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Degradation of dentin-bonded interfaces treated with collagen cross-linking agents in a cariogenic oral environment: An *in situ* study

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ABSTRACT

Objectives: To evaluate the effect of treatment using collagen cross-linking agents as primer on resin–dentin bond interfaces subjected to cariogenic oral environment (COE).
Methods: Each of forty human teeth had two cavities (4 × 4 × 1.5 mm) prepared within enamel margins. These cavities were acid-etched and treated by the primers containing one of the following treatment agents (6.5% proanthocyanidins, 0.1% riboflavin-UVA activated light, 5% glutaraldehyde or distilled water as a control group). After that the cavities were bonded and restored with resin composite. One restoration for each tooth was tested immediately (IM) and another was included in an intra-oral palatal device that was placed in each mouth of ten adult volunteers for 14 days in COE. After 14 days, the teeth were removed and each restoration was sectioned to obtain a slice for Knoop microhardness (KHN) and resin–dentin bonded sticks for microtensile bond strength (μTBS) and nanoleakage (NL) evaluation. Data were evaluated by two-way ANOVA and Tukey's tests ($\alpha = 0.05$).
Results: After 14 days in a COE, the KHN was reduced for all groups, except for the glutaraldehyde group; however, the proanthocyanidins group retained the highest KHN in IM and after COE ($p < 0.05$). The μTBS was not reduced after COE for the proanthocyanidins and glutaraldehyde groups, however only the proanthocyanidins treatment did not increase the NL after COE ($p > 0.05$).
Conclusion: The *in situ* study model seems to be a suitable short-term methodology to investigate the degradation of the bonding interfaces under a more realistic condition. Under COE, the proanthocyanidins and glutaraldehyde treatments produced stable interfaces that are worth further clinical investigation.

1. Introduction

The hybrid structure formed during the dental bonding procedure occurs by demineralization of the dentin surface, followed by infiltration and subsequent polymerization of monomers around the collagen fibrils [1]. Therefore, to achieve effective and stable bonding, the preservation of dentin collagen is critical, since collagen represents the major organic component of the dentin matrix.

Unfortunately this is not an easy task. During bonding procedures, the demineralized collagen fibrils are not completely infiltrated by resin monomers, [2,3] and these denuded collagen fibrils are more prone to degradation. Fluctuations of acidity produced by different pHs of foods and drinks as well as that induced by bacterial acids may increase the amount of exposed organic matrix to be broken-down by bacterially derived enzymes. Additionally, host-derived enzymes [4] such as matrix metalloproteinases (MMPs) and cysteine cathepsins [5–8] present in the dentin matrix and in the gingival crevicular fluid, also play a role on resin–dentin bond degradation.

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The role of these host-derived proteases in the breakdown of the collagen matrices during the pathogenesis of dentin caries [9,10], periodontal disease [11] and degradation of resin–dentin bonded interfaces [12] has already been demonstrated [8,9]. Measures that enhance dentin resistance against collagenolytic activities have great potential for improving the longevity of the dentin bonding. In this context, collagen cross-linking agents have been investigated as dentin biomodifiers.

These crosslinking agents can interact with various extracellular matrix components inducing increases in the mechanical properties of the tissue, decreasing the biodegradation rates and possibly inducing mineral nucleation [13–15], which make them a promising solution for preservation of resin–dentin bonded interfaces [16,17].

However, most of the studies that support the benefits of cross-linking agents are performed in laboratories, where the challenging conditions of the oral environment are barely reproduced. Although randomized clinical studies are the best study design to evaluate both the performance and longevity of restorative materials, they are time demanding, costly and dependent on the approval by a local Ethics Committee. Under this scenario, the conduction of *in situ* studies may gather important information to the field, as it resembles the challenging clinical conditions that resin–dentin interfaces are prone better than *in vitro* studies. *In situ* studies may be considered as an intermediate stage between *in vitro* and clinical studies. Therefore, the aim of this study was to investigate the degradation of resin–dentin interfaces treated by different collagen cross-linking agents after *in situ* cariogenic challenge, using microhardness, microtensile bond strength and nanoleakage. The test null hypotheses were that after 14 days of exposure to an intra oral cariogenic environment, the Knoop hardness, μTBS and nanoleakage of resin–dentin interfaces treated by different collagen cross-linking agents did not change.

