QUANTITATIVE AND QUALITATIVE EVALUATION OF RESIN-DENTIN BOND STRENGTHS: IN VITRO EFFECTS OF SURFACE TREATMENT WITH METALLOPROTEINASE INHIBITORS

ABSTRACT

AIM: The objective of this study was to evaluate the use of MMPs inhibitors (chlorhexidine and EDTA) in bond strength and quality of the hybrid layer of adhesive restorations in normal dentin using two ethanol-based total-etch adhesive systems. **MATERIAL AND METHODS:** Thirty-two extracted human molars were coronally sectioned and randomly divided into 8 groups (n=4), depending on the surface pre-treatment and adhesive system used. The total-etch adhesive systems Single Bond 2 (2-step) and Adper Scotchbond Multi-Purpose Plus (3-step) were used as follows: 1) according to manufacturer's instructions (etching with 37% phosphoric acid (H_3PO_4) for 15 s); 2) etching with H_3PO_4 for 15 s, followed by 2% chlorhexidine for 120 s; 3) etching with 0.1 M EDTA for 60 s; 4) etching with 0.1 M EDTA followed by 2% chlorhexidine for 120 s. Teeth were incrementally restored with composite resin (Filtek Z350XT). After water storage for 24 h, teeth were double-sectioned, yielding stick specimens of 1.0 mm^2 bonded area, and then subjected to microtensile bond strength (MTBS) test at 0.5 mm/min. Additional specimens were gold-sputtered to be analyzed under scanning electron microscopy (SEM). Data (in Mega Pascal) were subjected to Kruskal-Wallis and Dunn ($p < 0.05$) tests. **RESULTS:** The etching protocol (37% H_3PO_4 or EDTA) interfered with hybrid layer formation, monomer penetration and the MTBS. Funnel shaped resin tags were observed when dentin was etched with 37% H_3PO_4. In these specimens, MTBS were also higher. EDTA conditioning produced thin hybrid layers and smaller MTBS, regardless the adhesive system used. Chlorhexidine application after conditioning resulted in no apparent differences between both evaluated techniques (37% H_3PO_4 or EDTA). **CONCLUSION:** The use of chlorhexidine as a MMP inhibitor does not alter immediate bond strength values and does not interfere with hybrid layer formation.

KEYWORDS

Dentin-Bonding agents. Metalloproteinases. Chlorexidine. EDTA.

TOTTI, Marcelo*
GOULART, Marcelo*
FAGUNDES, Laerte Ohse*
THOMÉ, Thaís**
CONCEIÇÃO, Ewerton Nochi***
COELHO DE SOUZA, Fábio Herrmann**
ERHARDT, Maria Carolina G.**

Fellow Research, Department of Conservative Dentistry, School of Dentistry. Federal University of Rio Grande do Sul, Porto Alegre, Brazil*
Adjunct Professor, Department of Conservative Dentistry, School of Dentistry. Federal University of Rio Grande do Sul, Porto Alegre, Brazil**
Associated Professor, Department of Conservative Dentistry, School of Dentistry. Federal University of Rio Grande do Sul, Porto Alegre, Brazil***
Correspondence: carolinabee@hotmail.com (ERHARDT MCG)

| **Received** 18 Mar 2014 **Received in revised form** 22 Mar 2014 **Accepted** 25 Mar 2014

INTRODUCTION

Important advances in Adhesive Dentistry have occurred in the last two decades.[1,2] These advances are essentially based on the bonding between two substrates of different nature: the dental substance and the adhesive based restorative material.

However, undesirable consequences (such as recurrent caries and marginal discoloration) are normally found in composite resin restorations after some time in clinical function.[3,4] Failures in monomer infiltration and/or incomplete polymerization may create defects in the adhesive layer, resulting in demineralized dentin areas with exposed collagen, surrounded by interfibrillar nanospaces filled by water , activating proteolytic enzymes from the dentin matrix called metalloproteinases (MMPs), which hydrolyzes actively the protein substrate.[5]

These collagenases are in the collagen matrix exercising its action in the presence of bacterial inhibitors, what lead us to conclude that the collagen destruction may occur by an endogenous pathway.[6-9]

Nowadays researches are focused on the development of bonding techniques which could improve substantially the tooth-restoration interface longevity through reduction of MMPs action.[10-13] These enzymes are Zn/Ca-dependents and are able to degrade the organic matrix of dentin after demineralization.[14] The enzymes with gelatinolytic capacity that fundamentally appear on the dentin matrix and carious lesions are MMP-2, 8, 9 e 20.[15] These endoproteases have capacity to degrade almost all the extra cellular matrix components of connective tissues, and inside the oral cavity, they play an important role in the progression of diseases like caries and periodontitis.[11]

