Improvements of zoledronate-containing primer on dentin bonding of an universal adhesive

W. Zenobi(1), V.P. Feitosa*(1,2), M.E.M. Moura(1), C. D’Arcangelo(3), L.K. Rodrigues(1), S. Sauro(1,4)

(1) - Post-Graduate Program in Dentistry - Department of Restorative Dentistry, Federal University of Ceará, Fortaleza, Brazil.

(2) - Paulo Picanço School of Dentistry, Fortaleza, Brazil.

(3) Unit of Restorative Dentistry, Department of Oral Science, Nano and Biotechnology, “G. D'Annunzio” University of Chieti, Italy

(4) - Dental Biomaterials – Departmento de Odontologia, Facultad de Ciencias de la Salud, CEU-Cardenal Herrera University, Alfara del Patriarca , 46115, Valencia, Spain.

***Corresponding author: (Dr. Victor Feitosa) victorpfeitosa@hotmail.com and victor.feitosa@facpp.edu.br**

Contact Address:

Dental School – Federal University of Ceara

Rua Monsenhor Furtado S/N,

Bairro Rodolfo Teófilo - ZIP CODE: 60430-325

Fortaleza – CE, Brazil

Phone: +55 85 33668412

Mobile: +55 85 999164512

ABSTRACT

Objectives. To evaluate the bonding ability and nanoleakage of an universal adhesive applied to dentin after zoledronate-containing primer (zol-primer) before and after mechanical load cycling.

Materials and Methods. Flat superficial dentin surfaces were exposed on human molars and ascribed to one of the following adhesion procedures (n=6): 1-*Adper Single Bond Universal* (SBU) applied in etch-and-rinse mode, 2- SBU applied as etch-and-rinse after the application of zol-primer, 3- SBU applied in self-etch strategy, and 4- SBU applied as self-etch after the use of zol-primer. Half of bonded teeth were processed for microtensile bond strength test after 24h and further were cut and surveyed after 200,000 mechanical cycles. Silver-impregnated specimens were assayed for interface nanoleakage by SEM. Data were analyzed by two-way ANOVA and Tukey's test ($p<0.05$).

Results. At immediate evaluation, the four presented similar bond strengths, whilst both groups bonded with etch-and-rinse technique had significant bond strength reduction after mechanical load ($p<0.05$), with higher drop for zol-primer group. No negative effects were found for self-etch strategy ($p>0.05$) in microtensile test. Lower nanoleakage was observed for etch-and-rinse specimens treated with zol-primer. However, noteworthy reduction of adhesive layer thickness was observed with the combination of zol-primer and self-etch strategy.

Conclusion. It can be concluded that zol-primer should not be used along with an universal adhesive in etch-and-rinse mode, but its application before self-etch application provides minor alterations.

Keywords: Bond strength; Dental adhesive; Zoledronic acid; Mechanical stress.

INTRODUCTION

The mechanism of bonding to enamel and dentin is essentially based on an exchange process in which minerals removed are replaced by resin monomers, that upon polymerization become micromechanically interlocked (1). Regarding dentin bonding, impregnation of synthetic monomers is challenging due to the humidity, permeability and physiologic hydrostatic pulpal pressure (2). After adhesive polymerization, exposed collagen is easily detected and represents an area prone to initial degradation. Collagen degradation is accelerated by proteolytic enzymes, the so-called matrix metalloproteinases (MMPs) and cysteine cathepsins (3,4).

Several MMP inhibitors have been investigated (6,3) such as galardin, batimastat and chlorhexidine, some have already been incorporated in adhesives (5,7). Synthetic MMP inhibitors should contain a functional group capable of chelating the zinc ions, which binds to the active site in the MMP molecule (~~8~~,9,2). A polyphosphonic acid such as zoledronate may provide additional inhibition of MMP ~~thanks~~ due to the ~~potential~~ likely ~~attaching~~ bond of zoledronate ~~with~~ with proteins (10). This~~ which may achieve~~ can induce dentin remineralization and reduce the activity of MMPs,~~ thus~~ improving the durability of adhesion, especially when associated with ion-~~-~~releasing~~e~~ adhesives (9).

Recent multi-mode self-etch adhesives (so-called universal adhesives) may be applied in etched or non-etched enamel and dentin. The longevity and bond strength of these materials have been studied and remain questionable (11,12). Indeed, zoledronate could improve the durability of dentin bonds created with universal adhesives, especially when dentin-etching is undertaken.