2. Material and methods

The study protocol was approved by the Local Ethics Committee Review Board under protocol number 314.563. Ten healthy adult volunteers (aged 21–30 years, female and male) were selected according to the following inclusion criteria: good general and oral health and normal salivary flow rate. Participants that took antibiotics for the last 2 months before the experiment or wearing prosthesis or orthodontic devices were not included in this study. All volunteers agreed to participate and signed an informed written consent.

A total of forty extracted, non-erupted human third molars were used. The teeth were collected after obtaining the patients' informed consent under a protocol approved by the previously described Ethics Committee previously described. Teeth free from cracks or any other kinds of structural defects were selected. The teeth were disinfected by storage in 10% buffered formalin solution, pH 7, for 7 days [18] and stored in distilled water for up to 2 months after extraction.

2.1. Experimental design

This *in situ*, split-mouth study was designed for accumulation of a plaque-like biofilm on the restorations in a high cariogenic challenge promoted by sucrose exposure. This protocol was performed for 14 days. The factors under evaluation were: (1) three different collagen cross-linking agents (proanthocyanidins from grape seed extract, UVA-activated riboflavin, glutaraldehyde and distilled water as control); and (2) evaluation time—2 levels (immediate and 14 days after degradation in a cariogenic oral environment). Then, a total of eight experimental conditions were tested.

