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ABSTRACT

This study examined the effects of two deproteinization protocols on dentin microtensile bond strength (μTBS), *in situ* degree of conversion (DC) and interfacial nanoleakage (NL) of two self-adhesive resin cements (SARCs) after 24 h or 200,000 load cycles. One hundred fourteen third molars were distributed into six groups according to the type of cement and the strategy of deproteinization. Resin cements used were RelyX U200 (RU) and Maxcem Elite (ME) following the manufacturer´s instructions and after deproteinization protocols (NaOCl for 2 min or acid etching before NaOCl for 2 min). The bonded specimens were randomly divided and submitted to microtensile test after 24h or after 200,000 load cycles. Two slices from each subgroup were prepared for NL using ammoniacal silver nitrate solution, and analyzed through SEM. The *in situ* DC of three bonded-specimens from each group was measured through micro-Raman spectroscopy. Data was statistically analyzed by Two-way ANOVA and Tukey's test ($p<0.05$). Dentin deproteinization prior acid etching increased the μTBS of both cements at 24h, but no differences in RU groups were found after load cycling. Dentin deproteinization decreased the DC and NL of both cements. After load cycling, this technique was effective for ME, but did not affect the bond strength of RU. Dentin conditioning/deproteinization enhances the integrity of SARC-dentin interface thereby improving the longevity of dentin bond after load cycling.

KEYWORDS: Adhesion, Nanoleakage, Surface treatment, Dentin, Sodium hypochlorite, Luting cements.

1. INTRODUCTION

The clinical success of esthetic indirect restorations depends on the long-term bond stability between resin cements and dental tissue [1,2]. Composite cements may be classified as conventional resin cements or self-adhesive resin cements (SARCs), depending on the bonding strategy [3,4]. SARCs have been advocated to simplify clinically technique-sensitive multi-step procedures for luting indirect restorations. These cements are applied directly to the smear layer-covered without dentin pre-treatment using etchants or bonding primer [5,6].

Also, the use of adhesive procedures for restoration of endodontically treated teeth is quite common in daily practice. Among the possible restorative techniques, fiber-reinforced composite posts, which are adhesively luted into the canal, may be used in the treatment of endodontically treated teeth to be more conservative with the dental hard tissues [7,8]. However, usually, different substances are used together with the endodontic treatment. Among them, sodium hypochlorite (NaOCl) is the most common endodontic irrigant because of its antimicrobial characteristics during endodontic treatments [9-11]. NaOCl is also a nonspecific proteolytic agent able to oxidize and remove the organic components of dentin [12-15]. NaOCl pre-treatment used in combination with an acid etchant has been recently proved to improve the bonding of fiber post to radicular dentin when using conventional resin cements [16].

It is well known that SARCs do not completely dissolve the smear layer, and due to this “superficial” the interaction with the dentin, optimal bond strength may be impaired

[17,18]. Acidic functional monomers contained in SARCs urge low pH and hydrophilic properties in the beginning of the setting reaction. Thereafter, the negatively charged groups of the monomer bind to Ca^{2+} ions and to the dentin. Alongside, the alkalinity of the fillers provides further neutralization reaction of the functional monomers [19].

NaOCl may cause collagen removal by the deproteinization of the dentin etched with phosphoric acid [20], as well as on mineralized dentin [15,21]. Its use exposes the sub-superficial hydroxyapatite-rich dentin layer so enhancing the penetration and the chemical interactions between SARCs and the calcium of the dentin [22,23].

Therefore, the aim of this *in vitro* study was to assess the bonding performance of two SARCs after deproteinization of mineralized and phosphoric acid etched dentin surfaces through microtensile bond strength (μTBS) and interfacial nanoleakage (NL) tests in the immediate period or after load cycles. *In situ* degree of conversion (DC) assessment was also performed in the immediate time.

The following hypotheses were tested in this study: 1) dentin deproteinization improves the bonding performance of SARCs applied on etched or mineralized dentin; 2) the mechanical load cycles reduce the adhesion performance of both materials; 3) the DC of the SARCs is affected by the use of a deproteinization agent (NaOCl).

