A new concept in hybridization: Bromelain enzyme for deproteinizing dentin before application of adhesive system

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Abstract

Objective: The objective of this study is to assess the deproteinizing effect of bromelain enzyme and compare it with neodymium-doped yttrium aluminum garnet (Nd:YAG) laser and 10% sodium hypochlorite (NaOCl) by using scanning electron microscope (SEM) and polarized microscope. **Materials and Methods:** A total of 60 extracted human upper premolars were selected to be given standardized buccal and lingual class V cavities. The teeth were divided into three groups each one consisted of 20 teeth. Thirty teeth were recruited for SEM study and the other 30 for polarized microscope. Group 1: Teeth were deproteinized with Nd:YAG laser, Group 2: Teeth were deproteinized with bromelain enzyme and Group 3: Teeth were deproteinized with 10% NaOCl. **Results and Conclusions:** Application of bromelain enzyme has led to removing collagen network and significantly decreased the global leakage scores of the adhesive system.

Keywords: Adhesive system, bromelain enzyme, deproteinizing dentin

Introduction

Demineralization of dentin during conditioning with acids should be considered as a complex process restricted to the outermost dentin surface layer.[1] Acid etching of this area not only removes the smear layer, but in addition totally demineralizes top few micrometers, thus exposing the collagen fibers. Underneath this layer, a partially demineralized zone and then the dentin, which is not affected by conditioning.[2]

Studies using high resolution techniques showed that bonding agent of current adhesives failed to completely seal dentin from acid induced porosities. The size of the porosities in dentin were about 10-50 nm.[3] Neither low viscosity water compatible monomer mixtures nor microscopic restoration particles are able to close the pores. Therefore, dentin affected in its cohesive stability by acid etching will not be reinforced.[4] This zone of partially demineralized dentin with microcavities may be considered as a weak point in the attachment.[5] Similar studies in enamel showed no evidence for the formation of respective porosities or penetration paths.[6]

Prior to the description of the phenomenon of nanoleakage in 1994 numerous studies on the quality of restoration margins were carried out with dye penetration experiments.[7] The aim was detection and evaluation of marginal gaps. In these experiments, the penetration depths were evaluated by conventional light microscopy. The limited lateral resolution of this technique as well as the inadequate depth of focus did not allow detailed structure analysis within the hybrid layers. Therefore, interpretation of dye penetration experiments and their differentiation between marginal gaps only or a nanoleakage phenomenon was very difficult if not impossible. Under these conditions, individual restorations or materials may have been considered "leakage," only due to dye penetration within porous dentin, but in the absence of marginal gap. Therefore, such experiments should be conducted with techniques providing higher lateral resolutions than conventional light microscopy to differentiate between various paths of penetrations.[8,9]

The disadvantages generated by using 10% sodium hypochlorite (NaOCl) to deproteinize acid etched dentin like the formation of a fragility zone and cytotoxicity of the NaOCl; which are aggravated by the depth of dentin and the intolerable taste and odor have stimulated a new and different treatment philosophy to deproteinize dentin.[9]

The neodymium-doped yttrium aluminum garnet (Nd: YAG) laser is an excellent surgical instrument for tissue coagulation, vaporization and incision. The Nd: YAG laser is attracted mostly to pigmented tissue. The collagen network has ability to stain by special dye. So, if dentin was etched with acid and the partially demineralized dentin was stained with a special

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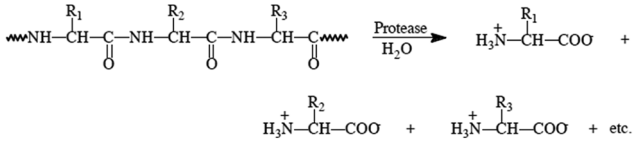
dye, which is selective for collagen network, the Nd: YAG laser with special parameters would ablate collagen network selectively and dentin would be deproteinized without affecting the mineralized tissue.[10]

The other new technique for removing collagen network is using some deproteinizing enzymes such as collagenase or bromelain enzyme.