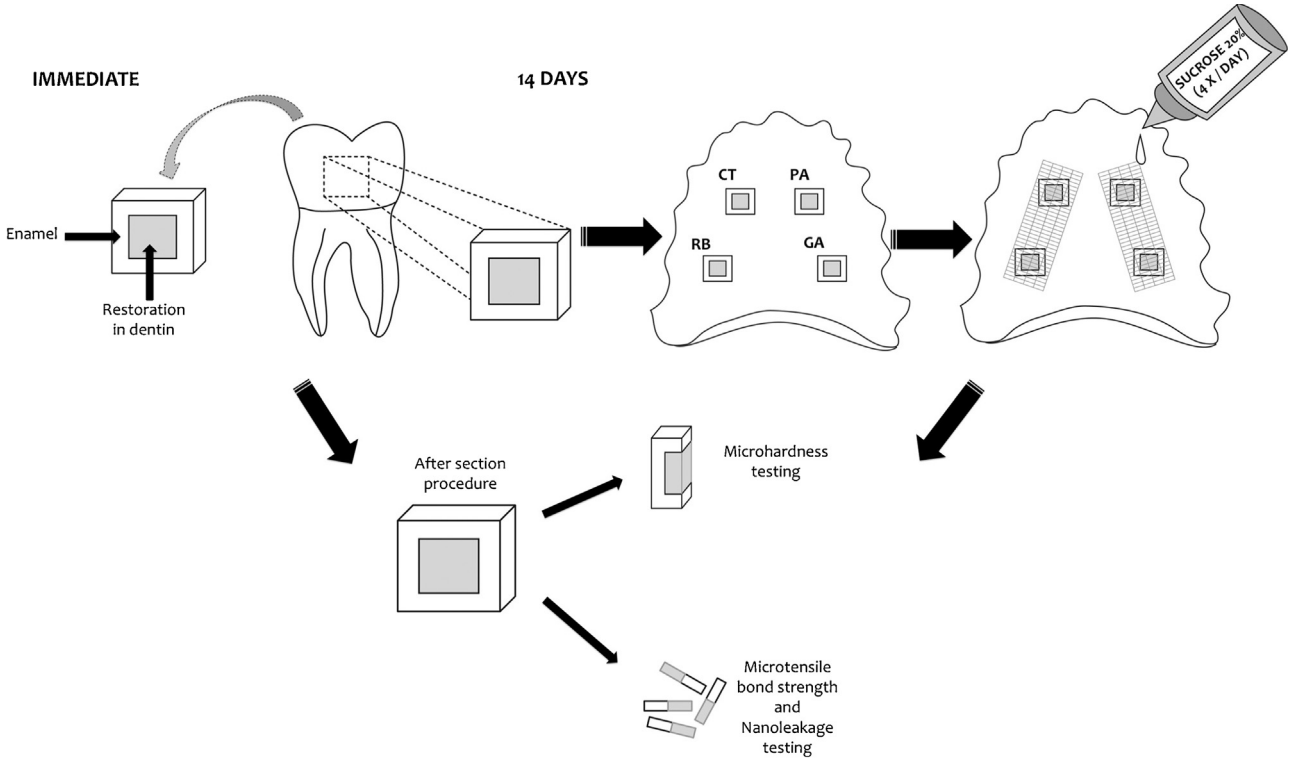

Fig. 1. Representation of experimental design used in this study. CT: control group; PA: proanthocyanidin group; RB: riboflavina group and GA: glutaraldehyde group.

2.2. Teeth preparation and bonding procedures

2.2.1. Microtensile bond strength (μTBS), microhardness and nanoleakage evaluation

Flat superficial enamel surfaces were exposed on each tooth after wet grinding the occlusal, buccal and lingual enamel on # 180-grit SiC paper. On each tooth, two dental blocks (6 × 6 × 3 mm) were obtained from the buccal and lingual surfaces. In each dental block, a standardized rectangular cavity was prepared (4 mm wide, 4 mm long, and 1.5 mm deep) with a carbide bur (# 330, KG Sorensen Ind. & Com. Ltda, Barueri, SP, Brazil), so that the axial wall was located in dentin and the thickness of enamel border ranged from 0.3 to 0.5 mm (Fig. 1). Teeth were then randomized by lottery and distributed within the different levels of the cross-linking agents ($n = 10$ specimens per group), so that the two cavities from the same tooth could be evaluated at 0 day and after 14 days in a paired design to reduce the intra-tooth variability.

All cavities were etched with 35% phosphoric acid gel for 15 s, water rinsed (30 s), air-dried (5 s) and kept slightly moist (details in Table 1). After that, the different collagen cross-linking primers (Table 1) were applied for 60 s under agitation. The riboflavin group was irradiated with UVA-light for 2 min (Philips, Hamburg, Germany; λ = 370 nm at 3 mW/cm^2) after the priming step with the cross-linking agent [17]. Following, the adhesive system was applied according to the manufacturer's instructions (Table 1) and the cavities were incrementally filled with a resin composite (Z250, 3M ESPE, Shade A3, batch number N549511) and with each increment light cured for 40 s. The light curing procedures were performed using a LED light curing unit (Radii Cal, SDI, Bayswater, Victoria, Australia) set at 1200 mW/cm^2. One of the cavity restorations from each tooth was tested immediately while the other one was placed in a palatal appliance for in situ challenge.

2.3. Palatal device preparation

For each volunteer, acrylic custom-made palatal devices were made with four sites (6.5 × 6.5 × 4 mm) in which the dental blocks from each group were positioned and fixed with wax (Fig. 1). To allow for plaque accumulation and for protection from mechanical disturbance, a plastic mesh was fixed to the acrylic resin, leaving a 1 mm space from the surface of the specimen [19,20]. Within each side of the palatal device, the positions of the specimens were randomly determined by lottery.

2.4. Intra-oral phase

During a 1-week lead-in period, and throughout the entire experimental phase, the volunteers brushed their teeth with a non-fluoride silica-based dentifrice formulation (Fleming, Ponta Grossa, PR, Brazil) prepared for this study. To provide a cariogenic challenge in all four specimens, each of the volunteers was instructed to remove the device and drip in a 20% sucrose solution (Fleming) onto all blocks four times a day (8 and 11 am and 3:30 and 7 pm) during 14 days [21,22]. Five minutes later, the device was re-inserted in the mouth.

