Evaluation of Chlorhexidine on the Quality of the Hybrid Layer in Noncarious Primary Teeth: An In Vitro Study

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ABSTRACT

It has recently been observed that chlorhexidine has the capacity to inhibit matrix metalloproteinase. Therefore, the object of this study was to assess the effect of chlorhexidine on the quality of the hybrid layer of noncarious primary teeth. In group 1, the teeth were subjected to acid-etching, chlorhexidine application, Single Bond adhesive insertion, and restoration with resin composite Z250. Group 2 received the same procedures, without the application of the antimicrobial agent. Twenty-five regions were examined by scanning electronic microscopy by blind examiners. The data obtained were statistically analyzed by the chi-square and student *t* tests at a 5% level of significance. The groups presented few interfacial gaps without statistically significant differences. Group 1 presented a larger number of areas with a visible hybrid layer (68%) vs group 2 (52%). The layer's thickness was 3.33 μm and 3.28 μm for groups 1 and 2, respectively (*P*=.94). The results showed that the clinical restorative protocol with the use of chlorhexidine application does not interfere significantly in the morphological characteristics of the hybrid layer.

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Metalloproteinases (**MMPs**) are a family of zinc-dependant proteolytic enzymes, responsible for degradation of the extracellular matrix of proteins.[1] and are found in dentin, dentinal fluid, and in total saliva.[2-4]

The enzymes MMP-2, MMP-3, MMP-8, MMP-9, MMP-14, and MMP-20 have been isolated in dental structures.[2-4] Recently, the expression of other metalloproteinases (1, 7, 10, 11, 13, 15, 16, 17, 19, 23, 24, and 25) were reported for the first time in odontoblasts and pulpal tissue.[5]

In dentinal tissue, MMPs 2, 8, 9 and 20 have been detected by enzymatic, polymerase chain reaction, and immunoelectrophoresis tests.[1,3,6] Sulkala et al. concluded that MMP-8 represents the major collagenase in human dentin.

Matrix metalloproteinases are enzymes capable of degrading the collagen of dentin. They can be activated by the lowering pH as a result of treating the tooth surface with primer and adhesive, or biochemistry of the carious process, leading to a greater degradation of the collagen fibrils and higher risk of nanoleakage.[6-9]

The bond between resin and dentin is created by mechanical adhesion between the hydrophobic and hydrophilic monomers and collagen fibrils, which forms the "hybrid layer."[10]

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Imperfect adhesive polymerization can result in bond defects. These areas are created by the discrepancy between the depth of dentinal demineralization and the depth of adhesive infiltration. Theoretically, they are located between the hybrid layer and the mineralized dentin. Morphologically, they consist of exposed collagen fibrils surrounded by nanometric-sized interfibrilar spaces filled with water—a process called nanoleakage. This defect may occur even in the absence of microleakage.[11]

It has also been reported that nanoleakage can be present in areas in which the water was incompletely removed from the dentin-resin interface[11]. This residual water can induce incomplete polymerization of the hydrophilic polymers of the adhesive, originating the nanoleakage. This defect is represented by the transmission electronic microscopy as a silver deposits oriented perpendicular to the surface of the hybrid layer, called as a morphological arborescent manifestation, which is predetermined by the orientation of the interfibrilar spaces towards the matrix of demineralized collagen fibrils. This absorption of water by the hydrophilic monomers contributes to degradation at the dentin-resin interface over the course of time[11].

In summary, the degradation of the dentin-adhesive interface is initially due to the incomplete removal of water from the dentin by solvents present in the adhesives. This leads to incomplete polymerization, generating unstable areas capable of undergoing hydrolysis. Moreover, it has been demonstrated that not all demineralized dentin is covered by the adhesive, resulting in areas of exposed collagen fibrils. These areas can be degraded by the matrix metalloproteinases, which are activated by the process of dentinal treatment with primer and adhesive.[7-9] Therefore, activation of the MMPs and degradation of the collagen compromise the quality of the hybrid layer and increase the occurrence of nanoleakage.