Bromelain is a protein, which functions as an enzyme and belongs to a subclass of enzymes known as proteolytic enzymes (proteases). The function of proteases is to catalyze the hydrolysis of proteins to give amino acids.



The aim of this study was to compare the efficacy of removing collagen network by using different methods such as NaOCl, Nd: YAG laser and bromelain enzyme and studying the nanoleakage effect of each one.

Materials and Methods

Sample preparation

A total of 60 extracted human upper premolars free of caries, restorations, cracks or obvious defects had been cleaned and stored in 50% ethanol at 8°C for a maximum of 1 month following extraction in order to avoid microbial contamination. This storage medium was chosen because it produces little change in dentin permeability. Prior to the experiments, the teeth were placed in water for 24 h at 20°C.[9] The teeth were divided randomly into two categories, 30 teeth were used for scanning electron microscope (SEM) study while the other 30 were used for nanoleakage study.

Cavity preparation

Standardized class V cavities were prepared on the buccal and lingual surfaces (3 mm high, 3 mm wide, 2 mm depth) using a high speed hand piece, which was adapted to the horizontal arm of a surveyor in such a way that the long axis of the bur will be perpendicular to that of the tooth, using a medium grain diamond bur No. 848, under water coolant. The outline of the cavity was drawn on the tooth surface with a 0.5 mechanical pencil using a matrix band with a pre-cut hole of 3 × 3 mm, which was fixed on the tooth with a retainer so that the gingival floor of the cavity was within the cemento-enamel junction.

The cavity form was completed with round bur No. 2, in a low speed hand piece using water coolant; the enamel margins were not beveled.

The teeth were randomly divided into three groups for each SEM study and nanoleakage study, each group was consisted of ten teeth, cavities per tooth were not inter related and the data were analyzed as if they were independent replica cavities for each of the treatments.

Groups according deproteinizing methods

- Group 1: Acid etch and Nd: YAG laser

Conditioning of enamel and dentin

The teeth were etched using the total etch technique (a 37% of phosphoric acid etchant) which was applied to the enamel and dentin beginning with the enamel margins for 15 s.

The cavities were thoroughly rinsed from phosphoric acid gel with water. The dentin surface was dried with an air syringe for 2 s to achieve a slightly moist surface (the surface is slightly glossy); however, no visible excess water should remain on the tooth surface.

Treatment with Nd: YAG laser

In order to remove the collagen network and interfering with the formation of conventional "hybrid layer," Van Geison stain was prepared by mixing 1% of aqueous fuchsin solution (9 cm^3), with saturated aqueous picric acid solution (50 cm^3) and distilled water (50 cm^3). A thin layer of Van Geison stain was placed on the internal surfaces of the cavity by using a bonding applicator, in a fine motion the dye was brushed gently into the dentin. The teeth were stabilized in their positions on the laboratory manikin and the manikin was fixed on the tray of the Nd: YAG device. The teeth were treated with Nd: YAG laser in a focus non-contact mode at its fundamental wavelength of 1064 nm, 10 MJ, for three pulses with water cooling system.[10]

- Group 2: Acid etch and bromelain enzyme

The same procedure as Group 1 was followed for conditioning of enamel and dentin with acid etch and then bromelain enzyme was applied on the acid etched dentin. The application of bromelain enzyme was done by using a disposable brush for a dwell time of 1 min and the bromelain enzyme was removed with 5 ml distilled water.

- Group 3: Acid etch and 10% NaOCl

The same procedure was followed as for Groups 1 and 2 for conditioning of enamel and dentin with acid etch, then 10% NaOCl solution was used instead of Nd: YAG laser and bromelain enzyme. The application of 10% NaOCl was done by using a disposable brush for a dwell time of 1 min and the NaOCl was removed with 5 ml distilled water.

Application of bonding agent

For nanoleakage study only, the excite bond was applied onto the conditioned tooth structure with a bonding applicator. In a light motion, the material was brushed gently into the dentin for 10 s.