All volunteers consumed city fluoridated water (0.6–0.8 mg F/l) and foods prepared with it. No restriction was made with regard to diet of the volunteers. They were instructed to wear the intraoral devices the whole time for 14 consecutive days, removing them only for dental hygiene and during the meals. The appliances were extra-orally brushed, except the restorations, and the volunteers were asked to brush carefully over the palatal area, to avoid disturbing the biofilm covering the mesh. They were asked to brush their teeth and appliance for up to 5 min. On the 15th day of the oral phase, around the 12 h after the last application of the

Table 1
Description of products, composition and application mode.

Product (Company)	Composition	Application mode
Scotchbond etchant (3 M ESPE, St. Paul, USA) batch number N261433	Phosphoric acid 35%, water and poly (vinyl alcohol).	Application on dentin surface. Wait 15 s. Rinsing for 10 s. Blot excess water using a cotton pellet
Single Bond Plus (3 M 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
Proanthocyanidin (PA) primer (Mega Natural Gold, Madera, USA) Batch number 05592502-01	Proanthocyanidin-Grape seed extract 6.5% weight, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured 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, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured applicator. After that, irradiation using UVA-light for 2 min (Philips, Hamburg, Germany; λ = 370 nm at 3 mW/cm^2). 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, deionized water.	After acid etching step, application for 60 s with gentle agitation using a fully satured 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 satured applicator. Gently air-drier for 5 s and kept slightly moist the surface

BisGMA: bisphenol a diglycidyl ether dimethacrylate, HEMA: 2-hydroxyethyl methacrylate. UDMA: diurethane dimethacrylate, EDMAB: ethyl 4-dimethyl aminobenzoate.

sucrose solution, the volunteers stopped wearing the intraoral devices. The restorations were then removed from the dental appliances and washed in tap water.

From this point on, both the restorations aged in the oral environment as well as the ones performed for immediate bonding were prepared in a similar manner. Each of the restorations was longitudinally sectioned to obtain a thin slice from the resin–dentin specimen for cross-sectional Knoop microhardness. The remaining of the restoration was used for resin–dentin μTBS and nanoleakage evaluation.

2.5. Cross-sectional microhardness

The thin restoration slice was embedded in acrylic resin with the cut surface being exposed, which receiving subsequent flattening and polishing with 1000, 1500, 2000, and 2500-grit SiC papers and 1 and 0.25 μm diamond pastes (Buehler, Lake Bluff, IL, USA) using a polish cloth. After ultrasonic cleaning, cross-sectional microhardness measurements were made in dentin with a microhardness tester (HMV-2, Shimadzu, Tokyo, Japan) equipped with a Knoop indenter (KHN) under a 15 g load for 5 s. Three lines of three indentations on each specimen were made, one line being 20 μm distant from the restoration margin and the other two lines being 100 and 200 μm distant, respectively. The indentations were made at the following depths from the enamel–dentin junction: 5, 15, and 25 μm [22].

2.6. Microtensile bond strength evaluation

The remaining part of the restoration was longitudinally sectioned in both "*x*" and "*y*" directions across the bonded interface with a diamond saw. This procedure was performed to obtain resin–dentin sticks with a cross-sectional area of approximately 0.8 mm^2. The cross-sectional area of each stick was measured with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) to the nearest 0.01 mm. 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 (Model 5565, Instron, Canton, OH, USA) at a crosshead speed of 0.5 mm/min. The failure modes were evaluated under stereomicroscopy at 100× magnification and classified as cohesive (within dentin or resin composite), adhesive (failure at resin/dentin interface), or adhesive/mixed (failure at resin/dentin interface with partial cohesive failure of the neighboring substrates). Approximately 15–20 resin-bonded sticks could be obtained per tooth including the pre-test failures. Usually 13–18 resin-bonded sticks were tested in this method.