Furthermore, zoledronate may improve the resistance of partially demineralized collagen fibrils to mechanical stress. However, to our knowledge, this combination (zoledronate+universal adhesive) has never been investigated so far.

Therefore, the objective of this study was to assess the effect of an experimental primer containing 7% zoledronic acid (Zol-primer), applied prior to one universal adhesive both in etch-and-rinse (ER) and self-etch (SE) techniques, on the microtensile bond strength (μTBS) to dentin before and after mechanical cycling load challenge. The two study hypotheses to be tested were that the use of the Zol-primer applied along with universal adhesive: (i) provides significant resistance of dentin bonds after load cycling, and (ii) induces no change on silver nanoleakage.

MATERIALS AND METHODS

Preparation of Experimental Zol-primer

The formulation of the zol-primer used has been described by Tezvergil-Mutluay et al. 2014 (9), and was formulated with deionized water (50 vol%) saturated (7 mg/mL) with zoledronate [1-hydroxy-2-(1H-imidazol-1-yl) ethane-1,1-diyl]bis-phosphonic acid (MW 290; Santa Cruz Biotechnology, Santa Cruz, USA) and absolute ethanol (50 vol%) (pH adjusted to 6.8 with 0.1 M NaOH).

Sample preparation

Twenty-four third molars recently extracted for surgical reasons in the service of Dentistry and Maxillofacial Surgery Hospital General Universitario de Valencia were stored in deionized water (pH 7.4) at 4° C prior to use, under a protocol approved by the Biomedical Research Ethics Committee of the University and Polytechnic La Fe Hospital in Valencia, with registration No. 2014/00487/PI. Medium dentin specimens were obtained by removing the roots 2 mm below cemento–enamel junction (CEJ) and with a parallel cut at 2 mm above CEJ using a slow-speed water-cooled diamond saw (Isomet, Buehler, Lake Bluff, USA). The dentin surface was wet-polished with 600-grit SiC papers for 1 min to create a standard smear layer prior to bonding procedures (13,14).

Experimental design and bonding procedures

Dentin specimens were divided randomly by using Microsoft Excel (Windows) randomization into two principal groups (n=12) based on the bonding technique used: SE and ER. Universal adhesive employed was Scotchbond Universal (3M-ESPE, St. Paul, USA). The composition and application procedures are listed in Table 1. Resin composite build-ups were constructed in 3 horizontal layers (2-mm thick) up to 6 mm with Spectrum ® TPH® resin composite (Dentsply, Denver, USA). Photoactivation of the resin-based materials was performed using LED curing unit DB85 (Dabi Atlante, Ribeirao Preto, Brazil). The output intensity was monitored with a Demetron Radiometer (Model 100, Demetron Research, Danbury, USA) to maintain a minimal light output intensity of 1000 mW/cm^2 throughout all experiments. All materials were used following the manufacturers' recommendations. During to the bonding procedures, the samples from each main group were divided into subgroups (n=6), regarding the use or not of Zol-primer before adhesive application. Specimens of each principal group were divided into another sub-groups (n=3), based on the challenge test: Control: water immersion for 24h and MCL: mechanical-cycling load regimen. Spreading of specimens division is presented in Figure 1.

Mechanical-cycling challenge

The resin-bonded specimens were submitted to the mechanical cycling load executed using Chewing Simulator CS-4 (SD Mechatronik, Feldkirchen-Westerham, Germany) which has a stainless steel tip of 4 mm in diameter in contact with the central part of the restored specimens. All resin-bonded specimens were submitted to 200,000 mechanical cycles under a load of 30 N, at a rate of 2 Hz for one week (15).

Microtensile bond strength (µTBS) and Fracture type analysis

After 24h immersion in distilled water, resin-bonded specimens were sectioned in resin-dentin sticks (0.9 mm x 0.9 mm) for microtensile bond strength testing. Sticks from the most peripheral area presenting remaining enamel were excluded. The sticks were attached to a jig with a cyanoacrylate cement (Super Bonder gel, Loctite, Henkel Corp., Rocky Hill, USA) and tested to tensile failure in a universal testing machine (DL2000, EMIC, Sao Jose do Rio Preto, Brazil) with a 500-N load cell and 0.5 mm/min cross-head speed. The exact cross-sectional area of each tested stick was measured with a digital caliper. The µTBS results were calculated and expressed in MPa. The µTBS values obtained from the sticks of the same resin-bonded tooth were averaged. Mean bond strength of each individual tooth was used as one unit for statistical analysis. Three resin-bonded teeth (n = 3) were evaluated for each sub-group. The µTBS data were statistically analyzed by two-way ANOVA (presence of zol-primer and aging regimen) and Tukey's post-hoc test at 5% significance level. Subsequent to the µTBS testing, the mode of failure of each fractured stick was determined using a stereomicroscope (Olympus SZ 40-50; Tokyo, Japan) at 100X magnification. The fractures were classified as adhesive, mixed, cohesive in composite or cohesive in dentin.