Recent studies have shown that the release and activation of these endogenous enzymes, matrix metalloproteinases, during dentinal bonding may be responsible for the in vitro disappearance of collagen fibrils from the hybrid layer with time.[9]

Efforts were made to find a substance that inhibits the action of these proteinases. One substance that has shown desirable antiproteolytic properties is chlorhexidine.[7,13,14]

Based on the hypothesis that chlorhexidine could be an MMP inhibitor, enhancing the quality of the hybrid layer, the purpose of this study was to analyze by scan ning microscopy the quality of the hybrid layer formed on normal dentin at the dentin-resin interface in primary teeth when the application of chlorhexidine was included in the clinical protocol.

METHODS

This study was submitted and approved by the Ethics Committee of the Catholic University of Brasilia (Brasilia, Federal District, Brazil), being a randomized control trial formed by ten sound primary first or second molars.

These teeth were extracted at the final stage of rhizolysis, and had the root portion removed. The teeth were cleaned immediately after extraction and stored in an aqueous solution of 0.2% thymol, at 4°C, for up to 30 days. The coronal portion was separated from the root using carbide bur no. 330 (KG Sorensen, Barueri, Sao Paulo, Brazil) at high speed (350,000 rpm; Turbina Extra Torque 605, Kavo do Brasil SA, Joinville, SC, Brazil) at the cementoenamel junction. The pulp tissue was removed with a dental explorer.

Standardized radiographs of the teeth were taken, and the total dentin thickness (dentin-enamel junction to the pulp) measured. Half of this measurement was transferred to the preparation bur to obtain standardized dental depth preparations. The class I preparations were made with carbide bur no. 330 (KG Sorensen, Barueri, Sao Paulo, Brazil) at high speed (350,000 rpm; Turbina Extra Torque 605, Kavo do Brasil SA, Joinville, SC, Brazil).

The specimens were randomly divided into 2 groups (group 1 and group 2), with five teeth in each. All materials were used according to manufacturers' recommendations.

For group 1, The dentinal surface of each tooth was: etched with 35% phosphoric acid (3M ESPE, St. Paul, Minn) for 15 seconds, thoroughly washed with water for 15 seconds, coated with an aqueous solution of 2% chlorhexidine (Cav Clean, Dentsply, New York, NY) and left on for 30 seconds[14], and were lightly dried with absorbent paper to maintain the dentinal surface humid. A layer of the adhesive Adper Single Bond 2 (3M ESPE) was applied with a microbrush; another layer was applied and gently dried for 5 seconds. Polymerization was performed for 20 seconds. Filtek Z250 B1 (3M ESPE), was applied in 2 x 2 mm increments and polymerized for 20 seconds per increment, until the cavity was completely filled. This gradual polymerization technique was used to reduce polymerization shrinkage. Polymerization was performed with a second generation LED type light polymerizing appliance (DMC, Santo André, São Paulo, Brazil) with a light intensity of 600 mW/cm2.

After this procedure, final polymerization was performed for 40 seconds. This was confirmed by measurement with a radiometer (model no. 100, Demetron Research Corp, Danbury, Conn) before each sample was polymerized.

For group 2, each tooth was submitted to the same procedures with the exception of chlorhexidine application. Therefore, after acid-etching and washing the cavity, the tooth was gently dried with absorbent paper and the adhesive procedures were performed.

The samples were prepared in a single stage and in random sequence. After this, they were stored in water at 22°C until the moment of fracture and metallization.

The restorative material/dentin interface was exposed by fracturing specimens with a surgical blade[15] longitudinally, in the mesiodistal direction, obtaining a buccal and a lingual half.

The samples were prepared for later analysis by scanning electronic microscopy (model DSM 962, ZEISS, Oberkochen, Germany) with acceleration voltage of 15 Kv.

The hybrid layer thickness and the interfacial gap width were measured in 5 random sites in each tooth. The first interfacial gap measurement was made at about 300 μm from the crown's center, and the remainder were made at intervals of approximately 100 μm from the initial measurement. This methodological approach agreed with Telles et al.[15]

The depths of the gaps were measured at 1,500X magnification. The condition of the dentinal tubules was assessed and classified as partially filled and empty. The hybrid layer thickness was assessed at 4,000X magnification. Only visible hybrid layer areas were considered.