2.7. Nanoleakage evaluation

Two resin-bonded sticks from each restoration, not used for microtensile testing, were randomly selected for nanoleakage evaluation. The sticks were immersed in 50 wt% ammoniacal silver nitrate solution in total darkness for 24 h. Thereafter, they were rinsed thoroughly in distilled water, and immersed in a photo-developing solution for 8 h under fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface. Specimens were polished using 1000-, 1500-, 2000- and 2500-grit SiC papers and 1 and 0.25 μm diamond pastes (Buehler Ltd., Lake Bluff, IL, USA) on polishing clothes. They were ultrasonically cleaned, air-dried, mounted on stubs and coated with evaporated 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 from each specimen. The first image was obtained in the center of the stick, while the further two were obtained 0.3 mm left and 0.3 mm right from the first picture. A total of six images were obtained per tooth at each period (3 images × 2 resin-bonded sticks). Thus, for each experimental condition, 60 images were evaluated per group (6 images × 10 teeth) [23]. A blinded author to the experimental conditions took the pictures. The relative percentage of silver nitrate uptake within the hybrid layer was measured in all pictures using the ImageTool 3.0 software (Department of Dental Diagnostic Science, University of Texas Health Science Center, San Antonio, USA).

2.8. Statistical analysis

The microhardness (KHN), μTBS (MPa) and nanoleakage (%) data from the same experimental unit (tooth) were averaged for statistical purposes at each storage time interval. The resin-bonded sticks with premature and cohesive failures were not included in the tooth mean due to their low frequency of occurrence in this experiment.

The Kolmogorov–Smirnov test was employed to assess whether the data from each test (microhardness, μTBS and nanoleakage) followed a normal distribution. Barlett's test was performed to determine if the assumption of equal variances was valid. After observing the data normality and equality of the variances, the data from microhardness (KHN), μTBS (MPa) and nanoleakage (%) were subjected to two-way repeated measures ANOVA (cross-linking agents and evaluation time) and Tukey's test for pair wise comparisons ($\alpha = 0.05$).

3. Results

3.1. Microhardness

Significant decreases in the microhardness values were observed after 14 days in a cariogenic oral environment for all groups, except the glutaraldehyde group (Table 2; $p = 0.002$). The proanthocyanidins group showed the highest microhardness values in the immediate period and after 14 days of cariogenic challenge. The control group (distilled water) showed the lowest microhardness values in both evaluation periods ($p = 0.002$).

3.2. Microtensile bond strength

The mean cross-sectional area was $0.8 \pm 0.12\ mm^2$ and no difference among groups was detected (data not shown; $p > 0.05$). Most of the failures were mixed (data not shown). None of the cross-linking primers affected the immediate μTBS which ranged from 34.1 to 39.2 MPa (Table 3; $p > 0.05$), however after 14 days of cariogenic challenge the untreated controls and the teeth treated with riboflavin showed significant reductions in bond strength, only the proanthocyanidins and gluraldehyde groups showed stable μTBS ($p < 0.05$).

Table 2
Means and standard deviations of the microhardness (KHN) for all experimental groups.

Group	Immediate	14 days
Control	25.2 ± 4.3 c	16.7 ± 2.2 c
Proanthocyanidin	48.0 ± 12.2 a	33.6 ± 11.5 abc
Riboflavin	44.2 ± 6.8 ab	27.1 ± 4.0 bc
Glutaraldehyde	25.9 ± 1.9 bc	28.5 ± 4.7 bc

Means identified with the same letter are statistically similar (Tukey's test; $p > 0.05$).

Table 3
Means and standard deviations of the μTBS (MPa) for all experimental groups.

Group	Immediate	After 14 days
Control	39.2 ± 3.4 Aa	29.1 ± 3.7 Bb
Proanthocyanidin	36.4 ± 6.5 Aa	36.7 ± 7.1 Ba
Riboflavin	37.6 ± 3.7 Aa	29.7 ± 4.4 Bb
Glutaraldehyde	34.1 ± 2.5 Aa	30.5 ± 1.2 Ba

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

3.3. Nanoleakage

Nanoleakage–free interfaces were never seen (Fig. 2). The nanoleakage % in immediate groups varied from 5.9 to 6.7% (Table 4). After 14 days, nanoleakage increased significantly after 14 days in cariogenic oral environment ($p < 0.001$), except for the proanthocyanidins group.