Nanoleakage analysis

Two resin-dentin sticks were selected from each bonded tooth and storage condition during the cutting procedure. These sticks were immersed in 50wt% ammoniacal silver nitrate ($AgNO_3$ (aq)) solution in complete darkness for 24h (16). Subsequently, the specimens were rinsed with distilled water to remove the excess of silver nitrate and immersed in photo-developing solution for 8h under light to reduce silver ions into metallic silver grains. The silver-impregnated sticks were embedded in epoxy resin and polished using 600-, 1200-, 2000-grit SiC papers and diamond pastes (Buehler, Lake Bluff, IL, USA) with 1 and 0.25 µm particle sizes, and ultrasonically cleaned of 15min after each abrasive/polishing step. Specimens were finally air-dried, dehydrated overnight in silica gel under vacuum, coated with carbon and analyzed using SEM

(Inspect 50, FEI, Amsterdam, Netherlands) and observed in backscattered electron mode at 20 kV.

RESULTS

Microtensile bond strength testing (μTBS)

The two-way ANOVA showed significant interaction ($p<0.001$) between the specific treatments and the aging regimen. Tukey's test indicated no significant differences among all groups before load cycling ($p>0.05$), and both groups treated in self-etch mode provided stable bond strength after load cycling ($p>0.05$). Conversely, adhesive applied in etch-and-rinse mode depicted bond strength reduction after load cycling without zol-primer ($p=0.006$), and especially when zol-primer was applied adjunctively ($p<0.001$) reaching very low bond strength (8.0±2.1 MPa). All numeric values in means and standard deviations are presented in Table 2.

Scanning electron microscopy (SEM)

The nanoleakage results of the samples analyzed by SEM are shown in Figure 1~~2~~. Comparing the 24h group with and without zol-primer application, sparse silver impregnation was found only at the bottom of hybrid layer. When etch-and-rinse technique was employed, water channels were detected reaching adhesive-resin joint without zoledronate (Fig. 1~~2~~C). However, a silver-free zone at the top of adhesive layer was found when zol-primer was used (Fig. 1~~2~~D). After mechanical load cycling in self-etch mode, and increase of nanoleakage was observed at hybrid layer and bottom of adhesive layer, which was accompanied by a reduction of adhesive layer thickness only for zol-primer treated specimens (Fig. 1~~2~~F). This reduction of adhesive layer thickness was also detected when etch-and-rinse specimens without zol-primer were subjected to load cycling (Fig. 1~~2~~G). In addition, gaps were found at the top of adhesive layer. When zol-primer was combined with etch-and-rinse technique after load cycling (Fig. 1~~2~~H), some gaps were also observed and located at the hybrid layer. Water trees were observed along the entire interface of cycled etch-and-rinse specimens regardless the application of zoledronate.

DISCUSSION

According to the results obtained, the hypothesis that zoledronate affords dentin bond resistance after mechanical load needs to be rejected. Yet, the second hypothesis that zol-primer induces no changes in interfacial silver nanolakege must also be rejected. By analyzing the results of microtensile bond strength test after 24h storage in water, in both adhesion technique (SE and ER), the application of zol-primer did not cause statistically significant alterations, thereby demonstrating full compatibility with the universal adhesive (ScotchBond Universal, 3M-ESPE) used in the study without jeopardizing initial bond strength. When comparing the results obtained after load cycling, the pre-treatment was only effective in self-etch strategy.

The application mode (self-etch or etch-and-rinse) also did not affect the initial dentin bond strength of universal adhesives, what are in agreement with previous investigations (17–19). The action of phosphoric-acid etching or acidic functional monomers plays a fundamental role on smear layer demineralization/modification and demineralization of underlying dentin in order to create spaces for resin monomers infiltration (4,20). In dentin, more hydrophilic monomers may penetrate the network of exposed collagen fibrils, thereby creating the interdifusion zone (hybrid layer) after in situ polymerization (9,21–24).

