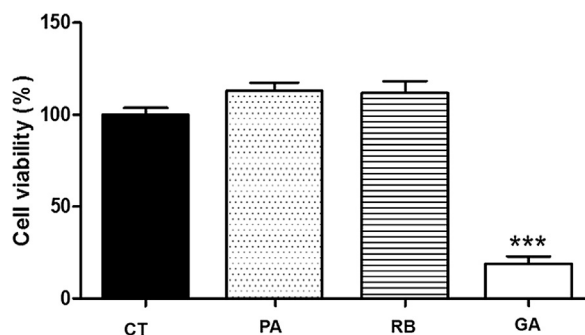


Fig. 3 – Graphic representation of the effect of on 3T3 cells after 24 h of exposure in the MTT assay. *Statistical difference compared to the other groups (Tukey's test; $p < 0.001$).**

concentrations (6.5 wt%). Perhaps the only substrate that the proanthocyanidin has for bonding is collagen when applied as an aqueous primer, before applying the adhesive [36,37,44,47]. Therefore, by the time the adhesive is applied, there is no longer proanthocyanidin available to compete for binding with the free radicals generated during the adhesive's polymerization.

It is quite difficult to produce nanoleakage-free interfaces. Nanoleakage reveals the locations of defects at the resin–dentin interface that could work as the pathway for the resin–dentin bonds' degradation over time. These defects result in areas in which resin failed to infiltrate, residual water/solvent had not been displaced by adhesive resin [55] or fluid transudation occurred through the dentin to the adhesive layer [21,56–60]. Although these nanometer-sized spaces are too small to allow for bacterial penetration, they are large enough to work as channels for water sorption [18,20,61] and leaching of uncured water-soluble monomers. A low pH created by uncured acidic monomers, water and bacterial oral fluids absorbed for gradient, can lead to the cleavage of the prodomain of MMP molecules and thus facilitate their functional activity [62,63]. The neutralization this acidic environment by the dentinal buffering mechanisms or through the salivary buffer systems, allows the pH-activated MMPs to cleave matrix components [62,64]. The insoluble collagen fibrils are slowly broken down into gelatin peptides that are in turn broken down by gelatinases to smaller peptides and amino acids that elute from the hybrid layers. This mass is replaced by water. Thus, higher silver nitrate uptake equates with the increase in water uptake that follows loss of collagen and resin stability [65]. The increase in nanoleakage is consistent with the reported degradation of collagen fibrils in hybrid layers [66,67].

We cannot expect the application of aqueous solutions of cross-linkers to produce nanoleakage-free interfaces, since the protocols tested herein cannot alter the hydrophilic nature of the adhesive, improve water/solvent evaporation or produce less permeable adhesive interfaces. Thus, the similar nanoleakage among the experimental and control groups, both immediately and at 18 months, can be seen with enthusiasm. This is evidence that the infiltration of monomers, solvent evaporation and polymerization of the adhesive were not jeopardized by the preliminary step of collagen cross-linking. If this had occurred, a more pronounced and intense nanoleakage, like those observed in the poorly polymerized conditions of the adhesive interfaces [17,19,68], would have been observed.

Interestingly, the application of proanthocyanidin and riboflavin produced interfaces that were less prone to nanoleakage over time for both adhesives. For SB system, the proanthocyanidin group did not show a significant increase of nanoleakage over time. The exogenous cross-link of the dentin matrix by proanthocyanidin leads to dehydration on the collagen fibrils, which decreases the swelling ratio and the water absorption [6,12,45]. This suggests that the application of proanthocyanidin can minimize the risk of collagen network collapse resulting from air drying and therefore minimize the technique sensitivity of the wet bonding [6,43].

All previous attempts to inhibit MMPs with different chemicals required that these inhibitors bind to MMPs irreversibly;

however, there is no evidence of such long-term binding. As riboflavin—the unique cross-linking that is applied—is associated with high energy of UVA light, we speculate that, despite an immediate limited effect in terms of MMP inhibition, this effect could be maintained for a long time.

Obviously, we cannot rule out the fact that the biodegradation of the resin–dentin interface is quite complex and involves a cascade of events, starting with the extraction of resins that have infiltrated the dentin matrix, followed by an enzymatic attack on exposed collagen fibrils [69,70]. Proteases such as metalloproteinases (MMPs) and cysteine cathepsins are thought to be responsible for enzymatic degradation of the collagen fibrils via hydrolysis [71].

All of the cross-linking agents reduced or eliminated the MMPs' activity in the dentin-bonding interface [8,15,35]. This is the first laboratory study that demonstrated such inhibition potential *in situ* for these tested agents. On the dentin collagen, the triple-helical conformation makes the interstitial collagens resistant to most proteinases; however, when collagenases bind to collagen, they unwind the triple-helix, allowing single peptides to enter the substrate binding and catalytic domains [72]. Cross-linking agents produce cross-links in collagen that stiffen it enough that it cannot unwind its structure. In addition, cross-linking agents can cross-link proteases, which directly interferes with their molecular mobility [73]. They can inactivate C-terminal telopeptidases, thereby maintaining the telopeptides' ability to sterically block collagenase binding to the critical peptide bond [73,74]. Hence, it is reasonable to expect that cross-linking agents are capable of inhibiting the MMP activity. This is in agreement with previous findings reporting that cross-linked collagen exhibited reduced digestibility by collagenase [8,12,15,28,75]. It worth to mention that, *in situ* zymography used in the present study was recently validated by Almaydi et al. [76]. The technique was used because it provides the localization of MMP activity within the tissue without the need for MMP isolation and separation [77,78].

Among all of the cross-linking agents, proanthocyanidin was the most effective agent in reducing the MMP activity. Again, the very effective chemical interaction of proanthocyanidin and high reticulation ability throughout the whole collagen length [36] make it very effective. It is speculated that proanthocyanidin has stronger interaction ability with collagen than glutaraldehyde and riboflavin do. Also, the dehydration of the collagen fibrils and the hydrophobic effect (as previously discussed) produced by proanthocyanidin result in less water and fluid sorption, better adhesive infiltration and consequently fewer denuded fibrils within the hybrid layer. Altogether, these factors result in a hybrid layer that is less prone to the MMPs' activity [4,62].

5. Conclusions

Under a standardized application time of 60s, the use of all of the cross-linking primers before applying the adhesive was effective in minimizing the degradation of the μ TBS of both adhesives, without jeopardizing the polymerization of the resin monomers. The stable μ TBS of the adhesives can be attributed to the reduced *in situ* collagenolytic activity of

the MMPs, which was much more pronounced with the use of proanthocyanidin. Glutaraldehyde reduced cell viability significantly and should be avoided for clinical use.

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