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In vitro biological and adhesive properties of universal adhesive systems on sound and caries-affected dentine: 18 months

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ABSTRACT

The present study aims to evaluate antimicrobial activity, cytotoxicity, as well as resin-dentine microtensile bond strength, nanoleakage and degree of conversion of six universal adhesives, on sound and caries-affected dentine after 18 months. The adhesives Prime&Bond Active (PBA), Scotchbond Universal (SBU), Tetric N-Bond Universal (TNU), Ambar Universal (AMU), Clearfil Universal Bond Quick (CUQ) and One Coat 7 Universal (OCU) were used. Antimicrobial activity was evaluated against Streptococcus mutans. For cytotoxicity, methyltetrazolium assay was used, after 24 h exposure of osteoblast-like cells line to the adhesive's dilution of 1, 0.1, and 0.01 v/v %. After, the adhesives were applied in etch-and-rinse or self-etch strategies on sound or caries-affected dentine surfaces, resin composites restorations were constructed. Then, the specimens were sectioned to obtain sticks to be evaluated in microtensile bond strength and nanoleakage after 24 h and 18 months, and degree of conversion after 24 h. ANOVA and Tukey's test were applied ($\alpha = 0.05$). For antimicrobial activity, CUQ showed higher values than all adhesives. For cytotoxicity, the PBA, AMU, CUQ and OCU adhesives presented cytotoxicity in different dilutions. For microtensile bond strength, OCU presented the lowest values, regardless of time, dentine or strategy. For nanoleakage, differences were observed among adhesives depending on time, dentine or strategy. For degree of conversion, TNU presented the highest values, while PBA and OCU presented the lowest values. Worst values of microtensile bond strength and nanoleakage were always obtained in caries-affected dentine after 18 months. Thus, not all universal adhesives behave the same in terms of antimicrobial activity and cytotoxicity. However, the majority showed worst results when applied in caries-affected dentine, mainly after 18 months.

Clinical relevance. Universal adhesive systems may have differences in their biological and adhesive properties, both on sound, but mainly in caries-affected dentine after 18 months.

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1. Introduction

Clinicians are constantly exposed to making decisions about the treatment of deep caries. Currently, partial caries excavation strategy is presented as more minimally invasive than total caries excavation when deep dentine carious lesions are treated, to preserve dental structure avoiding the risk of pulp injuries [1,2]. Thus, the deeper but remineralizable zone of caries-affected dentine is preserved, being the superficial caries-infected dentine removed [3–5].

Caries-affected dentine presents characteristics such as low mineral content, and an increased porosity [6]. However, the presence of an altered pattern of organic matrix of the collagen fibrils and non-collagenous proteins is the most challenging characteristic, since it determines a higher humidity, determining a decrease in the mechanical properties of the dentine [7,8]. Thus, hybrid layers created on caries-affected dentine, owing to higher enzymatic activation in response to pH oscillations during caries progression [9,10], present a faster degradation in comparison with hybrid layers created on sound dentine [11–14].

On the other hand, the production of lactic acid by *Streptococcus mutans* (S. mutans) during the caries process and the interaction of this acid with dentine, produces a reduction in its mechanical properties, especially in dentine fracture resistance to chewing forces [15]. In this sense, since most of the studies are focused on the adhesive behavior on sound dentine, and the information of adhesion to caries-affected dentine is very limited, it is important to investigate the adhesive process on caries-affected dentine, which is a challenging and clinically relevant substrate in dental practice [16,17].

Moreover, to provide a more simplified alternative of adhesion to dentine, universal adhesive systems were introduced in the dental market [18], whose advantage is the ability to be used in a one-step self-etch or two-step etch-and-rinse application mode. Thus, universal adhesives are currently widely used due to the wide facility of use as well as great versatility by the clinicians. Unfortunately, little is known about cytotoxicity and antibacterial capacity of these universal adhesives, and on the other hand, their bonding performance to caries-affected dentine in long term water storage. Therefore, studies that evaluate cytotoxic and antibacterial properties, as well as bonding properties on resin-caries-affected dentine interface over time are needed.

Accordingly, the aim of the present study was to evaluate the antimicrobial activity and cytotoxicity, as well as resin-dentine microtensile bond strength, nanoleakage and *in situ* degree of conversion on sound and caries-affected dentine of several universal adhesives, after 24 h and after 18 months of water storage. The null hypotheses to be tested were: (1) all universal adhesives present cytotoxicity, (2) no adhesive has antimicrobial activity, (3) microtensile bond strength, nanoleakage and *in situ* degree of conversion do not change when the adhesives are applied on sound or caries-affected dentine, (4) microtensile bond strength and nanoleakage do not change between 24 h and 18 months of water storage.

2. Materials and methods

Six universal adhesive systems were used in this study: Prime&Bond Active (PBA; Dentsply-Sirona, Konstanz, Baden-Württemberg, Germany), Scotchbond Universal (SBU; 3 M Oral Care, St. Paul, Minnesota, USA), Tetric N-Bond Universal (TNU; Ivoclar Vivadent, Schaan, Liechnstein), Ambar Universal (AMU; FGM Prod. Odont. Ltda, Joinville, SC, Brazil), Clearfil Universal Bond Quick (CUQ; Kuraray Noritake Dental Inc, Kita-Ku, Osaka, Japan) and One Coat 7 Universal (OCU; Coltene/Whaledent AG, Altstätten, Switzerland). The properties evaluated were antimicrobial activity (AMA) and cytotoxicity (CTX), as well as resin-dentine microtensile bond strength (μTBS), nanoleakage (NL) and *in situ* degree of conversion (DC) on sound and caries-affected dentine. The batch number and composition of six adhesives tested are listed in Table 1.

2.1. In vitro antimicrobial activity

Pure culture was obtained by culturing *S. mutans* ATCC 25175 in brain heart infusion broth (BHI, Difco Laboratories, Detroit, MI, USA) for 72 h at 37°C [19]. Then, 100 μL of the bacterial suspension was swabbed onto BHI to create the lawn [20,21]. In order to measure *S. mutans* sensitivity to the evaluated universal adhesives, disk diffusion method was used. Filter paper discs of 6 mm diameter were prepared from Whatman filter paper No. 1 (Sigma–Aldrich, Munich, Germany), placed in a Petri dish and sterilized in a hot air oven at 160 °C for 2 h. After that, the discs were moistened with 20 μL of each universal adhesive, evaporating the solvent and placed immediately over the plates. The plates were incubated in an anaerobic jar (5% CO_2) for 48 h at 37 °C. With a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) the inhibition zones (mm) were measured to the nearest 0.1 mm. Three samples of each universal adhesive were evaluated [22].