Dentin bonding agents based on the total etching technique are able to reactivate endogenous MMPs in the etched dentin, submitting the collagen fibers to proteolytic degradation early months after performance of adhesive procedures.[16] pH values between 2.3 and 5 are effective to activate salivary gelatinases in a process described as acid activation.[10,17] Then, the existence of proteolytic activity after the application of a two-step conventional adhesive (pH= 4.3) can be explained by the increase of the MMPs activation, what represents a reduction of the adhesive effectiveness of these materials.[11] Notwithstanding, decreased proteolytic capacity of these enzymes is able to reactivate through a clinic activation process from additional pro-enzymes. Thus, MMPs inhibitors may, in similar way, lose activity along time.[18]

Ethylenediaminetetraacetic acid (EDTA) is a potent MMPs inhibitor, because it is able to remove and neutralize them.[9] Besides, this acid does not demineralize collagen fibers in depth, as ordinarily observed with phosphoric acid,

but it only dissolves the extrafibrillar calcium, allowing its total infiltration by the monomers, which protects from the MMPs collagenolytic action of dentin matrix when recover it.[19]

Chlorhexidine also presents MMPs inhibitor properties, even in low concentrations . MMPs inhibition (mainly -2, -8 e -9) is possibly result from the chelating zinc-dependent property.[15] Despite chlorhexidine had become one of the most popular MMPs inhibitor to prevent degradation of dentin-bonding adhesive interfaces,[12] little is known about its interaction with EDTA when used to acid-etch dentin.

Therefore, the aim of this study was to evaluate the influence of the use of MMPs inhibiting substances (chlorhexidine and EDTA) in the bond strength and in the quality of hybrid layer of adhesive restorations performed in sound dentin using two ethanol-based conventional adhesive systems (3- and 2-step). The null hypothesis tested was that the use of these MMPs inhibitors does not decrease the immediate bond strength when compared to those obtained with bond systems applied according to the manufacturer's instructions.

MATERIAL AND METHODS

This study was approved by the Research Committee and the Ethics Research Committee of the School of Dentistry of Federal University of Rio Grande do Sul (UFRGS).

Thirty-two freshly extracted sound human molars were selected, and stored in 0.1% (weight) thymol suspension for disinfection until the moment of use. Occlusal enamel was removed in the amelo-dentinal junction by a perpendicular cut along the dental axis with a double face diamond disc (*KG Sorensen Ind. e Com. Ltda, Barueri, SP, Brazil*). After dentinal surface exposure, the occlusal face of each tooth was polished with silicon carbide sandpaper of decreasing grain #150, #320, #400 and #600 (*Saint-Gobain Adesivos Ltda., Jundiaí, SP, Brasil*) coupled on a mechanical polishing (*APL-4, AROTEC S.A., Cotia, SP, Brazil*), under constant irrigation.

The samples were initially randomized into four groups, according to the following surface treatments: H_3PO_4 – Dentin surfaces were etched with 37% phosphoric acid during 15 s, washed by 15 s and gently dried with absorbent papers; H_3PO_4 + 2% chlorhexidine – Dentin surfaces were etched with 37% phosphoric acid during 15 s and washed by 15 s; 2% chlorhexidine solution was actively applied for 120 s, and subsequently dried with absorbing papers; EDTA 0.1 M – Dentin surfaces were etched with EDTA 0.1 M during 60 s, washed by 15 s and gently dried with absorbent papers; EDTA 0.1 M + 2% chlorhexidine 2% - Dentin surfaces were etched with EDTA 0.1 M during 60 s and washed by 15 s; 2% chlorhexidine solution

was actively applied during 120 s, and after dried with absorbing papers.

Filtek Z350XT (3M Dental Products Division, St. Paul, MN, USA) composite resin was inserted in 3 increments of 2 mm over the surface previously treated surface; each increment was photoactivated during 40 seconds with Optilight Max device (*Gnatus Equipamentos Médico-Odontológicos Ltda., Ribeirão Preto, SP, Brazil*).