2. MATERIALS AND METHODS

2.1. Tooth preparation

One hundred and fourteen freshly human non-carious third molars were extracted and used in this study under a protocol number 339.789 approved by the Ethics Committee of the Federal University of Ceará (UFC; Ceará, Brazil). The teeth were stored in 0.01% thymol solution at 4°C and used in a period no longer than 1 month

after extraction. A flat dentin surface was created by removing the occlusal enamel using a water-cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, USA). A parallel cut was also performed 4 mm underneath the cementum enamel junction (CEJ) to remove the roots. Presence of enamel remnants on the dentin surface was evaluated using an 80x light microscope (Leica DM 1000 – Leica Microsystems GmbH - Wetzlar, Germany). Dentin specimens were polished under water irrigation with a 600-grit silicon-carbide paper for 30s in order to produce a standardized smear layer.

2.2. Restorative procedure

Resin composite (Filtek Z350 - 3M ESPE, Seefeld, Germany) blocks (5.0 x 5.0 x 2.0 mm) were created using molds made of polyvinylsiloxane. The resin blocks were light-cured for 40 s (using 2mm incremental technique) with LED dental curing unit set at 1200 mW/cm^2 (DB 685; Dabi Atlante, Ribeirão Preto/SP, Brazil). The composite blocks were polished under water irrigation using a 400-grit silicon-carbide paper for 10s, and then ultra-sonicated in distilled water for 10 min. Before cementation, specimens were silanized (Prosil, FGM, Joinville, Brazil) following the manufacturer´s instructions of each cement used in this study (Table 1).

Two SARCs, RelyX U200 (3M ESPE, Seefeld, Germany) and MaxCem Elite (Kerr, Orange, USA), were used for the luting procedures on the resin blocks and dentin specimens. In accordance with the strategy of deproteinization, the specimens were distributed into six groups: RelyX U200 (RU) or MaxCem Elite (ME) following the manufacturer´s instructions (control); RU or ME after dentin deproteinization (RU - NaOCl) and; RU or ME after phosphoric acid etching and dentin deproteinization (RU – Etching + NaOCl) (Table 1). The etching procedure was realized using 37% H_3PO_4 Gel (Condac 37%, FGM, Joinville, Brazil) for 15 s, rinsed with air spray for 30s and dried with

absorbent paper. For the deproteinization step, dentin surface was treated with 5% NaOCl for 2 min under continuous rubbing, and finally rinsed for 30s with distilled water [24]. The cementation was performed as per manufacturer´s instructions (Table 1) and using a standardized pressure of 20 g/mm^2.

2.3. Specimens preparation and load cycling

After 24 hours in distilled water at 37°C, the specimens from each group were randomly divided in two sub-groups for the microtensile bond strength test (n=6): immediately and after load cycling. Cycled specimens were adapted in auto-polymerizing acrylic resin moulds with a thin layer of silicone used to simulate periodontal ligament [25]. The excess silicone was removed with a spatula at the CEJ and the specimens underwent 200,000 mechanical load cycles at 60 N, and 2 Hz frequency using the chewing simulator CS-4 (SD Mechatronik, Westernham, Germany).

Specimens were finally sectioned in both “x” and “y” directions, across the bonded interface, using a diamond blade in an Minitom cutting-machine (Struers A/S, Copenhagen, Denmark) to obtain sticks with cross-sectional areas of 0.9 mm$^{2.}$

2.4. Microtensile bond strength test (µTBS)

The sticks were measured individually with a digital caliper (Absolute Digimatic, Mitutoyo, Tokyo, Japan) and tested using a universal testing machine (EMIC DL 2000, São José dos Pinhais, Brazil) at crosshead speed of 0.5 mm/min.

Fracture analysis was performed using a stereo microscope (Leica DM 1000 – Leica Microsystems GmbH - Wetzlar, Germany) at 80X magnification and classified according to the failure mode as adhesive/mixed (M) when the failure occurred at the

resin–cement or cement-dentin interface, cohesive in cement (CC), cohesive in dentin (CD) or cohesive in resin composite (CR).