2.2. In vitro cytotoxicity

2.2.1. Cell culture

To determine cytotoxicity of universal adhesives, osteoblast-like cell line Saos-2 (ATCC® HTB-85™) was used. The osteoblast-like cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Dreieich, Germany) supplemented with 10% Fetal Bovine Serum (FBS; BI Biological Industries) and 1% penicillin-streptomycin solution (Thermo Fisher Scientific), in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C [23]. Medium was changed every 2–3 days. After reaching confluence, the cells were washed with Dulbecco's phosphate buffered saline (PBS; Thermo Fisher Scientific) and detached with trypsin-EDTA 0.05% for 5 min (Thermo Fisher Scientific). A cell fraction was stained with a trypan blue solution (Sigma–Aldrich, Munich, Germany) and counted in a hemocytometer, for plating the intended cell number.

2.2.2. Cell stimulation

All stimulation experiments were performed in 96-well plates in triplicate, and repeated at least three times. After that, 10,000 cells per well were seeded, with a 70% confluence 24 h after seeding. Cells were incubated with three different dilutions (1, 0.1, and 0.01 v/v%) of each universal adhesive in 100 μl cell culture medium for 24 h at 37 °C [23]. Incubation with plain culture medium was used as 100% viability control, while 20% methanol was used as an apoptosis control.

2.2.3. Assessment of cytotoxicity

Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific) was used for cell cytotoxicity determination. After stimulation for 24 h, the medium was removed and replaced with 100 μL of fresh culture medium. Ten μL of 12 mM MTT stock solution were added to each well and incubated at 37 °C for 2 h. After that, 100 μL of SDS-HCl solution were added to each well and thoroughly mixed by pipetting. The microplate was incubated at 37 °C for 4 h, followed by the measurement of absorbance at 490 nm with a 670 nm-correction wavelength in a microplate reader. Cell viability was calculated and normalized to control experiments (=100%) [24].

Table 1
Universal adhesive system (batch number), composition ([a]) and application mode.

Universal adhesive system (batch number) and pH	Composition ([a])	Etch-and-rinse mode	Self-etch mode
Prime&Bond Active (**PBA** - Dentsply-Sirona, Konstanz, Baden-Württemberg, Germany) (1,703,000,452) pH = ~2.5	Phosphoric acid modified acrylate resin, multifunctional acrylate, bifunctional acrylate, acidic acrylate, isopropanol, water, initiator, stabilizer (10-MDP and PENTA)	1. Apply phosphoric acid for 15 s. 2. Remove gel with vigorous water spray and rinse conditioned areas thoroughly for 15 s. 3. Remove rinsing water completely by blowing gently with an air syringe or blot dry. Do not desiccate dentine. 4. Apply adhesive to completely wet the surfaces to be treated. Avoid pooling of the adhesive. 5. Keep the adhesive slightly agitated for 20 s. 6. Disperse adhesive and remove solvent with clean, dry air from an air-water syringe. Treat every surface with a moderate air flow for at least 5 s until a glossy and uniform layer results. 7. Light cure for 10 s at 1200 mW/cm^2	1. Apply adhesive to completely wet the surfaces to be treated. Avoid pooling of the adhesive. 2. Keep the adhesive slightly agitated for 20 s. 3. Disperse adhesive and remove solvent with clean, dry air from an air-water syringe. Treat every surface with a moderate air flow for at least 5 s until a glossy and uniform layer results. 4. Light cure for 10 s at 1200 mW/cm^2
Scotchbond Universal (**SBU** - 3 M Oral Care, St. Paul, Minnesota, USA) (691,954) pH = 2.7	10-MDP, 2-HEMA, BisGMA, DCDMA, MPTMS, VP-copolymer, fumed silica, ethanol, water, photoinitiators	1. Apply etchant for 15 s. 2. Rinse thoroughly for 15 s. 3. Blot excess water. 4. Apply actively the adhesive to the entire surface with a microbrush for 20 s. 5. Direct a gentle stream of air over the liquid for about 5 s until it no longer moves and the solvent is evaporated completely. 6. Light-cure for 10 s at 1200 mW/cm^2.	1. Apply actively the adhesive to the entire surface with a microbrush for 20 s. 2. Direct a gentle stream of air over the liquid for about 5 s until it no longer moves and the solvent is evaporated completely. 3. Light-cure for 10 s at 1200 mW/cm^2.
Tetric N-Bond Universal (**TNU** - Ivoclar Vivadent, Schaan, Liechnstein) (W15554) pH = 2.5–3.0	Ethanol, phosphonic acid acrylate, Bis-GMA, HEMA, UDMA, diphenyl (2,4,6-trimethylbenzoyl) phosphine oxide	1. Apply phosphoric acid gel onto the dentine for 15 s. 2. Rinse thoroughly with a vigorous stream of water for at least 5 s. 3. Gently air dry, keep dentine visible moist. 4. Scrub one coat of adhesive for 20 s 5. Gently air thin for 5 s. 6. Light-cure for 10 s at 1200 mW/cm^2.	1. Scrub one coat of adhesive for 20 s 2. Gently air thin for 5 s. 3. Light-cure for 10 s at 1200 mW/cm^2.
Ambar Universal (**AMU** - FGM Prod. Odontológicos, Joinville, Santa Catarina, Brazil) (310,516) pH = 2.6–3.0	10-MDP, HEMA, UDMA, methacrylic monomers, photoinitiators, coinitiators, stabilizers, silica nanoparticles and ethanol	1. Apply phosphoric acid for 15 s. 2. Wash the surface with plenty of water and dry the cavity so that the dentine does not get dehydrated, but without the accumulation of water on the surface. 3. Apply a first layer vigorously rubbing the adhesive with the micro applicator for 10 s. 4. Next, apply a second layer of adhesive for 10 s, spreading the product. 5. Evaporate excess solvent by thoroughly air-drying with an air syringe for 10 s. 6. Light cure for 10 s at 1200 mW/cm^2.	1. Apply a first layer vigorously rubbing the adhesive with the micro applicator for 10 s. 2. Next, apply a second layer of adhesive for 10 s, spreading the product. 3. Evaporate excess solvent by thoroughly air-drying with an air syringe for 10 s. 4. Light cure for 10 s at 1200 mW/cm^2.
Clearfil Universal Bond *Quick* (**CUQ** - Kuraray Noritake Dental Inc, Kita-Ku, Osaka, Japan) (CD0011) pH = 2.3	10-MDP, 2-HEMA, BisGMA, hydrophilic amide methacrylate, MPTMS, NaF, colloidal silica, photoinitiators	1. Apply phosphoric acid for 10 s. 2. Rinse and dry the surface. 3. Apply the adhesive with a rubbing motion on the surface with the applicator brush. No waiting time is required. 4. Dry the entire cavity wall sufficiently by blowing mild air for more than 5 s until the adhesive does not move. 5. Light cure for 10 s at 1200 mW/cm^2.	1. Apply the adhesive with a rubbing motion on the surface with the applicator brush. No waiting time is required. 2. Dry the entire cavity wall sufficiently by blowing mild air for more than 5 s until the adhesive does not move. 3. Light cure for 10 s at 1200 mW/cm^2.
One Coat 7 Universal (**OCU** - Coltène Whaledent, AG, Altstätten, Switzerland) (H82402) pH = 2.8	10-MDP, HEMA, UDMA, methacrylated polyacrylic acid, other methacrylates, photoinitiators, ethanol, water	1. Apply etchant for 15 s. 2. Rinse for 10 s. Air dry to remove excess water. 3. Dispense a drop of adhesive and rub it onto the dentine with a disposable dental brush for 20 s. 4. Blow gently with oil-free compressed air for 5 s. 5. Light cure for 10 s at 1200 mW/cm^2.	1. Dispense a drop of adhesive and rub it onto the dentine with a disposable dental brush for 20 s. 2. Blow gently with oil-free compressed air for 5 s. 3. Light cure for 10 s at 1200 mW/cm^2.