After the storage period (24 h in water at 37˚C) the restored teeth were sectioned vertically (*EXTEC ® Labcut 1010 - Low Speed Diamond Saw, Extec Corp., Enfield, CT, USA*) into slices and posteriorly cut in sticks with adhesive area about 1 mm^2. The specimens were fixed in a microtensile testing device (*Odeme Equipamentos Médico e Odontológicos LTDA, Joaçaba, SC, Brazil*) with cyanoacrylate-based adhesive (*Super Bonder, Henkel Loctite Adesivos LTDA, Itapevi, SP, Brazil*) and tensioned until fracture in a Universal Testing Machine (*EMIC DL500, EMIC LTDA, São José dos Pinhais, PR, Brazil*) at 0.5 mm/min speed. Fractured sticks were carefully removed from the microtensile device and the bonded area was measured with a digital caliper (*Mitutoyo MDC Lite 293, Mitutoyo Sul Americana São Paulo, SP, Brazil*). Bond strength values were analyzed by Kruskal-Wallis test with significance level of 0.05.

Fractured specimens were examined in an optical microscope (*SZ 51 Olympus Optical do Brazil Ltda, São Paulo, SP, Brazil*) in 40X magnify to determine the fracture type, that were classified in cohesive in dentin, resin, adhesive or mixed.

Additional bonded slices from each experimental group were analyzed through scanning electron microscopy (SEM). Specimens were polished with silicon carbide sandpaper of decreasing abrasiveness, treated with 37% phosphoric acid for 30s and 10% sodium hypochlorite during 2 minutes, to evidence the resin infiltration inside the dentin. Finally, specimens were gold-sputtered (*Med 10, Balzers Liechtenstein*), and bonded interfaces were analyzed with scanning electron microscopy (*JEOL JSM 5600LV, JEOL, Japan*) in 1000, 1500, 2000, 2500 and 4000 X magnifications.

RESULTS

Table 1 illustrates the bond strength results (MPa) obtained after the microtensile test in each experimental group. Because of non-normal distribution data, a non-parametric analysis was carried out using the Kriskal-Wallis statistical tests ($p<0.001$), followed by the Dunn multiple comparison test.

Higher bond strength values were obtained by the experimental groups which received H_3PO_4 acid etching. The application of chlorhexidine did not interfere on the bond strength values, no matter the etching method

used: phosphoric acid (G1, G2, G3, G4) or EDTA (G5, G6, G7, G8), for both adhesive systems tested.

Table 2 displays the distribution of fracture patterns in the different experimental groups. It is possible to observe that most specimens in all groups presented mixed fractures.

From experimental groups treated with phosphoric acid (Figure 1) a homogeneous thick hybrid layer with good interaction with dentinal tissue was obtained, also presenting good penetration of resin monomers inside the dentinal tubules. On the other hand, experimental groups treated with EDTA (Figure 2), presented a thin hybrid layer and poor integration with subjacent dentin, as the penetration of resin monomers inside the dentinal tubules were much more discrete. The comparison among groups also shows that, no matter the adhesive system used, the hybrid layer and resin tags formation is higher in those groups in which had 35% phosphoric acid as etching agent. It is also possible to observe that the use of chlorhexidine does not influence hybrid layer formation, confirming that the hybrid layer formation and resin tag penetration is clearly determined by the etching protocol adopted.

DISCUSSION

The objective of this study was to evaluate the use of MMPs inhibitors (chlorhexidine and EDTA) in bond strength and quality of the hybrid layer of adhesive restorations in sound dentin using two ethanol-based total-etch adhesive systems (3- and 2-step). The null hypothesis of this study was partially refused, since EDTA decreased the immediate bond strength between adhesives and dentin; but for chorhexidine the null hypothesis was confirmed, because its use did not change the immediate bond strength between the substrates.

Regarding the use of chorhexidine previously to the adhesive, it is possible to verify by the obtained results of this study that its use did not change the bond strength values, and was only affected when the etching protocol was modified. The obtained results are in accordance with previously published studies.[10-13]

SEM images (Figures 1 and 2) suggest no changes in the immediate bond strength – when the use of chorhexidine is carried out previously to the adhesive protocol – once resin tag formation and hybrid layer thickness pattern are unalterated, only affected when the etching protocol is altered.

Chlorhexidine is able to inhibit the MMPs action through a chelating mechanism, and it is believed that the application time of 30 seconds is able to impregnate the collagen fibrils to provide a true inhibition effect. Even this relation of inactivation of enzymes by the chlorhexidine needs to be better elucidated, it

seems to be one of the keys to achieve an effective improvement in the durability of bond between the composite and the dental element by preserving of hybrid layer and consequent stability of bond strength.[11]

Table 1. Microtensile bond strength values obtained in the experimental groups (MPa).