Each site was analyzed by 2 calibrated examiners, and consensus was reached for each measurement. The examiners were blind to group assignment at the time of microscopic evaluation.

The results were statistically analyzed, via chi-square and student *t* tests at a level of significance of 5%, to test the hypothesis that there was no difference in the quality of the hybrid layer when using chlorhexidine compared to the control group (group 2).

RESULTS

Of the 25 points analyzed in group 2, 3 (12%) presented gaps, while in group 1, 6 (24%) areas presented this defect (*P*=.46). The mean gap width value observed was 3.87 (±2.42) and 4.03 (±3.68) for groups 1 and 2, respectively (*P*=.93; Figure 1).

The condition of the dentinal tubules was also examined at 1,500X magnification (Table 1; Figures 1-3). It was not possible to perform analysis on 2 points and on 3 points in groups 1 and 2, respectively. This occurred because the type of fracture made it impossible to analyze the tubules' contents. The condition of the tubules was not shown to be significant for groups 1 and 2 (*P*=.56).

The hybrid layer was visible in 17 (68%) and 13 (52%) of the 25 points examined in groups 1 and 2, respectively. All the teeth presented at least 1 region with visible hybrid layer formation (Figures 4-6). The mean and standard deviation of the hybrid layer thickness is shown in Table 2.

Table 1. Analysis of Dentinal Tubules

	Group 1 (with chlorhexidine)		Group 2 (without chlorhexidine)	
	Frequency	%	Frequency	%
Empty	3	13	2	9
Partially filled	20	87	20	91
Total	23	100	22	100

Table 2. Means and Standard Deviations of the Hybrid Layer Thickness (μM)*

	Mean±(SD)
Group 1 (with chlorhexidine)	3.33±1.90
Group 2 (without chlorhexidine)	3.28±2.22

* *P*=.94

DISCUSSION

This in vitro study was designed to examine the quality of the hybrid layer when chlorhexidine was applied. 2 groups of teeth received the same dentin bonding procedures, with the exception of the chlorhexidine application. Only teeth in the final stage of rhizolysis were collected to ascertain a certain pattern of tooth age between the 2 groups assessed. This design reduced the influence of the tooth-related biases such as tooth-age, since with aging, there is a decrease in the number and diameter of the dentinal tubules, due to continuous intratubular dentin mineralization, which interferes with the degree of dentinal permeability.[16] To reduce bias related to dentin depth, all the teeth received Class I preparations. This minimized the variations in the dentinal structures when the buccal, lingual, or other surfaces were exposed.[17]

Different procedures used for cavity preparation produce different smear layers. Consequently, the use of water-abrasive papers or diamond disks for cutting the dentin create different surfaces from those obtained in the clinic, and could produce different types of smear layers. This altered substrate could affect results that are not comparable with the clinical situation.[18] To eliminate this bias and better simulate the in vivo situation, tooth preparation was performed with a carbide bur under constant water irrigation.

The depths of the cavities prepared in the present investigation were standardized regarding exposure of mean area of the dentin so to create similar mechanical properties and, similar bond strength.[19]

It is important to emphasize that 5 areas per tooth were examined, resulting in 25 regions examined per group. The sample size was compatible with and adequate for the proposed objective.[15,17,18]

Perfect dentin-adhesive interface sealing is mainly achieved by the absence of interfacial gaps, since this is an irreversible process that could lead to microleakage. Analysis of these defects demonstrated gaps in groups 1 and 2, with no statistical significant differences (*P*=.46). In these regions, the dentinal tubules were shown to be empty and, therefore, lacking the formation of resin tags (Figure 1). In these areas, hybridization was not satisfactory: the acid-etching process caused dentin demineralization and the adhesive was not able to penetrate the entire region attacked, as demonstrated by the appearance of the dentinal tubules. Therefore, the dentin-