[a] 10-MDP = methacryloyloxydecyl dihydrogen phosphate; PENTA = dipentaerythritol penta acrylate monophosphate; HEMA = 2-hydroxyethyl methacrylate; UDMA = urethane dimethacrylate; MPTMS = γ-methacryloxypropyl trimethoxysilane; DCDMA = Decamethylene dimethacrylate, VP-copolymer = Methacrylate-modified polyalkenoic acid copolymer.

2.3. Teeth preparation

This study was approved by the Institutional Ethics Committee from the State University of Ponta Grossa (Ponta Grossa/PR/Brazil;State protocol 2.399.496). Two hundred and sixteen extracted human third molars, caries free, collected from patients with age ranging from 18 to 30 years old were used. Teeth were disinfected in 0.5% chloramine, stored in distilled water and used within 3 months after extraction. In order to expose a flat dentine surface on each tooth, the occlusal was wet grinding with 180-grit SiC paper. The exposed dentine surfaces were

polished with 600-grit SiC paper for 60 s to standardize the smear layer.

2.3.1. Microbiological caries induction

Half of the teeth (n = 108) were sterilized and each tooth was individually immersed in a falcon tube containing an artificial caries solution. The solution contained 9.25 g of brain heart infusion culture supplemented with 1.25 g of yeast extract, 5.0 g of sucrose, in 250 mL of distilled water and 100 μL of primary culture of *S. mutans* (ATCC 25175), with the pH around 4.0. The specimens were incubated in an anaerobic jar (5% CO_2) at 37 °C. Every 48 h, the specimens were transferred to another falcon tube containing a new artificial caries solution. After 14 days, the specimens were sterilized and washed in deionized water [25]. After that, the dentine surface was exposed by grounding the surrounding enamel of all teeth with a diamond bur n° 4137 (KG Sorensen, Barueri, SP, Brazil). Then, the occlusal dentine surfaces were further polished with 600-grit SiC paper for 30 s to standardize the smear layer and simulated caries-affected dentine. Caries-affected dentine substrate was confirmed in a pilot study (data not shown) by a microhardness test, obtaining similar values to those shown in a previous study by Marquezan et al. [25], and lower values than sound dentine.

2.4. Bonding procedures

The universal adhesives were applied in etch-and-rinse (ER) or self-etch (SE) mode, as per manufacturer' instructions (Table 1). In ER mode, the dentine surface was acid-etched with 37% phosphoric acid (Condac, FGM Prod. Odont. Ltda, Joinville, SC, Brazil). After that, 3 resin composite increments (Opallis, FGM Prod. Odont. Ltda, Joinville, SC, Brazil) of 1.0 mm of thickness each were individually applied and light activated for 40 s with a LED light source at 1000 mW/cm^2 (VALO, Ultradent Products, South Jordan, UT, USA) on the bonded surfaces. A single operator carried out all bonding procedures. The bonded teeth were stored in distilled water at 37 °C for 24 h. Nine teeth were used for each experimental group.

After that, 96 teeth (48 for sound dentine and 48 for caries-affected dentine) were longitudinally sectioned in "x" direction across the bonded interface with a diamond saw in a cutting machine (IsoMet 1000; Buehler, Lake Bluff, USA), under water cooling at 300 rpm to obtain resin-dentine slices with a thickness of approximately 1.2 mm^2 for nanoleakage and *in situ* degree of conversion test. On the other hand, 120 teeth (60 for sound dentine and 60 for caries-affected dentine) were longitudinally sectioned in both "x'' and "y'' directions across the bonded interface to obtain resin-dentine bonded sticks with a cross-sectional area of approximately 0.8 mm^2. The number of premature failures (PF) per tooth during specimen preparation was recorded. The cross-sectional area of each resin-dentine bonded stick was measured with the digital caliper to the nearest 0.01 mm and recorded for subsequent calculation of the microtensile bond strength values (Absolute Digimatic, Mitutoyo, Tokyo, Japan).

2.5. Microtensile bond strength

Each resin-dentine bonded stick was attached to a modified device for microtensile bond strength test with cyanoacrylate resin (IC-Gel, bSi Inc., Atascadero, CA, USA) and subjected to a tensile force in a universal testing machine (Kratos, São Paulo, SP, Brazil) at 0.5 mm/min. The failure mode was evaluated under an optical microscope (SZH-131, Olympus; Tokyo, Japan) at 40x and classified as cohesive in dentine (failure exclusive within cohesive dentine – CD); cohesive in resin (failure exclusive within cohesive resin – CR); adhesive (failure at resin/dentine interface – A), or mixed (failure at resin/dentine interface that included cohesive failure of the neighboring substrates, M). The number of premature failures (PF) was recorded, but it was not included in the average mean bond strength.