Host-derived matrix metalloproteinases (MMPs), found both in saliva and in dentin, have been shown to be involved on the degradation of the unprotected collagen fibrils within the hybrid layer (Chaussain-Miller *et al.*, 2006; Hannas *et al.*, 2007). The durability of dentin bonding agents is affected by the degradation of the resin compounds occurring *via* hydrolysis of sub-optimally polymerized hydrophilic resins and degradation of collagen matrices by matrix metalloproteinases (MMPs) and cysteine cathepsins (Tjaderhane 2013). Nevertheless, current attempts to extend the longevity of resin–dentin bonds via incorporation of matrix metalloproteinase inhibitors (Carillho et al. 2010) lack to address the mineral phase re-deposition in demineralized dentin collagen, thereby providing fossilization of MMPs (Brackett et al. 2011).

The use of bioactive materials in order to remineralize collagen fibrils may provide a feasible strategy to extend the longevity of resin–dentin interface (Profeta et al. 2012). A poly(phosphonic acid) such as zoledronate may provide MMP inhibition (Sulkala et al., 2001) due to its chelation ability (Tezvergil-Mutluay *et al.*, 2014). The combination of these two agents (bioactive materials and MMP inhibitiors) might represent a further method for the inhibition of endogenous proteases within the resin-dentin interface as recently demonstrated by zoledronate-containing primer and ion-releasing resins in the investigation of Tezvergil-Mutluay and collaborators (2014). Concerning the activation of MMPs, when dentin is completely demineralized by phosphoric acid, a severe release of the calcium and phosphates necessary for MMP activation is warranted (Tezvergil-Mutluay *et al.*, 2014). Phosphoric acid etching in etch-and-rinse adhesion strategy affords higher exposure of collagen fibrils which would hypothetically promote more suitable substrate for the action of zoledronate.

Nevertheless, according to results in Table 2, the use of the zol-primer cannot be indicated in association with etch-and-rinse adhesive technique due to the striking decrease of bond strength after mechanical load cycling. Contrariwise, zol-primer does not impair the bond strength when used prior to the self-etch strategy. This shortcoming is dependent on adhesive technique. Zoledronate can bind to collagen fibrils (Ryan et al., 2007) which may jeopardize monomers infiltration in exposed dentin when used in acid-etched dentin before adhesive application, as observed in the present outcomes (Table 2). Such results are reported herein for the first time, once the present study is the first to survey the combination of zoledronate-primer with universal adhesives in terms of bonding performance. After load cycling, this negative effect was more noteworthy due to the breakdown of resin-sparse demineralized fibrils. Lower concentration of zoledronate or the usage of other etch-and-rinse adhesives could surpass this effect, thereby demonstrating the advantages of zol-primer over longer time period of aging.

In the present study, Single Bond Universal (3M-ESPE) was employed. Its specific composition contains 10-MDP acidic functional monomer, which possesses a 10-carbon hydrophobic extending spacer chain and demonstrates chemical adhesion to the hydroxyapatite, forming stable calcium phosphate salts without causing intense demineralization (32). The chemical adhesion formed by 10-MDP is more stable in water than that formed by other monomers used in further self-etch adhesives, such as 4-META or Phenyl-P (33). The 10-MDP monomer is adsorbed on the surface of the hydroxyapatite forming a multiple layered structure (nanolayering) that provides high durability for dentin/enamel bonds (Yoshihara et al., 2011).

When comparing the images of nanoleakage (Figure ~~2~~1), when self-etch technique was used, low silver infiltration located only at the bottom of hybrid layer was found, regardless the presence of zoledronate (immediate images). After load cycling, both self-etch groups depicted increased nanoleakage in the hybrid layer, and particularly for zoledronate treated speicmens, the thickness of adhesive layer had a notable reduction, suggesting a lower degree of polymerization and a lower modulus of elasticity. Indeed, this might result in higher degradation over time. One may speculate that the high chelating activity of zoledronate is responsible for the capture of ions H^+ during amine co-initiating photo-activation of camphoroquinone which may alter the degree of conversion (Feitosa et al. 2012).

For etch-and-rinse technique, we observed greater nanoleakage than in self-etch groups thanks to the action of phosphoric acid etching resulting in a greater degree of demineralization and removal of smear plugs, thereby increasing dentin permeability (Tay-Sauro). When the experimental zoledronate-containing primer was used, the bisphosphonate tends to bind mineralized tissue in peritubular dentin, partially occluding dentin tubules and reducing permeability. In fact, decreasing the permeability, overall nanoleakage is reduced which explains why the detection of silver nitrate did not occur in the upper portion of the adhesive layer of zol-primer treated specimens. Furthermore, the presence of zoledronate on etched dentin may interfere with the infiltration of adhesive monomers around collagen fibrils. Therefore, after load cycling, the area with higher presence of water (weak zone) was at the hybrid layer (Figs. 1~~2~~D and 1~~2~~H), which explains the gaps in this part of the interface (Fig. 1~~2~~H).