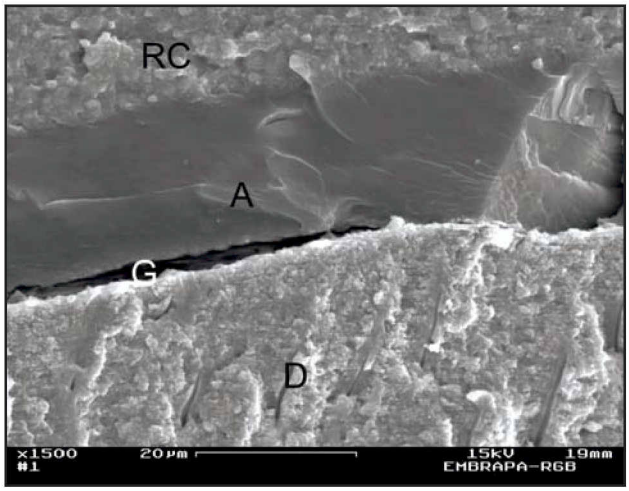

Figure 1. Gap (G), partially filled dentinal tubules, in a sample from group 1, site D (A=adhesive; D=dentin; RC=resin composite).

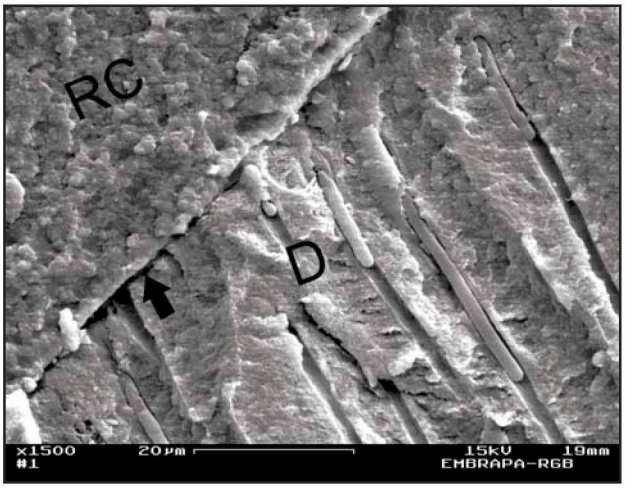

Figure 2. Partially filled dentinal tubules, hybrid layer (arrow) in a sample from group 2, site C (D=dentin; RC=resin composite).

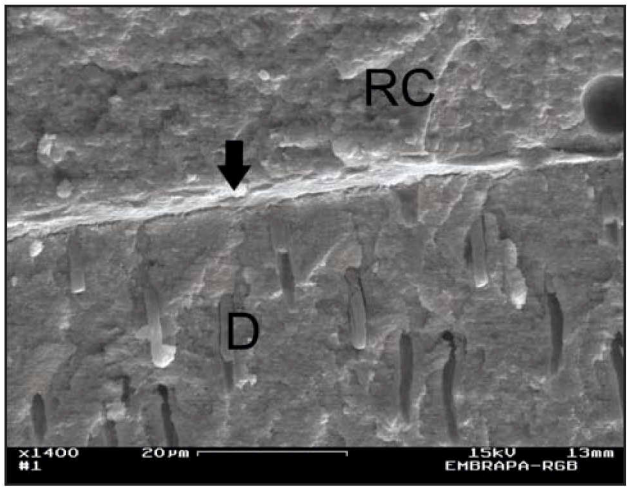

Figure 3. Partially filled dentinal tubules, hybrid layer (arrow) in a sample from group 1, site C (D=dentin; RC=resin composite).