GROUPS	N	Median	25%	75%	Dunn (5%)
1. H_3PO_4 + Adper Single Bond	20	21.74	16.64	26.57	A
2. H_3PO_4 + SBMP	20	19.09	14.35	24.13	A
3. H_3PO_4 + CHX + Adper Single Bond	20	16.36	13.86	23.96	A
4. H_3PO_4 + CHX + SBMP	20	20.33	16.10	25.93	A
5. EDTA + Adper Single Bond	20	13.82	13.03	20.79	B
6. EDTA + SBMP	20	8.31	6.87	10.34	C
7. EDTA + CHX + Adper Single Bond	17	13.64	11.20	20.05	B
8. EDTA + CHX + SBMP	18	9.09	7.71	12.22	C

Equal letters represent groups with no significant statistically difference ($P \leq 0.05$)

Table 2. Distribution of fractured specimens in experimental groups (as percentage).

GROUPS	CD	CR	M	A
1. H_3PO_4 + Adper Single Bond	12.5%	18.8%	65.6%	3.1%
2. H_3PO_4 + SBMP	6.5%	12.9%	74.2%	6.5%
3. H_3PO_4 + CHX + Adper Single Bond	4.8%	23.8%	66.7%	4.8%
4. H_3PO_4 + CHX + SBMP	17.6%	17.6%	44.1%	14.7%
5. EDTA + Adper Single Bond	27.3%	13.6%	59.1%	0.0%
6. EDTA + SBMP	9.5%	33.3%	52.4%	4.8%
7. EDTA + CHX + Adper Single Bond	0.0%	23.5%	64.7%	11.8%
8. EDTA + CHX + SBMP	16.7%	11.1%	66.7%	5.6%

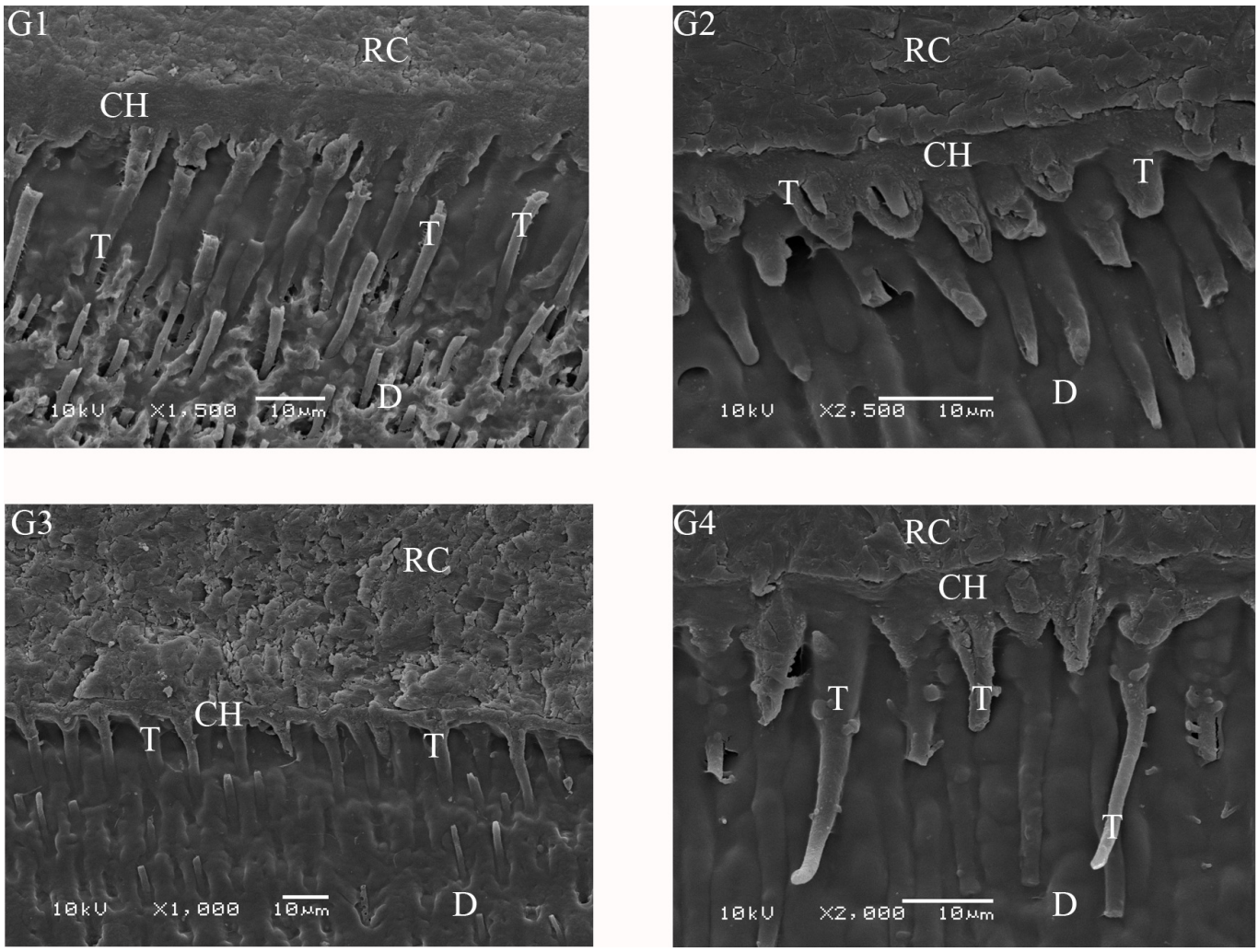
CD: cohesive fracture in dentin; CR: cohesive fracture in composite resin; M: mixed fracture; and A: adhesive fracture.