2.5. Interfacial nanoleakage evaluation (NL)

For interfacial nanoleakage evaluation, two resin-dentin sticks from each sub-group were used. They were placed in ammoniacal silver nitrate solution in darkness for 24 h, rinsed thoroughly in distilled water, and immersed in photodeveloping solution for 8 h under a fluorescent light to reduce silver ions into metallic silver grains within voids along the bonded interface [26].

Specimens were polished with a 1200-, 2500- and 4000-grit SiC paper and 1 µm diamond paste (Buehler Ltd, Lake Bluff, USA), and ultrasonically cleaned for 3 min between each polishing step. Thereafter, they were mounted on aluminum stubs, air-dried and gold sputter coated for analysis in a field emission gun scanning electron microscope (FEG-SEM) (Quanta FEG, FEI, Amsterdam, Netherlands).

2.6. Degree of Conversion (DC)

The degree of conversion at the bonded interfaces was measured by micro-Raman spectroscopy. Three further specimens for sub-group were prepared as previously described and cut in 1 mm slices. These were polished using 2500- and 4000-grit SiC papers and then ultra-sonicated for 3 min between each polishing procedure. Spectra were acquired from each specimen at the center of the cement layer (n=3). Raman spectra were collected using Xplora micro-Raman (Horiba, Paris, France) in the range of 1590–1670 cm^{-1} using the 638 nm laser emission wavelength, with 5 seconds acquisition time and 10 accumulations. The diameter of laser beam used over the specimen was 1 µm and the analysis was performed with 100x magnification lens.

A small amount of uncured resin cement from each material was also obtained and its spectrum was used as unpolymerized reference. The % DC was calculated according to the two-frequency technique using the net peak absorbance areas of the aliphatic C=C stretching vibrations at 1635 cm^{-1} as analytical frequency and the aromatic C⋯C stretching vibrations at 1610 cm^{-1} as internal reference.

2.7. Statistical analysis

The experimental unit in the current study was the tooth. The microtensile bond strength values of all sticks from the same tooth were averaged for statistical purposes. All data were submitted to Kolmogorov-Smirnov normality test. After passing this test, the microtensile bond strength (MPa) data were subjected to Three-way (cement vs. strategy of deproteinization vs. cycling) ANOVA, Two-way (cement vs. strategy of deproteinization) ANOVA and Tukey's *post-hoc* test with α = 0.05. For DC, data were statistically analyzed using Two-way ANOVA (cement vs. strategy of deproteinization) and Tukey's test with α = 0.05. The interfacial nanoleakage was only evaluated qualitatively.

3. RESULTS

3.1. Microtensile bond strength test (μTBS)

Data passed normality test (p=0.456) and equal variance test (p=0.399). The Three-way ANOVA demonstrated that load cycling had no significant effect on any variable (p=0.217). Therefore, two Two-way ANOVA tests were employed separately for the immediate and load cycled data. These tests showed that the interaction was statistically significant for cement and strategy of deproteinization for immediate (p<0.001) and cycled (p=0.001) subgroups. The μTBS means, standard deviations and

number of specimens tested are shown in Table 2. The use of the dentin deproteinization associated or not to etching significantly increased the bond strength of both cements in the immediate group. RU showed higher bond strength values than ME, however, after etching and NaOCl, both groups showed the same results (Table 2 – Part A).

After load cycling, the results are different for both SARCs. While for ME, the use of deproteinization associated or not to etching showed higher bond strength than ME control. For RU, no significant differences were observed between control and deproteinization groups, and the two are higher than deproteinization associated to etching (Table 2 – Part B).

The distribution of failure modes and pre-test failures for each group are summarized in Figure 1. All experimental groups showed a high incidence of cement cohesive failures. Cohesive fractures within the dentin were not observed for all groups.

3.2. Interfacial nanoleakage (NL)

Representative SEM images of the adhesive interface produced in all conditions tested are depicted in Figure 2 and Figure 3. For both cements observed after 24 h, when dentin was deproteinized associated or not to prior etching (Figure 2B, 2C, 2E and 2F), minimal silver nitrate deposition along the interface was observed. After load cycling, for ME, the deproteinization (Figure 3E) showed the lowest amount of silver impregnation followed by deproteinization associated to etching (Etching + NaOCl – Figure 3F). For RU, there was lower amount of silver for both experimental protocols (Figure 3B and 3C) than RU control (Figure 3A). The ME control group showed a large gap at the interface for immediate and load cycled control groups (Figure 2D and Figure 3D).