2.6. Nanoleakage

The resin-dentine bonded slices were immersed in 50 wt% ammoniacal silver nitrate solution in total darkness for 24 h, rinsed with distilled water, and immersed in photo developing solution for 8 h under fluorescent light. After that, specimens were placed on metallic stubs, polished with 1000-, 1500-, 2000- and 2500-grit SiC paper and 1 and 0.25 μm diamond paste (Buehler Ltd., Lake Bluff, IL, USA), ultrasonically cleaned for 8 min, air dried and gold sputter coated (MED 010, Balzers Union, Balzers, Liechtenstein). Then, the resin-dentine interfaces were observed in a scanning electron microscope in the backscattered mode at 15 kV (VEGA 3 TESCAN, Shimadzu, Tokyo, Japan).

Three pictures were taken of each specimen, one picture in the center of the resin-dentine slice and the other two pictures 0.3 mm to the left and right of the first one. As two resin-dentine slices per tooth were evaluated and a total of five teeth were used for each experimental condition, a total of 30 images were evaluated per group. A technician who was blinded to the experimental conditions under evaluation took them all. The relative percentage of nanoleakage within the adhesive and hybrid layer areas was measured in all pictures using the public domain Image J software [26].

2.7. In situ degree of conversion within adhesive/hybrid layers

All resin-dentine bonded slices selected for this test were wet polished using 1500; 2000; 2500 and 4000-grit SiC paper for 30 s each, ultrasonically cleaned for 10 min and positioned into micro-Raman spectrometer (Bruker Optik GmbH, Ettlingen, Baden-Württemberg, Germany), that was calibrated for zero and then for coefficient values using a silicon specimen. Specimens were analyzed using the following parameters: 20-mW Neon laser with 532-nm wavelength, spatial resolution of ≈3 μm, spectral resolution ≈5 cm^{-1}, accumulation time of 30 s with 6 co-additions, and magnification of 100× (Olympus UK, London, UK) to beam diameter of ≈1 μm. The spectra were taken at the resin-dentine bonded interface, in the middle of the hybrid layer within the intertubular dentine, at three different sites for each specimen and the values averaged for statistical purposes. Spectra of uncured universal adhesives were taken as reference. Post-processing of spectra was performed using the dedicated Opus Spectroscopy Software version 6.5 (Bruker Optik GmbH, Ettlingen, Baden-Württemberg, Germany). The ratio of the double-bond content of monomer to polymer in the adhesive was calculated according to the following formula: DC (%) = $(1 - R_{cured}/R_{uncured})$ x 100, where R is the ratio of aliphatic and aromatic peak areas at 1639 cm^{-1} and 1609 cm^{-1} in cured and uncured adhesives.

2.8. Statistical analysis

The data was analyzed using the Kolmogorov-Smirnov test to assess normal distribution, as well as the Bartlett's test for equality of variances to determine if the assumption of equal variances was valid. After confirming the normality of the data distribution and the equality of the variances, data for the cytotoxicity (%) was subjected to a one-way ANOVA (adhesive) and Tukey's test for each dilution evaluated and Dunn's test for comparison with the control group. Data for microbiological test (mm) were subjected to a one-way ANOVA (adhesive). The μTBS (MPa) and nanoleakage (%) data were subjected to four-way ANOVA (adhesive vs. dentine type vs. strategy vs. storage time). The *in situ* degree of conversion (%) data was submitted to three-way ANOVA (adhesive vs. dentine type vs. strategy). After that, Tukey's post hoc test was used for pair-wise comparisons (α = 0.05) using the Statistica for Windows software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. In vitro antimicrobial activity

The results of antimicrobial activity against *S. mutans* for the different universal adhesive systems are shown in Table 2. CUQ showed antibacterial properties against *S. mutans* significantly higher than all other universal adhesives (Table 2; $p < 0.01$).

3.2. In vitro cytotoxicity

For dilution of 1 v/v%, no differences were observed among all adhesive systems (Table 2; $p > 0.05$). For dilution of 0.1 v/v%, PBA and AMU showed significantly high cytotoxicity against cells (Table 2; $p < 0.01$). For dilution of 0.01 v/v%, CUQ showed significantly higher cytotoxicity than SBU, TNU and OCU (Table 2; $p < 0.05$). When compared with the viability control (culture medium), dilution of 1 v/v % showed cytotoxicity in all adhesive groups (Table 2; $p < 0.05$). For dilution of 0.1 v/v%, PBA, AMU, CUQ and OCU showed cytotoxicity when compared with viability control (Table 2; $p < 0.05$). On the other hand, dilution of 0.01 v/v% showed no differences among all adhesive systems and viability control (Table 2; $p > 0.05$).

3.3. Microtensile bond strength testing (μTBS)

For all comparisons, sound dentine showed significantly higher μTBS values than caries-affected dentine (Table 3; $p < 0.01$). Also, significantly lower μTBS values were observed when 18 months of water storage were compared with immediate time (Table 3; $p < 0.01$). On the other hand, when self-etch and etch-and-rinse strategies were compared, no significant differences between them were observed (Table 3; $p > 0.05$).

Regarding adhesive systems, on sound dentine, higher μTBS values were observed for PBA, SBU, TNU and AMU, when compared to OCU (Table 3; $p < 0.01$; both times and strategies). Actually, OCU showed significantly lower μTBS than the majority of all other universal adhesives, mainly after 18 months of water storage in both strategies (Table 3; $p < 0.01$).

On caries-affected dentine, in the immediate time, TNU and PBA showed significantly higher μTBS than SBU, CUQ and OCU (Table 3; $p < 0.01$). Although the decreasing of the μTBS values for all adhesives and strategies was observed after 18 months of water storage, TNU and AMU showed significantly higher μTBS values than CUQ and OCU after 18 months (Table 3; $p < 0.01$).

3.4. Nanoleakage evaluation

For most of the comparisons, caries-affected dentine showed significantly higher nanoleakage values than sound dentine (Fig. 2 and Table 4; $p < 0.01$). Also, significantly higher nanoleakage values were observed for all adhesives when 18 months of water storage were compared with immediate time (Figs. 1 and 2 and Table 4; $p < 0.01$). On the other hand, when self-etch and etch-and-rinse strategies were compared, no significant differences between them were observed (Figs. 1 and 2 and Table 4; $p > 0.05$).

Regarding adhesive systems, on sound dentine, higher nanoleakage values were observed for PBA when compared with most of the adhesives in the immediate time and after 18 months of storage (Fig. 1 and Table 4; $p < 0.01$). On caries-affected dentine in the immediate time, AMU showed significantly lower nanoleakage than SBU, CUQ and OCU in both strategies (Fig. 2 and Table 4; $p < 0.01$). Despite the increase of the nanoleakage values for all adhesives and strategies, after 18 month of water storage, PBA and CUQ showed significantly higher nanoleakage values than TNU and SBU (Fig. 2 and Table 4; $p < 0.01$).