By observing the specimens created by etch-and-rinse technique without zol-primer treament (control), the presence of water (nanoleakage) at the top of adhesive layer was found (Fig. 1~~2~~C). In this case, the absence of zoledronate and its reduction on dentin permeability resulted in more permeable adhesive layer prone to osmosis, explaining the flow of water through almost the entire thickness of adhesive layer (water trees). Clearly, hydrolysis as well as incomplete polymerization occur due to water seepage turning this part of the interface (top of adhesive layer) more susceptible to degradation and fractures, particularly after load cycling (Fig. 1~~2~~G).

In the present study, important adverse effects on the adhesive resin were noted after mechanical stress when etch-and-rinse strategy was employed, especially when adjuctive use of zol-primer was undertaken. However, several investigations (Feitosa et al. 2012, Brackett et al. 2011) demonstrated the barriers of etch-and-rinse technique, such as lack of optimal solvent evaporation, adequate polymerization and impaired dentin sealing (Sauro et al 2010). Nevertheless, current literature (Muñoz et al. 2015) suggests higher durability of dentin bonds for the self-etch application of universal adhesives, which may favour the adjuctive use of zol-primer. Indeed, future studies might focus on reduced concentrations of zoledronate in the primer as well as the combination with more dentin bonding agents. Certainly, the incorporation of MMP inhibitors (i.e. zoledronate) into dental adhesives may be one of the suitable strategies to improve the durability of resin-dentin bonds.

CONCLUSION

Zoledronate-containing primer does impair the bond durability of an universal adhesive applied in etch-and-rinse mode after mechanical load cycling. However, minor alterations on water impregnation in the interface suggest reduced dentin permeability. Moreover, zoledronate treatment had no adverse effects when the adhesive is applied in self-etch technique.