3.3. Degree of Conversion (DC)

The two-way ANOVA demonstrated that the control groups exhibited the highest DC, which was significantly different from the other groups ($p<0.05$). The mean and standard deviation values of the DC are presented in Figure 4.

For RU, the deproteinization associated to etching significantly decreased the DC when compared to only NaOCl. For ME, there were no significant differences between the use of solely NaOCl or NaOCl with prior acid etching. However, it worth mentioning that, when comparing both materials after the same treatments, RU presented higher DC than ME in all conditions, except for NaOCl with prior acid etching groups in which there were no significant differences between two cements.

4. DISCUSSION

According to the present results, both strategies of dentin deproteinization improved the immediate performance of SARCs; thus, the first hypothesis needs to be accepted. For the mechanical loaded specimens, the use of NaOCl, regardless the use of prior phosphoric-acid etching improved the bond strength of Maxcem Elite and had no effect on RelyX U200. Therefore, the second hypothesis needs to be rejected. The third hypothesis is accepted since the deproteinization significantly decreased the DC for both groups.

The use of solely NaOCl alters significantly the mineral content of root dentin, increasing the Ca/P ratio of dentin surface [27], thereby providing more mineralized tissue similar to enamel which could improve the chemical interaction of the SARCs with calcium. NaOCl can penetrate the apatite-encapsulated collagen matrix and remove the organic phase from mineralized dentin [15,21]. Two minutes application of 5% NaOCl treatment was applied as it affects the organization of collagen and glycosaminoglycans

in mineralized and partially demineralized dentin [13], improving the bonding results between dentin and self-adhesive resin materials [24,28].

The bonding mechanism of SARCs to dentin relies on mild etching along with shallow formation of an interdiffusion zone as well as on the chemical reaction of acidic functional monomers with calcium and hydroxyapatite [19,29]. Previous reports demonstrated that SARCs perform worse than conventional resin cements on luting indirect fillings to dental crown [3,30]. The increase in the overall mineral content and the reduction in water content due deproteinization may be advantageous for the hydrophobic SARC to chemically interact with hydroxyapatite and enhance the surface wettability, thereby improving the adhesion [31] and reducing the nanoleakage. This could explain the higher bond strength values and the minimal silver nitrate deposition at 24h along the interface observed with deproteinization treatment associated or not with etching.

Some studies suggest that SARCs have limited capacity to diffuse and decalcify the underlying dentin effectively [18,32]. When NaOCl solution is applied on smear layer-covered dentin, the mineral ratio increases and the smear layer is thinned due to dissolution of its collagen portion [21]. This NaOCl treated smear layer with less organic components may ease the bonding performance of SARCs, especially Maxcem Elite which contains the acidic functional monomer GPDM (glycerol-phosphate di-methacrylate) which has limited interaction with the smear layer-covered dentin. Furthermore, the pretreatment with NaOCl may increase the hydrophilicity of the substrate, thereby favoring the interaction with more hydrophilic resin cements such as Maxcem Elite.

Although the benefits of deproteinization for both SARCs, there are some important differences between Maxcem Elite and RelyX U200. Both SARCs are acidic initially after mixing in order to demineralize the dentin and approach neutral pH after curing [19,29]. Maxcem Elite maintains its low pH, whilst the pH of RelyX U200 increases after 24 h [33,34]. In the case such a low pH is maintained for a long period, for instance with Maxcem Elite, a negative effect might occur on the adhesion between cement and dentin [35,36]. Indeed, the gaps (Figures 2D and 3D) found within the interface of Maxcem could be due to the effect of such a stronger etching, which may have affected the optimal interaction of the cement to the dentin.