3.5. In situ degree of conversion within adhesive/hybrid layers

The results of *in situ* degree of conversion after 24 h are shown in Table 5. Usually, sound dentine showed significantly higher values of *in situ* degree of conversion than caries-affected dentine (Table 5; $p < 0.01$). No significant differences between self-etch and etch-and-rinse strategies were observed (Table 5; $p > 0.05$). On sound and caries-affected dentine, PBA and OCU showed significantly lower *in situ* degree of conversion than other universal adhesives (Table 5; $p < 0.05$).

4. Discussion

One of the objectives of this study was to evaluate the antimicrobial effect against *S. mutans* of several universal adhesive systems. As mentioned in the ecological plaque hypothesis, *S. mutans* is a gram-positive bacteria, that can be found in most caries lesions, presenting a preponderant role in pathogenesis of carious process, because several virulence factors that are related to its cariogenic capacity, such as adhesion and biofilm formation properties, and the possibility of producing acids [27]. Thus, the elimination or at least reduction of this bacteria is often related to preventing and stopping the progression of the carious process [28]. In this context, an adhesive system with antimicrobial properties would be a comparative advantage and it is one of the great challenges of adhesive restorative dentistry today [29].

In the current study it was observed that CUQ showed antibacterial properties against *S. mutans* significantly higher than all other universal adhesives. This could be explained by the presence of sodium fluoride (NaF) in the composition of CUQ, which has a widely proven antibacterial effect, decreasing the overall gene expression level in *S. mutans*, and inducing the expression of genes involved in some metabolic transporters which implies specific cellular internalization of sugars [30]. The interesting thing is that, despite the fact that CUQ was applied on 180 μm thick filter paper discs, which could have hindered the diffusion of sodium fluoride, it was possible to observe a great antibacterial effect. In this sense, this result may be due to the thinness of the adhesive layer when the universal adhesive system is applied on the dentine surface, could reflect in a similar way what happens in clinical

Table 2
Means and standard deviation for bacterial inhibition halo sizes (mm) against *S. mutans* ([a]), as well as means and standard error of viable cells (%) obtained in each experimental condition, in osteoblast-like cells ([b],[c]).

Adhesive	Bacterial inhibition (mm)[a]	Citotoxicity for dilutions (%)[b],[c]		
		1	0.1	0.01
Prime&Bond Active	10.41 ± 0.06[b]	1.86 ± 0.58 A ≠[b]	30.22 ± 3.82 e ≠	96.29 ± 6.16 [AB] =
Scotchbond Universal	10.21 ± 0.85[b]	0.66 ± 0.16 A ≠	102.52 ± 4.88 ab =	109.95 ± 3.66 [A] =
Tetric N-Bond Universal	7.62 ± 0.11 [cd]	0.96 ± 0.05 A ≠	113.18 ± 2.65 a =	114.55 ± 2.84 [A] =
Ambar Universal	8.42 ± 0.22 [cd]	1.40 ± 0.47 A ≠	34.94 ± 3.61 de ≠	102.76 ± 6.30 [AB] =
Clearfil Universal Bond *Quick*	25.31 ± 2.46[a]	2.21 ± 0.73 A ≠	71.90 ± 5.46 c ≠	85.31 ± 7.84 [B] =
One Coat 7 Universal	9.49 ± 0.38 [bc]	1.73 ± 0.30 A ≠	65.01 ± 4.42 cd ≠	110.39 ± 3.83 [A] =

[a] Means identified with the same superscript lower case letter are statistically similar. (Tukey's test, p ≥ 0.05).
[b] Comparisons are valid only within dilution. Means identified with the same capital, lowercase or superscript capital letter are statistically similar. (Tukey's test, p ≥ 0.05).
[c] Means identified with equals sign (=) are statistically similar with viability control (100%). (Dunn's test, p ≥ 0.05).

Table 3
Means and standard deviations of microtensile bond strength (MPa) obtained in each experimental condition, after immediate (24 h) and 18 months of water storage ([a]).

Adhesive	Sound dentine				Caries-affected dentine			
	Immediate		18-month		Immediate		18-month	
	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch
Prime&Bond Active	50.1 ± 4.9 A	43.7 ± 2.8 AB	29.4 ± 2.8 D	29.5 ± 2.6 D	25.1 ± 2.2 D	23.3 ± 1.1 D	17.7 ± 1.5 EF	18.1 ± 1.8 EF
Scotchbond Universal	44.7 ± 2.4 AB	39.3 ± 2.4 BC	28.2 ± 4.8 D	28.6 ± 1.8 D	21.3 ± 1.2 E	19.3 ± 2.3 E	16.0 ± 3.5 EF	15.5 ± 2.6 EF
Tetric N-Bond Universal	50.5 ± 5.9 A	45.8 ± 0.7 AB	32.1 ± 4.3 CD	30.4 ± 4.6 D	26.9 ± 4.9 D	27.1 ± 2.7 D	18.4 ± 3.0 E	18.2 ± 4.6 E
Ambar Universal	45.7 ± 4.7 AB	39.8 ± 4.6 BC	30.8 ± 5.2 D	27.9 ± 4.6 D	28.1 ± 1.4 D	24.3 ± 2.0 DE	18.5 ± 1.2 E	18.7 ± 1.3 E
Clearfil Universal Bond *Quick*	42.6 ± 2.3 BC	40.5 ± 0.9 BC	27.3 ± 2.9 D	25.3 ± 2.3 DE	21.1 ± 4.2 E	22.4 ± 4.2 E	14.0 ± 2.0 F	12.7 ± 0.8 F
One Coat 7 Universal	38.2 ± 3.1 C	31.2 ± 4.6 D	21.9 ± 1.8 E	22.3 ± 2.2 E	20.8 ± 2.2 E	18.8 ± 0.8 E	12.9 ± 0.8 F	10.7 ± 1.1 F

[a] Means identified with the same letter are statistically similar. (Tukey's test, $p \geq 0.05$).

Table 4
Means and standard deviations of nanoleakage (%) obtained in each experimental condition, after immediate (24 h) and 18 months of water storage ([a]).