The bonding was left for 10 s and then the excess was removed with the air stream free of water and oil. The bonding was light cured for 20 s.

Placing the restoration

For nanoleakage study only, the resin based composite (Solitaire, Heraeus Kulzer) was applied in three incremental horizontal layers. Each layer was separately light cured for 20 s from all surfaces to ensure complete polymerization. Before curing the final increment, a transparent matrix was placed to contour the restoration. The margins were finished and polished with sand paper disc.

Scanning electron microscope

The SEM was used to show the topography of the dentin substrate after deproteinizing the acid etched dentin with different techniques. 10 teeth from each group were prepared for scanning electron microscopy. The teeth were sectioned longitudinally (in the sagittal plane) into two halves, then sections were made all around the cavities by approximately 1 mm with a low-speed micro-engine and diamond wheel bur with a water cooling system. Specimens were air-dried and mounted on aluminum tubs. After undergoing sputtering with a 40 nm layer of gold, using Balzers SCD 050 apparatus, the wall surfaces of the treated samples were examined with a Jeol 6100 SEM at a magnification of × 2000). (Specifications were: resolution 4 nm [30 kV, WD 8 mm], accelerating voltage 0.3-30 kV, eucentric goniometer stage W/LaB6 filament, image framestore with digital image processing, 35 mm film camera, EDAX Genesis with thin window detector, kinetic energy. solid state back scattered electron detector). This showed the surface topography of the dentin substrate after treatment with different deproteinizing techniques.[10]

Ability to resist nanoleakage

The teeth were checked using a dissecting microscope to ensure that no flash of dental composite was left along margins. Root apices were sealed with solitaire composite and the entire teeth, except for the bonded interface and 1 mm of the teeth surfaces adjacent to the interface, were coated with two layers of nail varnish. the teeth were placed in a 50% (weight/volume) silver nitrate solution in total darkness for 24 h, rinsed in running water for 5 min, immersed in photo-developing solution and exposed to a fluorescent light for 8 h in order to reduce the silver ions to metallic silver.[2] After removal from the developing solution, the teeth were placed in running water for 5 min. The teeth were sectioned longitudinally across the bonded surface obtaining two sections for each sample, making a total of 20 specimens for each group. The sections were mounted on the glass slide using the resin adhesive. All the cut surfaces were polished with increasingly fine diamond pastes (3, 1 μm) obtaining a 4 μ thickness sections and then covered with slide covers. Examination was carried out in a polarized light microscope. Global leakage scores of each specimen were calculated as the percent of the total cut dentin surface that was penetrated by silver nitrate:

Global leakage score - P/L × 100

Where P - length of silver nitrate penetration along the resin/dentin interface and L - total length of dentinal cavity wall on the cut surface.[6]

Results

Scanning electron microscope study

Figure 1 shows a top view scanning electron micrograph of the acid-etched dentin surface after treatment with Nd: YAG laser. There is very small amount of collagen network covering the intertubular dentin and the peritubular dentin. The dentin substrate reveals microporosities similar to labyrinth membrane and the lateral branches among the dentinal tubules refer to the partially depletion of these branches from collagen fibers.

Figure 2 shows a top view scanning electron micrograph of the acid-etched dentin surface after treatment with bromelain enzyme for 1 min. There is no collagen network covering the intertubular dentin and the peritubular dentin. The dentin substrate appears as a labyrinth and the lateral branches among the dentinal tubules refer to the depletion of these branches from collagen fibers. The orifices of the dentinal tubules look wider than these tubules when Nd: YAG laser was used.

Figure 3 shows a top view of the acid-etched dentin surface after treatment with 10% NaOCl for 1 min. The collagen network that covers the intertubular and peritubular dentin is very prominent, distributed here and there throughout the dentin substrate. The orifices of the dentinal tubules seem unclear.

Nanoleakage study

All the samples in this study showed nanoleakage at the adhesive resin interface, but to a varying degree.