Maxcem Elite cement contains an amine-free redox initiator system while RelyX U200 has sodium sulfinate salts that prevent chemical incompatibility between acidic groups and self-curing components [37-39]. This may explain the highest *in situ* degree of conversion of RelyX U200 (75.7%). In NaOCl treated groups, the residual hypochlorite and oxygen species may induce incomplete polymerization of resin matrix. The oxygen released by NaOCl molecules may interfere with free radical propagation, inhibiting the polymerization of the cement as described in previous reports [40,41]. One may speculate that deproteinization creates a porous substrate with higher concentrations of OCl^- in such porous, resulting in localized decreased degree of conversion. The negative effect of NaOCl on polymerization may, conversely, be surpassed by the benefits of the improved chemical interaction of s SARCs to dentin resulting in overall better performance of these materials.

After load cycling, the results were different for both SARCs. While for ME, the use of deproteinization associated or not to etching showed higher bond strength than ME control, for RU, no significant differences were observed between control and only deproteinization treatment, maintained the bond strength values statistically similar

(p>0.05) (Table 2 – Part B). Nevertheless, it was observed significant increase of silver infiltration inside the luting interface for the RelyX U200 control group and gaps within the interface created using Maxcem Elite in control group (Figures 3A and 3D). However, there was little amount of silver uptake in experimental (NaOCl treated) groups, thereby demonstrating optimal interaction between deproteinized dentin substrate and the SARCs (Figure 3B, 3C, 3E and 3F). The decrease of nanoleakage for all materials suggests a possible long term benefit of the NaOCl pre-treatment to the indirect restorations luted with SARCs. As aforementioned, there is a potential smear-layer negative influence on the interaction SARCs with dentin. One may speculate that this influence plays a major role with Maxcem Elite than with RelyX U200 which could explain the notable higher improvements of dentin deproteinization for Maxcem Elite.

Increasing the number of steps is time-consuming and may reduce the attractiveness of these materials or pre-treatments. However, the long term benefits of dentin deproteinization, immediately and after chewing simulation, on bonding might justify and indicate such additional clinical step even considering the limitations of an in vitro methodology. Nevertheless, future investigations should be performed including the reduction of NaOCl concentration and application time, and focus on clinical trials.

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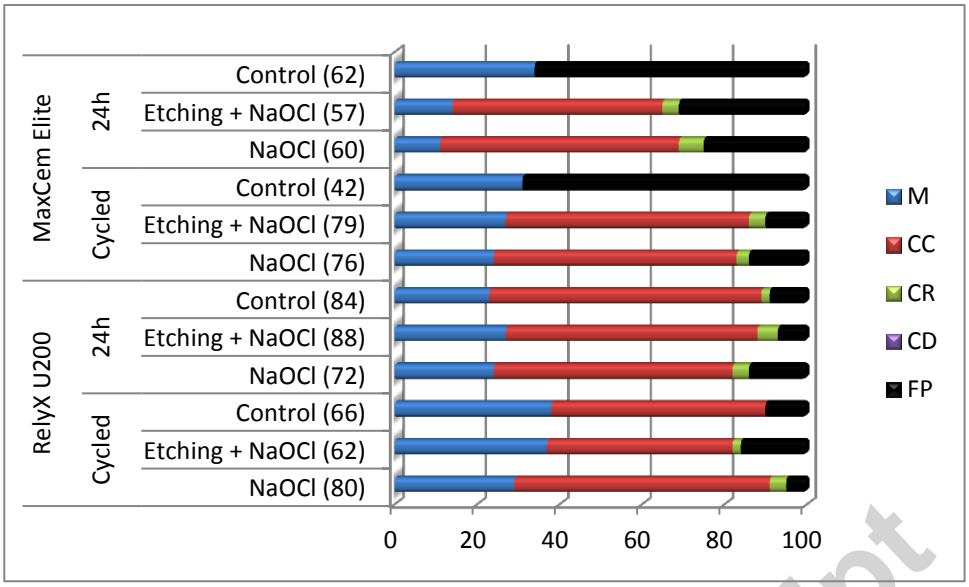

Figure 1: Percentage (%) distribution according to the pre-test failures and failure modes.