Adhesive	Sound dentine				Caries-affected dentine			
	Immediate		18-month		Immediate		18-month	
	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch
Prime&Bond Active	18.4 ± 2.7 C	17.9 ± 3.4 C	27.1 ± 6.5 DE	28.6 ± 3.5 DE	16.3 ± 2.3 BC	15.9 ± 4.3 BC	32.0 ± 3.4 E	29.7 ± 5.7 DE
Scotchbond Universal	13.5 ± 1.7 AB	12.8 ± 2.5 AB	17.3 ± 4.3 BC	14.2 ± 2.5 AB	18.1 ± 4.6 C	16.1 ± 3.3 BC	23.8 ± 4.4 CD	17.5 ± 8.5 BC
Tetric N-Bond Universal	11.9 ± 2.5 AB	11.1 ± 0.8 AB	19.2 ± 4.8 C	18.9 ± 2.6 C	15.3 ± 2.4 BC	14.3 ± 3.2 AB	21.8 ± 2.9 CD	17.2 ± 9.3 BC
Ambar Universal	15.8 ± 4.3 AB	10.6 ± 1.3 A	23.4 ± 3.8 C	20.9 ± 5.2 C	13.2 ± 4.1 AB	7.2 ± 2.7 A	28.2 ± 5.5 DE	22.3 ± 5.5 CD
Clearfil Universal Bond *Quick*	13.5 ± 2.9 AB	16.4 ± 5.2 BC	21.5 ± 5.5 C	27.6 ± 2.6 DE	20.0 ± 2.6 CD	25.2 ± 3.9 D	32.3 ± 5.9 E	32.5 ± 1.1 E
One Coat 7 Universal	17.7 ± 4.9 BC	13.9 ± 6.1 AB	21.9 ± 5.6 CD	17.7 ± 3.6 C	20.2 ± 5.8 D	20.7 ± 2.6 D	23.4 ± 4.1 CD	21.1 ± 4.0 CD

[a] Means identified with the same letter are statistically similar. (Tukey's test, $p \geq 0.05$).

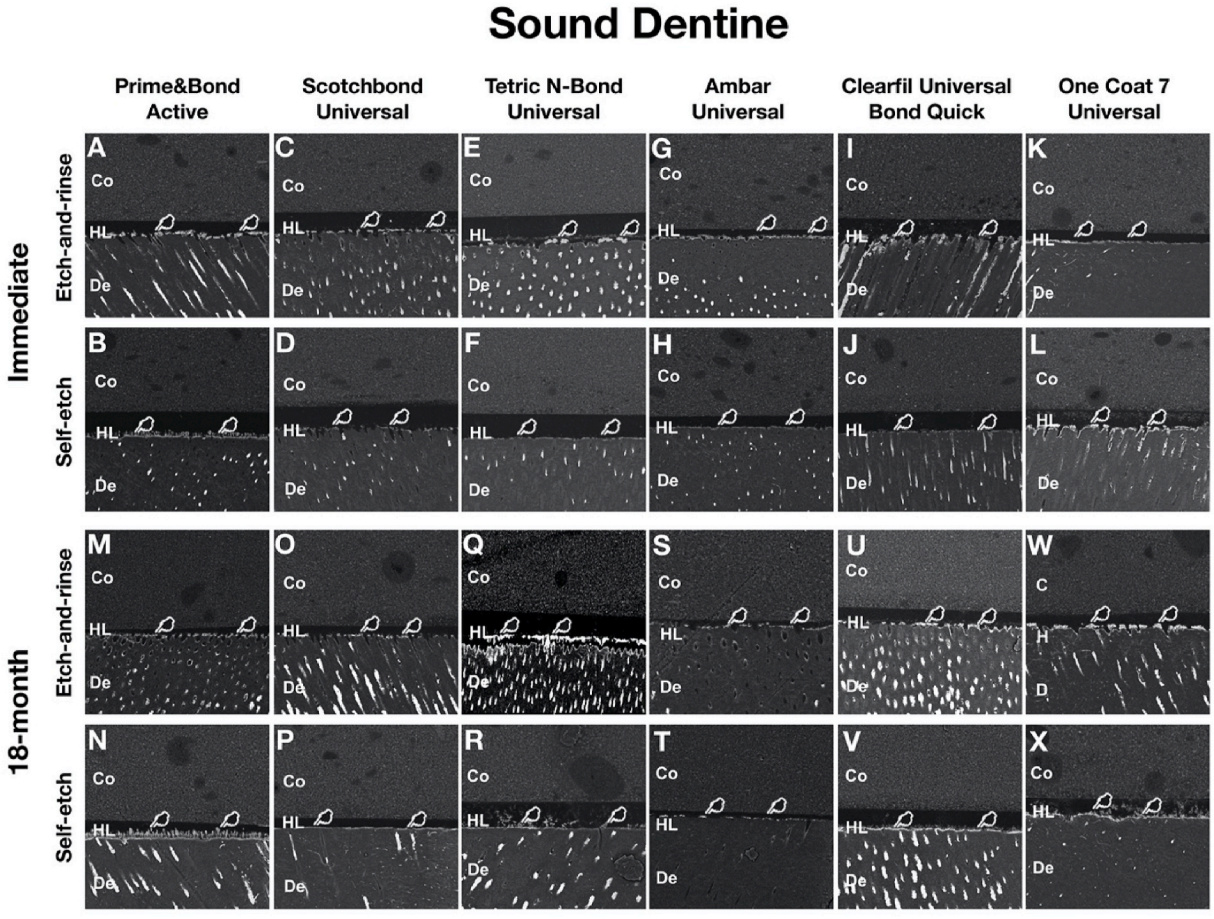

Fig. 1. Representative back-scattering SEM images of the resin-sound dentine interfaces obtained in each experimental condition, after 24 h (A–L) and after 18 months (M–X) of water storage (Co = composite; HL = hybrid layer and De = dentine).