Table 1 and Figure 4 show the mean values of the global leakage scores and the standard deviation after acid conditioning and treating with different deproteinizing techniques. In Group 2 when the bromelain enzyme was used after acid conditioning of dentin, the polarized light microscope showed the least amount of silver penetration; a very thin line or some few areas of silver deposition beneath the layer of bonding system.

In Group 1 when the Nd: YAG laser was used after acid conditioning of the dentin, the polarized light microscopy showed a relatively thick silver line with some tubules filled with silver deposition.

In Group 3 when the dentin was treated with 10% NaOCl after acid conditioning, the polarized light microscope showed

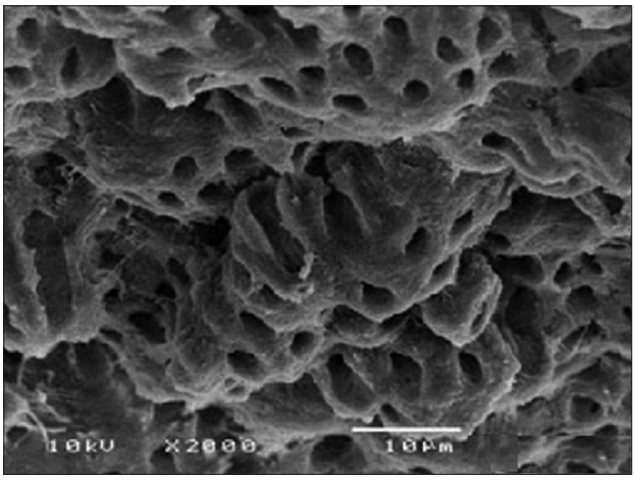


Figure 1: Scanning electron micrograph of the acid-etched dentin surface after treatment with neodymium-doped yttrium aluminum garnet laser

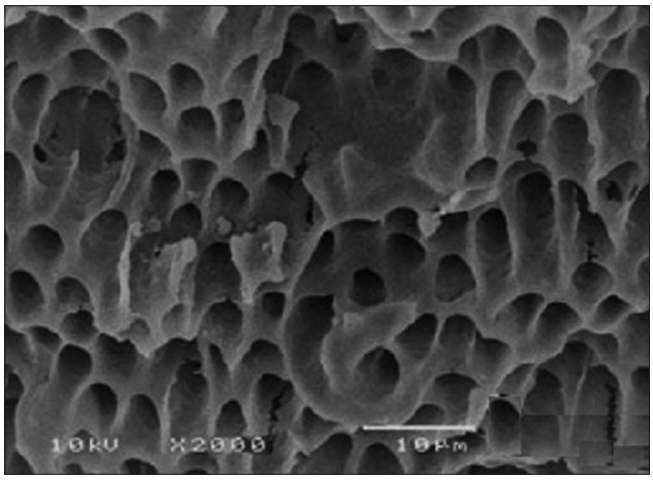


Figure 2: Scanning electron micrograph of the acid-etched dentin surface after treatment with bromelain enzyme

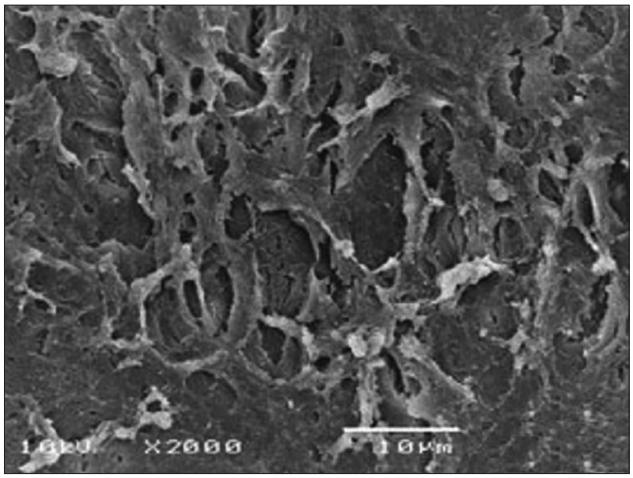


Figure 3: Scanning electron micrograph of the acid-etched dentin surface after treatment with 10% sodium hypochlorite