Several studies show that the previous application of chlorhexidine to the use of adhesive, after the application of phosphoric acid, seems to be effective in reduce the loss of bond strength along the time.[10] Carrilho et al.[10] (2007) defends the use of chlorhexidine as a primer of the adhesive system that should not be washed, because the exposed collagen fibrils due to the application of acid became impregnated by chlorhexidine and they are posteriorly sealed by the composite. This sealing may allow that chlorhexidine to remain retained, and hence continue inactivating the MMPs action along the time.[17]

Ethylenediaminetetraacetic acid (EDTA) is another powerful metalloproteinases inhibitor.[12] Some studies have tested acid etching with EDTA instead of the phosphoric acid,[12,20,21] because EDTA is able to remove hydroxyapatite without damage to the collagen matrix.[20] In the present study, the use of EDTA as etchant had the worst microtensile results (Table 1), independent of the use of chlorhexidine, as well as the adhesive system. These findings presented confirmation by the

qualitative evaluation of images through SEM (Figure 2), because it was observed the formation of a thin hybrid layer and little infiltration of resinous monomers inside the dentinal tubules.

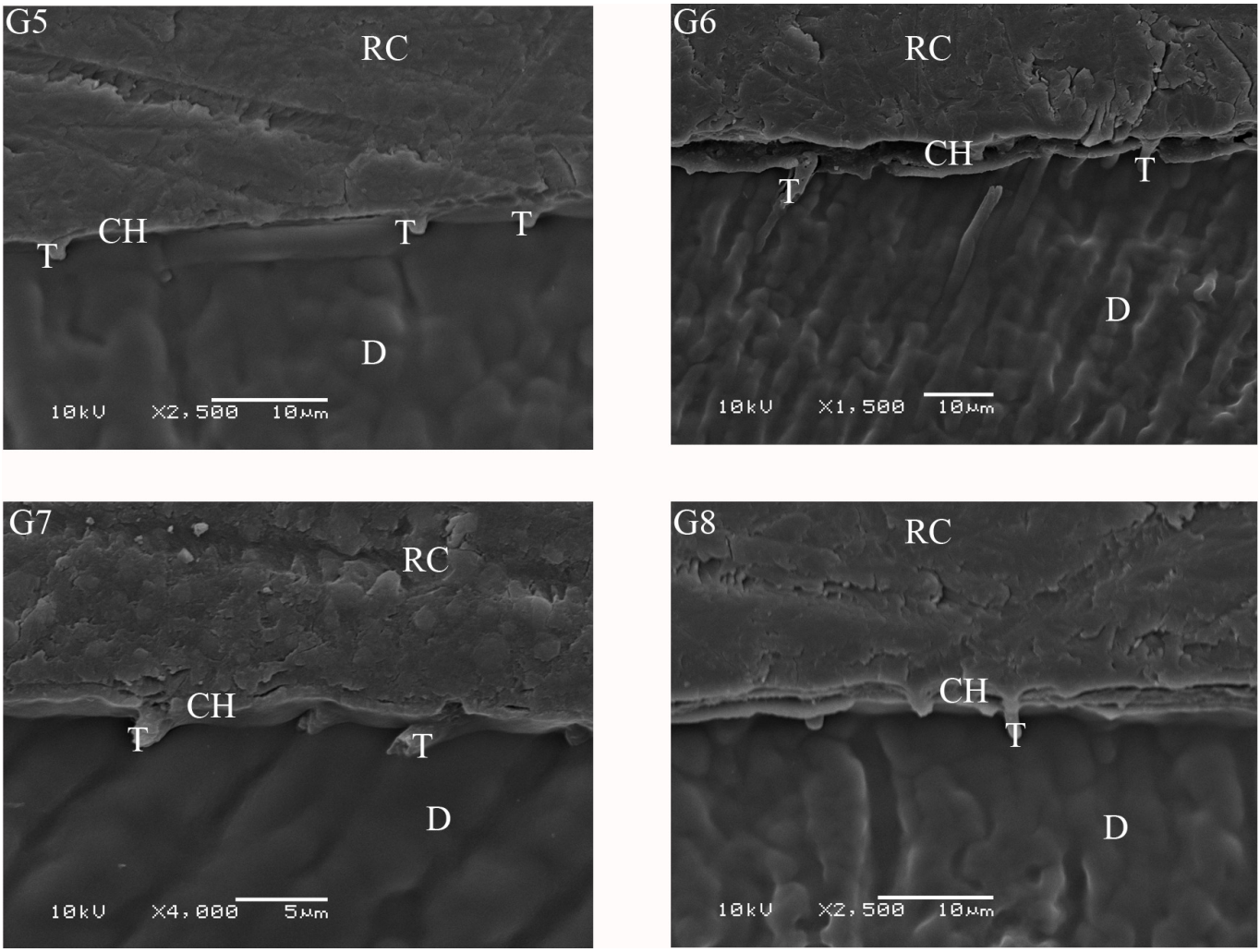
Figure 1. Comparison among the groups etched with H_3PO_4 (1, 2, 3 and 4). Thick hybrid layer (CH) with good tag penetration (T) in dentin (D). Group 1 (G1), group 2 (G2), group 3 (G3) and group 4 (G4).



Probably EDTA application did not provide an optimum smear layer removal, consequently not providing a suitable surface for proper resin tag and hybrid layer formation.[20] In the work carried out by Jacques and Hebling[20] (2005), it was observed that when EDTA was applied as acid agent with posterior use of self-etching adhesive, the immediate bond strength was higher; however, when the adhesive used was Single Bond (conventional 2-step) with EDTA as etchant, lower bond strength was obtained. According to the authors, it may be explained due to Single Bond´s low pH. Subsequent works performed by Osorio et al.[21] (2005) and Erhardt et al.[12] (2008) showed different results