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FiGURES

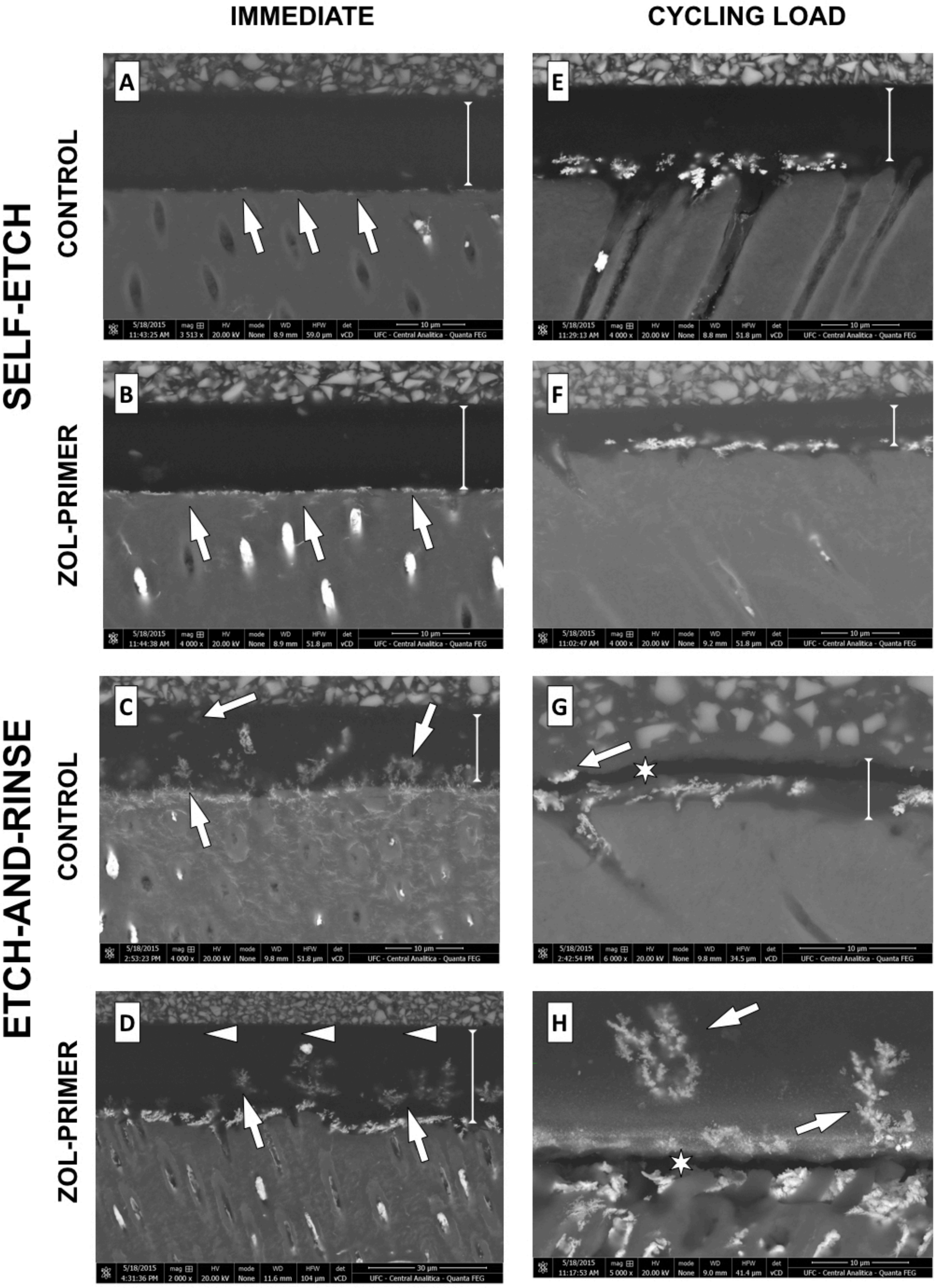

Figure 1. Scanning electron representative micrographs of resin-dentin specimens impregnated with silver nitrate. Figures A and B demonstrate low silver infiltration located only at the bottom of hybrid layer (arrows). When etch-and-rinse technique was employed (Figures C and D), water channels were detected (arrows) reaching adhesive-resin joint without zoledronate, but a well-

polymerized silver-free zone at the top of adhesive layer (arrowheads) was found when zol-primer was used. In this case, most nanoleakage was concentrated at hybrid layer. After mechanical load cycling in self-etch mode (Figs. E and F), and increase of nanoleakage was observed at hybrid layer and bottom of adhesive layer, which was accompanied by a reduction of adhesive layer thickness only for zol-primer treated specimens. Such reduction was also found when etch-and-rinse specimens without zol-primer were subjected to load cycling (Fig. G). Moreover, several gaps (asterisk) were present at the top of adhesive layer separating silver deposits (arrow). When zol-primer was combined with etch-and-rinse technique, gaps were located at the hybrid layer after load cycling (asterisk in Fig. H). Water trees were observed along the entire interface of cycled etch-and-rinse specimens regardless the application of zoledronate.

TABLES

Table 1 – Materials, compositions and application procedures

Materials	Composition	Application
Composite Resin **THP Spectrum®** **Dentsply SHADE A3**	Matrix : BisGMa, UDMA Filler : Barium Glass, Aluminium Boron Silicate, Pyralytic Silonized Silicia	Apply in 1–2 mm increments and light cure for 40 seconds
Adhesive **Scotchbond™ Universal**	6-MHP Phosphate Monomer, Dimethacrylate resins, HEMA, Vitrebond™ Copolymer, Filler, Ethanol, Water, Initiators, Silane	Self-etch Total- etch
Experimental **Zol-primer**	Zoledronate [1-hydroxy-2-(1H-imidazol-1-yl) ethane-1,1-diyl]bis-phosphonic acid, ethanol (50 vol%)	Apply one coat of zol primer for 15 s with gentle agitation. Gently air dry for 10 second.

BiSGMA: bisphenol A diglycidylmethacrylate; UDMA: urethane dimethacrylate; MDP: 10-methacryloyloxi decyl phophate; HEMA: hydroxyethylmethacrylate

Table 2 – Means (standard deviations) μTBS of each group in MPa.

μTBS (MPa)	Control	Load Cycled
Self-etch	26.4 (1.9) A,a	26.1 (4.1) A,a
Self-etch + Zol	29.7 (5.8) A,a	22.5 (4.6) AB,a
Etch-and-rinse	26.5 (4.4) A,a	18.8 (4.1) B,b
Etch-and-rinse + Zol	28.1 (4.2) A,a	8.0 (2.1) C,b

Different capital letters represent statistically significant differences in column among adhesives ($p<0.05$). Different lowercase letters depict significant difference in row between control and load cycling ($p<0.05$).