4. Discussion

The present study was the first that evaluated the application of collagen cross-linking agents in resin–dentin interfaces subjected to aging in the oral environment. So far, it was the first study that evaluated the incorporation of cross-linking agents in an *in situ* model.

After 14 days of cariogenic challenge in the oral environment one could observe significant reductions of the resin–dentin bond strength, and microhardness, as well as increase in the nanoleakage over that of the control group lead us to reject the null hypothesis. This means that the *in situ* model with a cariogenic challenge is a useful method to age the resin–dentin interfaces [22]. This is very relevant for further studies on dentin bonding, since this model better resembles the oral environment compared to *in vitro* studies and also provides clinically relevant information in a relatively short period of time [24].

The preservation of the resin–dentin interfaces from degradation was dependent on the type of cross-linking agents used. In the oral environment, the dynamic process of demineralization occurs with minerals being solubilized by organic acids produced by oral bacteria, and it is balanced by the buffering potential of the saliva that allows remineralization to occur [10,25]. The demineralization process may be followed by degradation of the exposed organic dentin matrix that mainly consists of type I collagen. If the balance between de- and remineralization is lost, pathological factors predominate [26]. This may induce several modifications of the dentin (reduction of mineral content, increase in micro- and nanoporosities due to changes in dentin collagen structure and distribution and noncollagenous protein), synergistically contributing to reductions in physical and mechanical properties of dentin [25].

The cariogenic environment promoted in our *in situ* model can explain the observed reductions in microhardness values after aging when compared to the immediate period. A higher rate of organic acids produced by oral bacteria induced more solubilization of mineral, which reduced the microhardness values. The control group exhibited the lowest microhardness mean as well as intense degradation of resin–dentin interface. This lower microhardness reflects lower mineral content in the intertubular dentin [27–29] and the biochemical and structural changes of the dentin matrix produced by these pH fluctuations that compromised the mechanical properties of the tissue [30].

Although the degradation of the hybrid layer has not yet been completely understood, it seems that the first stage of degradation involves the elution of the hydrophilic resins that infiltrated into dentin by water sorption and solubility phenomena [31]. Water sorption reduces the frictional forces between the polymer chains, which decreases the mechanical properties of the polymeric material due to swelling [32]. However, fourteen days is a very short period of time for to allow polymeric degradation. Perhaps, the demineralization of the enamel margins may have enhanced