from the present study and from those published by Jacques and Hebling[20] (2005).

Figure 2. Comparison among the groups etched with EDTA (5, 6, 7 and 8). Thinner hybrid layer (CH), with sparse tags penetration (T) in dentin (D). Group 5 (G5), group 6 (G6), group 7 (G7) and group 8 (G8).



It may be concluded that the use of chlorhexidine as a primer does not affect the immediate bond strength between dentin and composite, and does not have any negative effect on hybrid layer formation or monomer infiltration inside the dentinal tubules. New trends in Adhesive Dentistry is to integrate steps into the bonding technique, because as recent studies show, chlorhexidine application seems to be an effective MMPs inhibitor along the time . Therefore, one might expect to have this substance (or even other MMPs inhibiting substances) incorporated into adhesive´s formulations in order to reduce clinical steps.[20]

CONCLUSION

From the results obtained in the present study, it was possible to conclude that: 1. The

use of a chlorhexidine solution – in order to inhibit MMPs – applied as a primer after acid etching dos not interfere on the immediate bond strength, when conventional 3- and 2-step ethanol-base adhesive systems are used; 2. When EDTA was used as etchant, it did not provide good bond strength values, did not favor resin tag formation as well as was unable to provide homogeneous hybrid layers.

ACKNOWLEDGEMENTS

The authors acknowledge Prof. Dr. Marcus Vinicius Reis Só by equipment availability of in his laboratory. This Project has received financial support of Emergence Edict to Research (grant # 2011 – 5181) of UFRGS, and also the scholarship for scientific starting from the programs PROBITI-FAPERGS-UFRGS, PIBIC-CNPQ-UFRGS e PROBIC-FAPERGS-UFRGS.

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