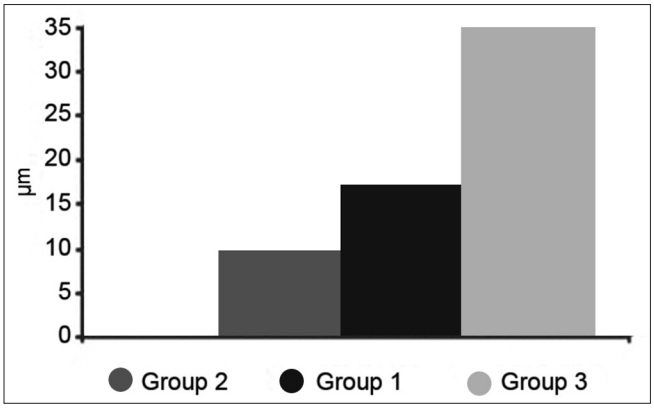


Figure 4: Mean of the global leakage scores of each treatment

thick areas of silver penetration; thick line of silver deposition involving the lower interface of the interdiffusion zone.

Table 2 shows the statistical analysis of the data by using the analysis of variance revealed that there was a highly significant difference ($P < 0.001$) in the global leakage scores among the three groups. Further analysis of the data was needed to examine the differences between all groups. Hence, the data were analyzed statistically by applying the Toukys multiple range test (HSD test).

Table 3 shows the HSD or honestly significant difference test, in which there are highly significant differences among all three groups.

Discussion

The longevity of composite fillings is, among other factors, dependent on the properties of the junction between tooth substances and filling materials. It has been shown that creating an interdiffusion zone between demineralized dentin and resin can reliably eliminate gap formation. This zone is called the hybrid layer. On the other hand, marginal discoloration, recurrent caries, post-operative sensitivity and finally a loss of restorations most frequent consequences of an insufficient or incorrect creation of the hybrid layer.

The majority of previous studies have been carried out to detect microleakage based on gap formations. Penetration of bacterial products, acids and even oral fluid at the interface between a restoration and the tooth has been regarded as detrimental to the longevity of the restorations. Reasons for this might be degradation of exposed collagen by hydrolysis. Garcia-Godoy and Finger in 1993[8] showed that with the restoration in place, the traditional microleakage method does not detect the exact location of leakages in 75% of the cases. It was found that the hybrid layer itself can be penetrated by tracers, such as silver nitrate. To distinguish this type of leakage within the hybrid layer from the typical microleakage, which is associated with gap formation, Sano *et al*., in 1995[6] introduced the term "nanoleakage". Penetration pathways in nanoleakage are porosities in a range <50 nm. These porosities are located

Table 1: Values of the global leakage scores of each treatment with mean and standard deviations

	1	2	3	4	5	6	7	8	9	10	Mean	SD
Group 1 Acid etch and Nd: YAG laser	17.4	16.5	17.7	16.8	16.9	16.9	17.5	17.3	17.1	17.2	16.2	±0.62
Group 2 Acid etch and bromelain enzyme	4.7	4.3	3.8	3.5	3.7	3.9	3.9	4.2	5.8	4.2	4.1	±0.21
Group 3 Acid etch and 10% NaOCl	31.2	31.4	30.1	31.8	30.2	30.3	30.4	32.2	31	31.2	30.1	±0.71

SD: Standard deviation; Nd: YAG: Neodymium-doped yttrium aluminum garnet

Table 2: Analysis of variance of global leakage scores

Source of variance	Sum of squares	df	Mean square	F	Sig
Between groups	3394.3	2	1697.672	5069.350	H.S
Within groups	9.0	27	0.335		
Total	3404.3	29			

Table 3: The results of Tukey's HSD test applied on the mean changes in values of the global leakage scores of each treatment

Groups	Difference between means	HSD value 5%	HSD value 1%	Sig
1 and 2	12.1	0.64	0.82	H.S
1 and 3	27			H.S
2 and 3	13.9			H.S

HSD: Honestly significant difference

between the untreated dentin and the superficial collagen-rich fibrous network and should have been penetrated by resin in order to reinforce demineralized dentin structures.