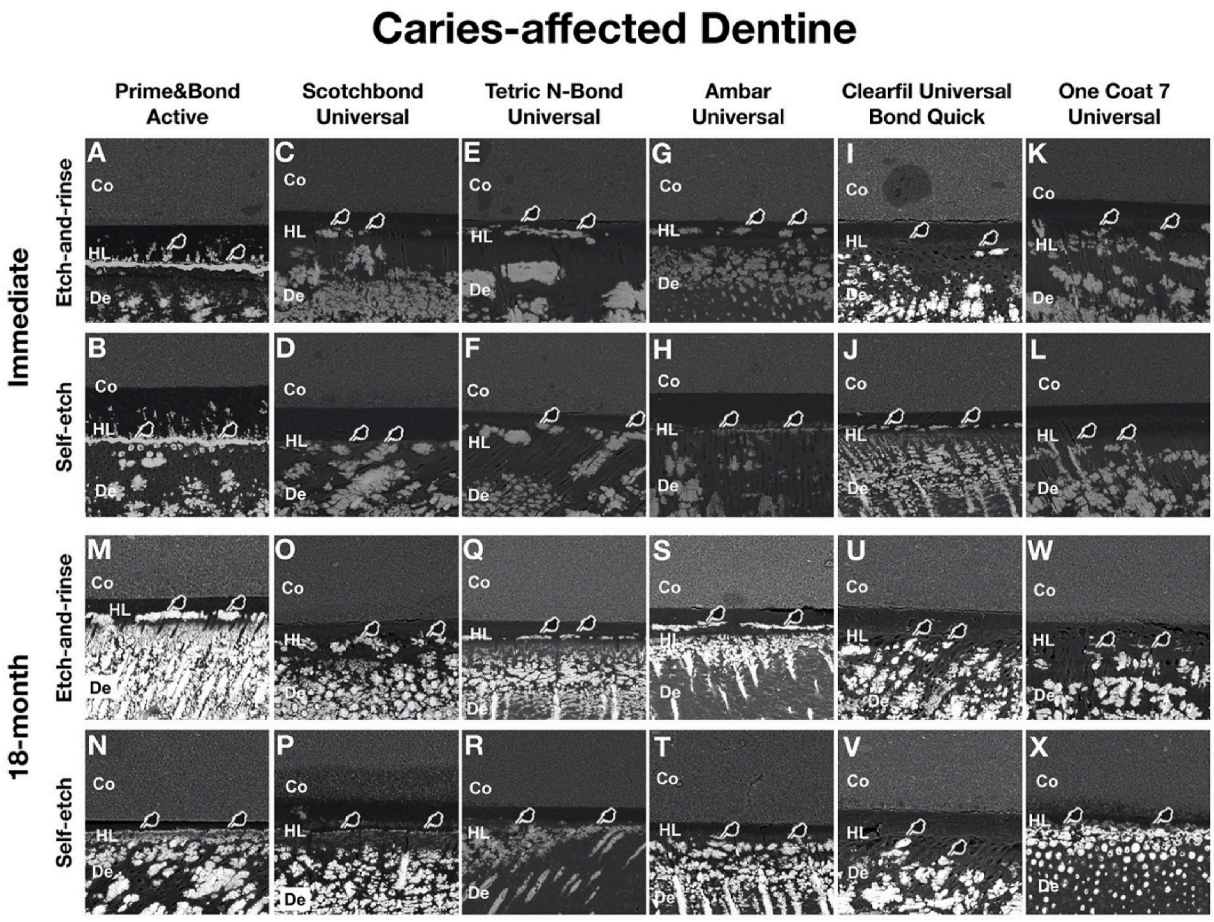

Fig. 2. Representative back-scattering SEM images of the resin-caries-affected dentine interfaces obtained in each experimental condition, after 24 h (A–L) and after 18 months (M–X) of water storage (Co = composite; HL = hybrid layer and De = dentine).

practice, where it could be generated a release of antibacterial monomers and help in the control of the carious process. This result is in agreement with a recent study that showed that CUQ present an important antibacterial activity against *S. mutans* (65.62–99.94% dead cells) [31].

On the other hand, it has been shown that many of the monomers that are part of the composition of adhesive systems, when applied in deep cavities and not properly polymerized, can diffuse through the dentine tubules, reaching the pulpal tissue, and potentially modifying pulp cell metabolism [32,33]. In this sense, universal adhesive systems could present different cytotoxicity due to the interaction of the some components with pulp cells, so the determination of the cytotoxicity of each product through cell viability tests is extremely significant, with the aim of help preserve pulp tissue health.

For 1 v/v% dilution, all adhesives were cytotoxically compared to the viability control (culture medium) which is in agreement with a recent study [24]. This result can be explained since universal adhesives contain MDP, which has been shown to exert effects comparable to those of TEGDMA concerning cytotoxicity, odontoblastic differentiation, and inflammatory response in human dental pulp cells [34].

On the other hand, PBA, AMU, CUQ and OCU showed significant differences when compared with the viability control in the 0.1 v/v% dilutions (30%, 35%, 72% and 65% of cell viability respectively). These percentages of cell viability agree with recent studies [24,35]. However, according to ISO 10993-5 standardization, if the viability is reduced to less than 70%, the material presents cytotoxic potential [36]; thus PBA, AMU and OCU showed cytotoxicity according to this ISO standardization. So, it is important to mention that, the evaluation of cytotoxicity shows that, by increasing the dilution, cytotoxicity will decrease, which is in agreement with a recent study in universal adhesives [37].

In the case of AMU and OCU, the high cytotoxicity may be due to the high percentages of UDMA (40% and 25% respectively) in the composition of these adhesives, a highly cytotoxic monomer [23]. In the case of OCU, it seems that the lower amount of UDMA (around 25%) can be responsible for the lower DC observed in the present study. The increase in the amount of UDMA is related to an improvement of the degree of conversion and polymerization rate of composites [38]. Also, the lowest

Table 5
Means and standard deviations of *in situ* degree of conversion (%) obtained in each experimental condition ([a]).

Adhesive	Sound Dentine		Caries-affected Dentine	
	Etch-and-rinse	Self-etch	Etch-and-rinse	Self-etch
Prime&Bond Active	60.96 ± 0.23 C	60.17 ± 2.38 C	54.48 ± 2.60 D	55.42 ± 2.16 D
Scotchbond Universal	70.83 ± 1.48 A	70.13 ± 1.27 A	62.22 ± 3.34 BC	62.71 ± 2.20 BC
Tetric N-Bond Universal	72.83 ± 1.26 A	70.34 ± 0.88 A	65.14 ± 0.65 B	65.04 ± 1.45 B
Ambar Universal	69.50 ± 0.86 AB	69.40 ± 2.76 AB	63.60 ± 3.43 BC	62.98 ± 4.60 BC
Clearfil Universal Bond *Quick*	72.46 ± 1.50 A	68.51 ± 2.51 AB	61.74 ± 0.61 C	60.26 ± 1.46 CD
One Coat 7 Universal	59.81 ± 3.02 C	60.59 ± 3.03 C	54.31 ± 2.13 D	55.76 ± 1.97 D

[a] Means identified with the same letter are statistically similar. (Tukey's test, $p \geq 0.05$).

degree of conversion of OCU was responsible for the lower immediate and 18 months bond strength, as previously showed by Siqueira et al. [39] and Costa et al. [40].