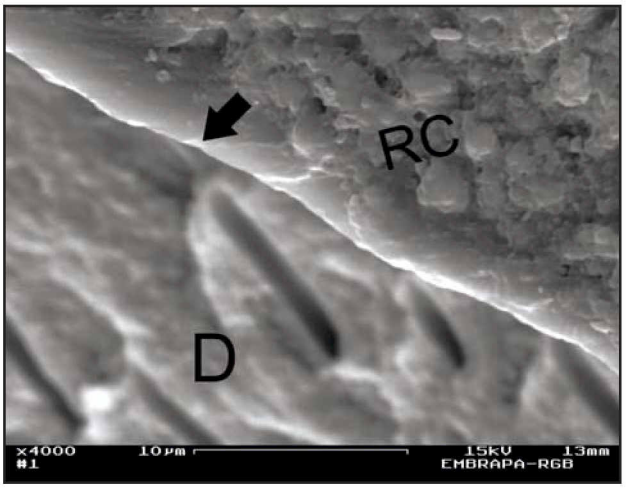

Figure 4. Visible hybrid layer (arrow) in a sample from group 1, site A; empty dentinal tubules (D=dentin; RC= resin composite).

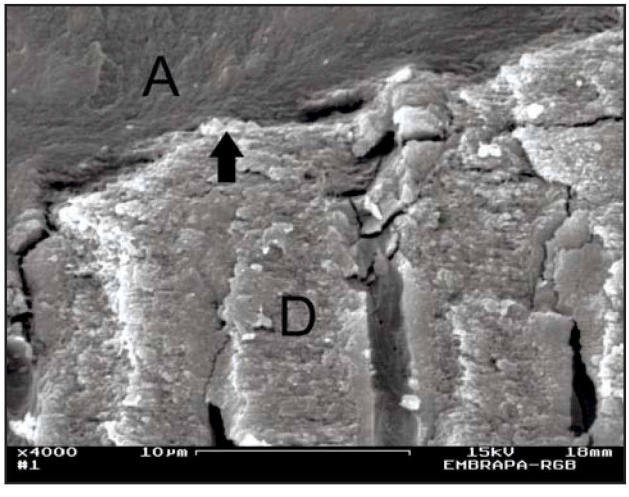

Figure 5. Visible hybrid layer (arrow) in a sample from group 2, site D; empty dentinal tubules (D=dentin; A=adhesive).

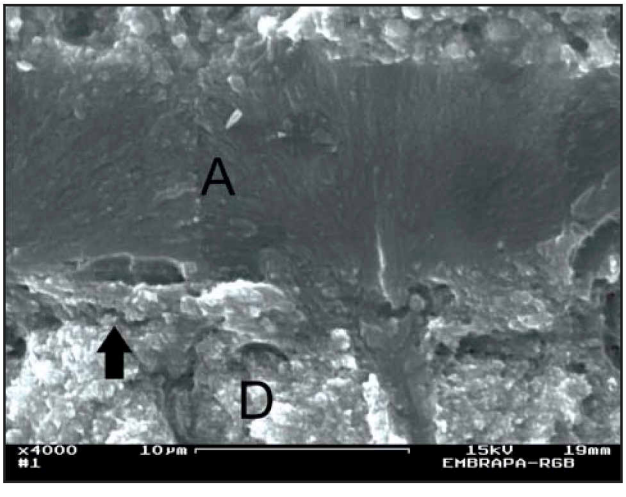

Figure 6. Visible hybrid layer (arrow), with resin tag formation, in a sample from group 1, site B (D=dentin; A=adhesive).

adhesive interface was not perfect and was susceptible to fracture by any mechanism that might cause stress at the interface, as had occurred with the fracture procedures or the vacuum of the scanning electron microscope.

The mean depth of these defects was slightly greater in group 2 (4.03 μm) than in group 1 (3.87 μm), although the difference was not significant (P=.93; Figure 1).

These values were much lower than the results reported by Telles et al.[15]: 9.9 μm for primary teeth treated with a single-step adhesive. The explanation could be the difference in the adhesive agents used. Hosoya[16] also found frequent gaps in the group of primary teeth treated with Single Bond adhesive.

Perfect dentin-resin interface sealing can be established by the adhesive completely filling the dentinal tubules and the demineralized collagen matrix.[21] When observing the contents of the dentinal tubules, 91% of group 2 areas were shown to be partially filled compared to 87% of group 1 areas (Table 1; Figures 1-3), but the difference was not statistically significant (P=.56).