*The numbers in parentheses indicate total number of specimens

M: mixed/adhesive; CC: cohesive in cement; CR: cohesive in resin; CD: cohesive in dentin

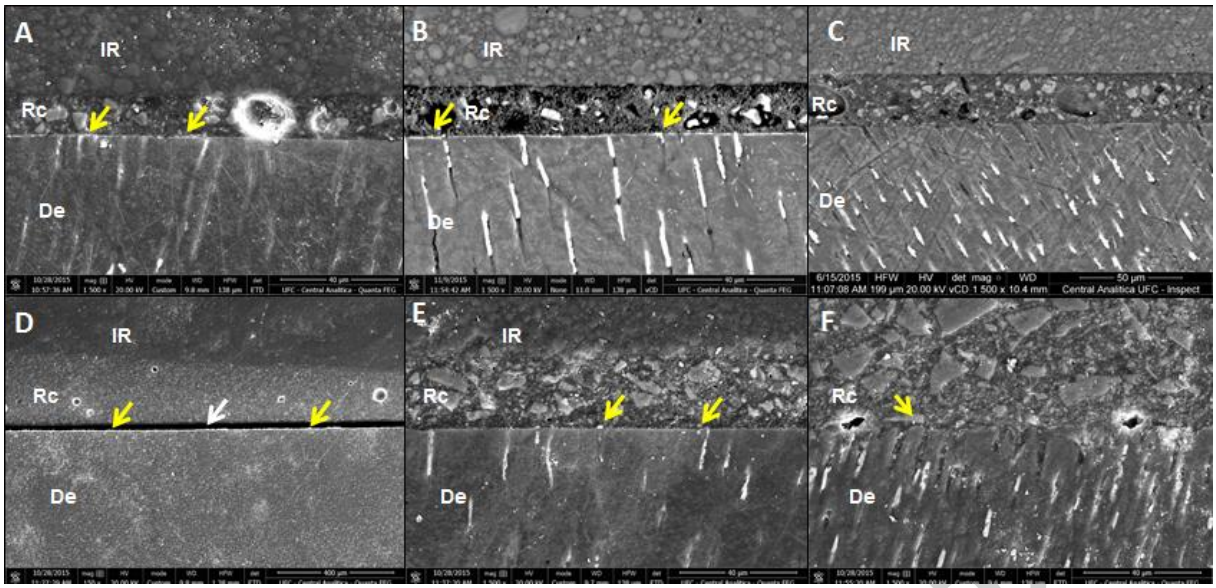

Figure 2 - Representative SEM images of the resin–dentin interfaces bonded with Relyx U200 (RU) and MaxCem Elite (ME) in the immediate groups without (Control: A and D) or with dentin deproteinization (NaOCl: B and E; Etching + NaOCl: C and F). Only few areas of silver nitrate uptake were observed within the adhesive interface (yellow arrows). For ME control group (D) a large gap can be seen (white arrow). (IR = indirect restoration; Rc = resin cement; and De = dentin).