In the case of PBA, the presence in its composition of dipentaerythritol penta acrylate monophosphate (PENTA), a very hydrophilic resin monomer, explains the lower cell viability, since recent studies have already demonstrated the cytotoxic potential of this monomer [41]. Also, the lower DC value observed in the present study for PBA could be co-responsible for increasing their cytotoxicity.

For instance, PENTA features a long main linear organic chain with four long lateral chains. This could reduce its mobility and flexibility which restricts the possibility of lateral functional monomers reacting [42]. The aforementioned causes that the conversion of monomers to polymers is diminished, showing lower degree of conversion values. In addition to that, PBA presents isopropanol as a solvent, which, being highly water-soluble [43], causes high solubility and water sorption of this adhesive system [24]. All this could explain the increase in nanoleakage values after 18 months of storage in water.

Regarding the microtensile bond strength to sound dentine, it was observed that when all universal adhesive systems were used in etch-and-rinse application mode, values between 38.2 and -50.5 (immediate time) and 21.9–32.1 MPa (after 18 months) were observed. Whereas when they were used in self-etch application mode, values between 31.2 and 45.8 (immediate time) and 22.3–30.4 MPa (after 18 months) were observed, in agreement with microtensile bond strength to sound dentine observed for Follak et al. [17]. On the other hand, on caries-affected dentine it was observed that when all universal adhesive systems were used in etch-and-rinse application mode, values between 20.8 and 28.1 (immediate time) and 12.9–18.5 MPa (after 18 months) were observed. Also, when they were used in self-etch application mode, values between 18.8 and 27.1 (immediate time) and 10.7–18.7 MPa (after 18 months) were observed. These results agree with previous literature where adhesion on caries-affected dentine was studied in etch-and-rinse or self-etch strategy [12,17,44].

However, a recent published study [17] showed a more pronounced reduction of microtensile bond strength for the universal adhesives on caries-affected dentine after 1-year of water storage, with values between 2.0 and 3.8 (etch-and-rinse) and between 3.2 and 7.7 MPa (self-etch). These lower values compared to those presented in this study, may be due to methodological differences, mainly the method of obtaining caries-affected dentine. While in the present study a caries induction microbiological model was used, in the Follak study's [17] a caries induction pH cycling model was used. Therefore, the authors of the present study speculated that in the present study a less attacked caries-affected dentine was performed when compared to the previous one. However, future studies need to be done to compare the microtensile bond strength in different caries-affected models.

As it was observed in this study, the bond strength values on caries-affected dentine are significantly lower when compared to sound dentine for all universal adhesive systems, both immediately and after 18 months of water storage, regardless of the adhesive strategy used. On the contrary, the nanoleakage values on caries-affected dentine are significantly higher when compared to sound dentine, both immediately and after 18 months of water storage, regardless of the adhesive strategy used, in agreement with some study [17,22,44]. Observe that, in the study published by Follak et al. [17], it was not possible to obtain specimens for nanoleakage evaluation when universal adhesives were applied in the caries-affected dentin. This is because caries-affected dentine represents a very challenging substrate, due to their increased porosity [6], increased dentine humidity and significantly reduced dentine mechanical properties [7,45]. Moreover, due to universal adhesives are simplified adhesives that act as semi-permeable membranes to this excess moisture, adhesive restorations on caries-affected dentine continue to be a challenge for clinicians.

Thus, the possibility of incorporating antimicrobial agents into adhesive systems, in addition to collagen crosslinker to improve the mechanical and adhesive characteristics of the hybrid layer on caries-affected dentine, should be studied in the future.

According to the application mode of universal adhesives, there is still controversy as to whether the etch-and-rinse or self-etch mode have better adhesive performance on caries-affected. Being 10-MDP a component of all universal adhesives, it has a very strong and stable chemical interaction with hydroxyapatite [46]. These insoluble salts of MDP-Ca protect collagen fibers, therefore, the conservation of Ca at the dentine-adhesive interface could favor this chemical bonding process [47]. Despite this theory, regarding the adhesive strategy on sound and caries-affected dentine, it was observed that when all universal adhesive systems were used in etch-and-rinse application mode, bond strength and nanoleakage values were similar to these in self-etch application mode. These results agree with previous studies when both adhesive strategies were evaluated [22,24,44]. However, at least one recently published study showed that, it was occurred more degradation in the dentin bonding interface when etch-and-rinse was used, instead self-etch approach [17]. A closer view regarding the results of the present study showed that for etch-and-rinse mode, the decrease in bond strength after 18 months, both in sound dentine and caries-affected dentine (37.3% and 35.7%, respectively), was slightly higher than in the self-etch mode (31.5% and 30.7%, respectively). The same phenomenon was observed in the nanoleakage values. Both methods reinforce the idea that the self-etch application mode could present more advantages than etch-and-rinse strategy for universal adhesives.

However, although all universal adhesives showed decreased bond strength and increased nanoleakage values after 18 months of water storage, regardless of the mode of application and substrate, some significant differences appeared between the universal adhesives tested, mainly OCU, PBA and CUQ. It is worth mentioning CUQ because it was recently launched for use on dentine without waiting for the adhesive to interact with the bonding substrate (the "no-waiting" concept) [48]. According to the manufacturer, the addition of a new multifunctional hydrophilic acrylamide amide monomer may allow a shortened application time because it may enhance the wetting of the dentine sub-surface [49].

Nevertheless, after 18-month of water storage, CUQ showed a significant decrease of the bond strength and increase of the nanoleakage results, mainly when bonding in the caries-affected substrate. Due to these results, it may be hypothesized that the "no-waiting" concept applied during CUQ application could be responsible for inadequate solvent evaporation of residual water and organic solvents [50] and could as well hinder an adequate infiltration of resinous monomers, mainly in the caries-affected substrate. Similar results in terms of dentine bonding degradation to CUQ were recently reported by Ahmed [51,52].

As limitations of this study, it is possible to mention that the inhibition halo *via* disc diffusion test is not a conclusive assay, since the test uses a single bacteria strain, and that hardly reflect results *in vivo* [53–55]. In addition, the caries microbiological induction models, intending to simulate caries-affected dentine that can be observed clinically, present differences when compared with natural caries-affected tissue [44]. In this context, new studies that incorporate other methods to evaluate antibacterial activity of adhesive systems are necessary.

5. Conclusions

The universal adhesives tested in this study showed a huge difference in their antimicrobial capacity and cytotoxicity which seems to depend on the chemistry and degree of conversion between their other components. However, the majority showed worse results in terms of bonding when applied in caries-affected dentine, mainly after 18 months of water storage.

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