Some authors suggest that when chlorhexidine is used after acid-etching, it could leave debris on the dentinal surface, making it difficult to form resin tags.[22] This was not observed in the present research, since the contents of the tubules did not differ significantly in the 2 groups. Similarly, Owens et al.[23] reported no differences between the surface that had been acid etched and the one that had chlorhexidine applied after this procedure.

The development of a hybrid layer, represented by a mixture of collagen fibrils and resinous monomers, is a determinant factor for the success of the restoration bonded to dentin. Therefore, this study examined the presence and thickness of the hybrid layer. Group 1 presented a larger number of areas with a visible hybrid layer (68%) compared to group 2 (52%).

Chlorhexidine may have helped clean the cavity, decrease the surface tension area, allowing better wettability and, consequently, contributing to better integration of the adhesive with the collagen fibrils, resulting in a hybrid layer of better quality. Pilo et al.[24] considered this antimicrobial a wetting agent that diminishes the sensitivity of the restorative technique. Chlorhexidine has a high affinity for dentinal structures, which is increased by acid-etching, and this could theoretically increase the bond strength of the adhesive to dentin.[25]

The hybrid layer thickness values in group 1 were shown to be slightly higher (3.33 μm) than those of group 2 (3.28 μm), but the difference also was not significant (P=.94; Table 2; Figures 4-6). In group 1, the hybrid layers were better delimited, thus facilitating the measurements. These values were a little higher than those reported by Telles et al.[15]: 2.5 μm, a with 1-step adhesive. Olmez et al,[26] however, found a hybrid layer with a thickness of 8.6 μm when using a 3-step adhesive. The difference could be due to the different methodologies of each research.

It was difficult to compare the results with data from the literature because of the limited number of articles on the subject. Only 3 studies were conducted with primary teeth, and none of them observed the quality of the hybrid layer by scanning electronic microscopy. Tulunoglu et al.[27] found a greater degree of marginal leakage when this antimicrobial agent was applied after acid-etching. This study, however, used 3-step dentinal adhesives that have different compositions, thus interfering in the dentin-adhesive interaction.

Vieira et al.,[28] found that the bond strength was significantly lower when chlorhexidine was used before acid-etching. Komori et al.[32], however, found no difference in the bond strength between groups treated or not with chlorhexidine, using the Single Bond adhesive. Recently, Hebling et al.[14], by means of transmission microscopy, detected on abnormal hybrid layer with progressive disintegration of the collagen fibril network in group 2 after acid etching. They concluded that using this antimicrobial agent as MMP inhibitor could prevent in vivo degradation of the hybrid layer.

As previously discussed, the MMPs present in mineralized dentin could be denatured by acid-etching and reactivated by treatment with dentinal adhesives. For the adhesive used in the present research, Mazzoni et al.[9] observed a reactivation of proteolytic activity of approximately 126%, which significantly contributed to the degradation of collagen fibrils.

According to the literature, the matrix metalloproteinases involved in this process may be MMP-2 and MMP-9, present in both demineralized and mineralized dentin, with the potential to degrade the organic matrix. In mineralized dentin, they are present in larger quantities and most are in the active form. In demineralized dentin, however, they are found in a lower quantity and in the inactive form,[3,29] but enzymes of this demineralized dentin may be reactivated by the adhesive.[9]

The technique with chlorhexidine application after acid-etching could prevent the degradation of collagen fibrils in the hybrid layer.[7] Carrilho et al. reported that autodegradation of collagen matrices can occur in resin-infiltrated dentin, but may be prevented by the application of a synthetic protease inhibitor, such as chlorhexidine.

For an initial assessment of the action of a substance, healthy teeth are ideal models since they avoid the influence of different stages of carious lesion progression. Nevertheless, having obtained these results, it is necessary to test chlorhexidine in carious teeth. Further research should be conducted to verify the characteristics of the hybrid layer of primary teeth, whether treated with chlorhexidine or not, after some period of storage in water.

CONCLUSION

Considering the limitations of this in vitro study, it can be concluded that the 2% chlorhexidine solution applied after acid-etching on the normal dentin of primary teeth did not influence the quality of the hybrid layer.

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