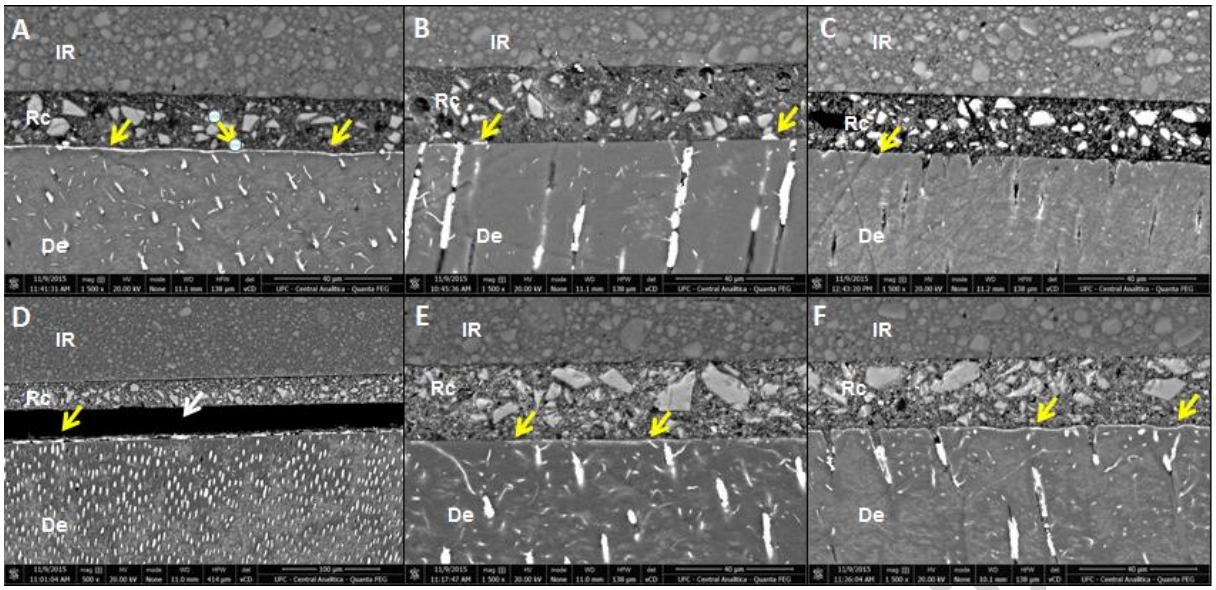

Figure 3 - Representative SEM images of the resin–dentin interfaces bonded with Relyx U200 (RU) and MaxCem Elite (ME) after load cycling without (Control: A and D) or with dentin deproteinization (NaOCl: B and E; Etching + NaOCl: C and F). RU control (A) showed a continuous line of silver nitrate uptake. When dentin surface was previously deproteinized, only few areas of silver nitrate uptake were observed within the adhesive interface (yellow arrows). ME control (D) presented a large gap like in immediate group (white arrow). (IR = indirect restoration; Rc = resin cement; and De = dentin).