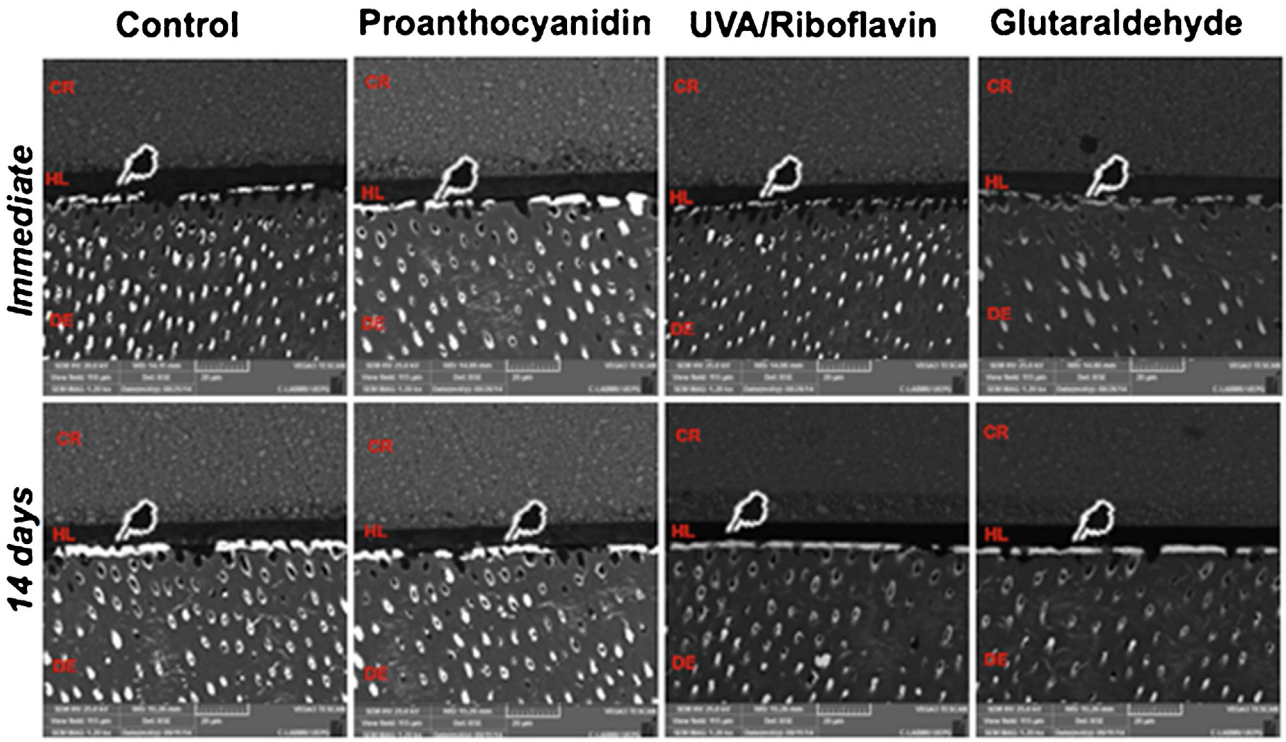

Fig. 2. Representative backscattered SEM photomicrographs of the resin-bonded interfaces for all experimental groups. After 14 days in cariogenic oral environment, the amount of silver penetration (*white hand*) increased significantly, except for the Proanthocyanidin group. CR: composite resin; HL: hybrid layer and DE: dentin.

Table 4
Means and standard deviations of nanoleakage (%) for all experimental groups.

Group	Immediate	After 14 days
Control	5.9 ± 2.4 Aa	13.8 ± 3.9 Bb
Proanthocyanidin	6.1 ± 1.9 Aa	9.3 ± 4.1 Ba
Riboflavin	6.3 ± 2.4 Aa	11.4 ± 1.4 Bb
Glutaraldehyde	6.7 ± 3.4 Aa	10.5 ± 2.0 Bb

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

gap formation at the interface and increased the flow of fluids and bacteria through the adhesive interface. This could have led to rapid undesirable consequences on the bond strength of the adhesive system.

It is known that the degradation of the organic component of the hybrid layer in resin–dentin interfaces is mediated by host-derived MMPs [9] and cysteine cathepsins [33,34]. The further demineralization of the collagen matrix in the acid environment might have led to the cleavage of prodomains of proteases, facilitating the functional activity of MMPs [9] in the cleavage of organic matrix components [9,35,36].

Curiously, both proanthocyanidin and riboflavin/UV treatment produced significant increase in microhardness values compared to the control group, and the glutaraldehyde group. Indeed, the exogenous collagen cross-linking agents can act as biomodifiers, resulting in dentin with higher mechanical strength, improved stability and reduced rate of biodegradation than those of natural tissues [13,14,17,37]. Glutaraldehyde is widely known as a fixation agent. Its interaction mechanism occurs through reactions from aldehyde groups containing in the glutaraldehyde with the ε-amino groups of lysyl (or hydroxysyl) residues on collagen [38,39]. However, this agent can promote rapid surface cross-linking of the tissue, generating a barrier that impedes its further diffusion into the tissue bulk [40,41]. This may have affected the depth of fixation, limiting its ability to increase the strength of the bulk of the dentin substrate.

Interestingly, glutaraldehyde-treated dentin did not exhibit reductions in microhardness after 14 days of cariogenic challenge. Actually, this could be expected due to a strong antibacterial activity [42–44] that could influence the bacterial growth, and consequently organic acid production and subsequent demineralization. Then, the synergic effect between the antibacterial activity (reducing indirectly the demineralization and exposed collagen matrix) and exogenous collagen cross-linking (increasing the stability of collagen fibrils and decreasing the MMPs potential activity), could explain the stable resin–dentin bond strength [45]. However, due to the fact that glutaraldehyde has been associated with a high cytotoxic potential in previous investigations [46–48] this agent seems to be the least acceptable cross-linking agent for incorporation into the bonding protocol.