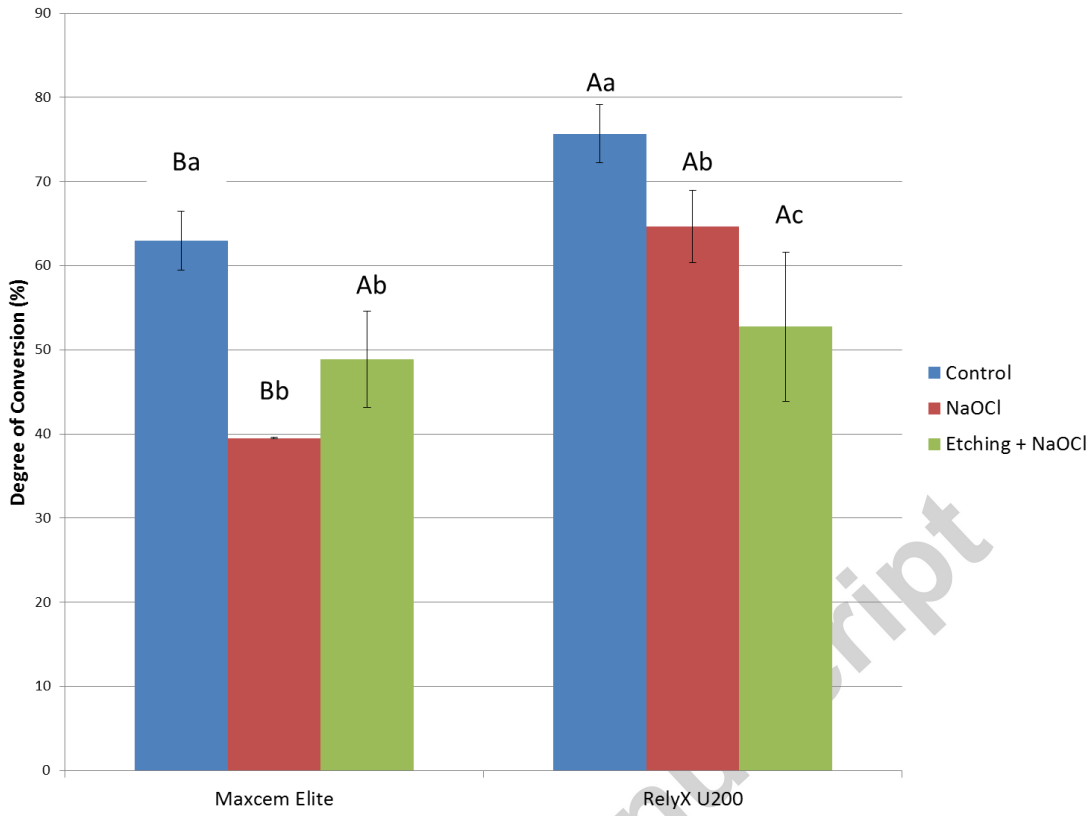

Figure 4. Degree of conversion (%) analysis represented by means and standard deviations for Maxcem Elite and RelyX U200. Different capital letters (comparing cements for the same treatment) and lower case letters (comparing treatments for the same cement) indicate statistical difference.

Table 1: Resin cements, their application protocols and strategy of cementation

	Composition	Control	Deproteinized
RelyX U200 (3M/ESPE, Seefeld, Germany)	Silane treated glass powder, substituted dimethacrylate, 1-benzyl- 5 -phenyl-barbic-acid, calcium salt, 1,12-dodecane dimethycrylate, sodium p-toluenesulfinate, silane treated silica, calcium hydroxide	1. Apply the silane for 60 s on the previously ragged surface of resin block and dry with air spray 2.Mix cement for 10 s and apply on silanized surface of resin blocks 3. Remove excess cement. Wait 3 min and light cure each surface/margin for 20 s	**Etching + NaOCl** 1. Apply 37% H_3PO_4 Gel (Condac 37%/FGM, Joinville, SC, Brazil) for 15 s 2. Rinsing with air spray 3. Dry with absorbent paper, keeping dentin moisture **NaOCl** 4. Apply 5% NaOCl under rubbing action for 2 min 5. Rinsing with air spray for 30 s 6. Dry with air spray 7. Apply the silane for 60 s on the previously ragged surface of resin block and dry with air spray 8.Mix cement for 10 s and apply on silanized surface of resin blocks 9. Remove excess cement. Wait 3 min and light cure each surface/margin for 20 s with LED dental curing unit set at 1200 mW/cm^2
MaxCem Elite (Kerr, Orange, USA)	Multifunctional dimethacrylates, Glyceroldimethacrylate Dihydrogen Phosphate (GPDM), proprietary Redox Initiators, photoinitiators, barium, fluoroaluminosilicate, fumed silica (66 wt%)	1. Apply the silane for 60 s on the previously ragged surface of resin block and dry with air spray 2. Place appropriate mixer on dual syringe cartridge. 3. Remove excess cement. Excess can be removed in gel state (gel state is achieved in 3 minutes) and light cure each surface/margin for 20 s	**Etching + NaOCl** 1. Apply 37% H_3PO_4 Gel (Condac 37%/FGM, Joinville, SC, Brazil) for 15 s 2. Rinsing with air spray 3. Dry with absorbent paper, keeping dentin moisture **NaOCl** 4. Apply 5% NaOCl under rubbing action for 2 min 5. Rinsing with air spray for 30 s 6. Dry with air spray 7. Apply the silane for 60 s on the previously ragged surface of resin block and dry with air spray 8. Place appropriate mixer on dual syringe cartridge and apply on silanized surface of resin blocks 9. Remove excess cement. Wait 3 min and light cure each surface/margin for 20 s with LED dental curing unit set at 1200 mW/cm^2

Table 2: Mean microtensile bond strengths (MPa) and standard derivations (SD) for the immediate and load cycled groups, as well as, statistical analysis (*)

μTBS (MPa)	**Immediate – Part A**		**Load cycled – Part B**	
	Maxcem Elite	**RelyX U200**	**Maxcem Elite**	**RelyX U200**
Control	3.87 (1.0) B,b	15.3 (2.3) A,c	1.6 (3.8) B,b	13.7 (6.2) A,a
NaOCl	21.2 (3.9) B,a	26.1 (3.3) A,a	24.9 (6.0) A,a	14.9 (3.5) B,a
Etching + NaOCl	23.3 (2.7) A,a	20.7 (3.2) A,b	24.7 (5.9) A,a	7.9 (6.1) B,a

*Different capital letters in row and lower case letters in column indicate statistical difference ($p<0.05$).