Although reductions of the microhardness values were observed for the proanthocyanidins group after 14 days of cariogenic challenge, the microhardness values remained above that untreated control. This fact can be explained by the very effective chemical interaction of proanthocyanidins with collagen matrices. The proanthocyanidins can interact with dentin collagen through four distinct mechanisms. Ionic interaction, hydrogen and hydrophobic bonding interactions along with covalent bonding produced with proline-rich proteins [13,49] are listed as the possible methods of interaction of proanthocyanidins with the organic substrate. Therefore, their ability to establish strong cross-linking with the proline-rich proteins, like collagen, demonstrates an extremely high affinity with this substrate [13,50].

The stabilization of collagen matrices due to increases of the biomechanical and biochemical properties of collagen [14,15,37,51] and the interaction of proanthocyanidins with non-collagenous proteins could have increased the ability for mineral nucleation [13], which would explain the high bond strength after 14 days of cariogenic challenge among the groups. Additionally, the inactivation of MMPs by proanthocyanidins made the resin–dentin interface more resistant toward the endogenous proteolytic activity [37,52,53].

The riboflavin (vitamin B2) associated with high energy of UVA-light (365 nm) generates free oxygen radicals. The reactive oxygen species can induce the formation of new covalent and strong cross-links within collagen [54]. This occurs through binding of the functional hydroxyl groups in riboflavin to proline and/or lysine in the collagen [55]. Therefore the increase of the dentin properties might have contributed to the increase of the microhardness in the immediate period.

In previous studies, riboflavin showed promising results for preservation of resin–dentin bonding interfaces. This was attributed to improvements of the collagen properties and its inhibitory potential for MMPs [17,56]. Unfortunately, this was not observed in the present study. Riboflavin depends on a physical method (UVA-light) to induce the exogenous cross-links in dental collagen. However, studies in the ophthalmology area report that the UVA irradiation is only absorbed into the superficial layers and therefore cross-linking does not occur in deeper areas [57,58], which may explain the lower performance in the present study. On the other hand, PA is a chemical agent with a good diffusion potential in thick tissues [46]. Thus, we speculate that the reason riboflavin-treated groups show a similar result as the control groups, where after solubilization of the mineral by cariogenic oral environment, the adjacent exposed collagen fibrils were prone to endogenous proteolytic activity by MMPs and cysteine cathepsins [25], contributing to the observable low resin–dentin bond strengths.

Nanoleakage was not affected by the exogenous collagen cross-linking in the immediate groups. Actually, it was not expected that the application of aqueous solutions of the cross-linkers would produce nanoleakage-free interfaces, since the protocols herein tested would not alter the hydrophilic nature of the adhesive, water/solvent evaporation or produce less permeable adhesive interfaces, which are all important parameters for nanoleakage [59–62]. However, interestingly, the application of PA produced resin–dentin interfaces less prone to nanoleakage after 14 days of cariogenic challenge. Apart from the cross-linking, the proanthocyanidins lead to dehydration of the collagen fibrils, with decrease in swelling and water absorption [52,63,64]. This can minimize the risk of collagen network from collapse resulted from air-drying [13,63], which would yield better adhesive infiltration and reduce the spaces for silver nitrate deposition in the nanoleakage test.

5. Conclusion

Although in situ model did not substitute the randomized clinical trials, in the present study it seems to be a suitable short-term methodology to investigate the degradation of the resin–dentin bonds. Under the protocol of 14 days of cariogenic oral environment, the use of proanthocyanidins as a primer after acid-etching produced stable resin–dentin interfaces and it is worth further clinical investigation. Glutaraldehyde was effective for stabilization of resin–dentin interfaces but its clinical use is of concern due to the cytotoxicity potential.

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