In order to quantify the amount of nanoleakage, Sano *et al*., in 1995[6] used silver nitrate to visualize the depth of dye penetration pathway within the hybrid layer. The silver nitrate staining is one of the most commonly used methods for leakage evaluation which provides a much sharper picture of penetration at tooth-restoration margins.[11] Gwinnett *et al*.,[12] speculated that the partially demineralized dentin zone is probably the most important morphological factor in achieving optimal bond strength, and that the outer collagen layer *per se* does not contribute to bond strength. An incomplete penetration of this zone should, therefore, decrease bond strength and increase dye penetration through nanometer spaces. As a consequence of this, the nanoleakage effect has been discussed to be one factor negatively affecting the quality of the bond.

Pashley *et al*., in 1993[13] introduced micropermeability test by placing a fluorescent dye into the pulp chamber of extracted teeth, this dye proved to have penetrated into dentin toward the interfacial region. These findings relate to nanoleakage and suggest possible pathways for permeability in the hybrid zone. Although the mechanisms leading to the nanoleakage phenomenon are not completely explored, it seems to be evident from our data that all groups showed nanoleakage at the adhesive resin interface, but to a varying degree, this is due, in part, to a discrepancy between the depth of etching and the depth of resin penetration. This results comply with Li *et al*., in 2000[11] who found that all their specimens tested showed nanoleakage at the adhesive-dentin interfaces and with Sano *et al*., in 1995[6] who found that even in the absence of marginal gaps, there was a varying amounts of penetration of silver ions through demineralized dentin "hybrid layer" into the underlying tubules. Furthermore, our results come with the agreement with Pioch *et al*., in 1999 and 2002[14,15] who found that all specimens used showed dye penetration (using 1% rhodamin-B solution as leaking material).

The application of bromelain enzyme on conditioned dentin significantly decreases the values of the global leakage score and gives the lowest values of global leakage scores. This is due to the ability of bromelain enzyme to remove the collagen network from acid etched dentin efficiently and this will lead to increase the diffusion potential of the monomer to the intact dentin and minimizing the nanoleakage. Furthermore removing of the collagen network from acid etched dentin substrate will make the chemical composition of dentin more similar to that of enamel by minimizing the organic component of dentin substrate and this will lead to the changing of the hydrophilic properties of the dentin. The use of Nd: YAG laser on the conditioned dentin had a decreasing effect on the global leakage scores but to a lesser degree. The application of 10% NaOCl for 1 min on the acid etched dentin will not remove the collagen network as it is with bromelain enzyme and Nd: YAG laser, so it gave the highest global leakage scores. This result came in disagree with the result of Pioch *et al*., in 2001[2] who found that nanoleakage can be reduced or prevented by using 10% NaOCl for 1 min. However, our results agree with that of Ferrari *et al*., in 2000[16] who found that the application of 10% NaOCl did not reduce the global leakage scores or improve the seal of the restoration. Groups 1 and 2 showed the lowest global leakage scores and there was a highly significant difference among groups.

The global leakage score value, which obtained from using bromelain enzyme was about 4 times less than that obtained from Nd: YAG laser and 7 times and half less than that obtained

from using 10% NaOCl as deproteinizing agent. The depletion of collagen from the surface of acid etched dentin results in:

- The permeability of dentin substrate will be increased due to the enlargement of dentinal tubules near the outer dentin surface; this will enhance the spreading and diffusing of adhesive monomers through dentin and this coincide with the explanation of Barbosa *et al*., in 1994[17] and Inaba *et al*., in 1995[18]
- The surface energy of the dentin will be improved, because the hydroxyapatite has a high surface energy substrate while collagen has a low energy surface and this will lead to enhance the diffusion of adhesive monomers through dentin. This description complied with our previous study also it came with the same line of that of de Castro *et al*., in 2000.[19,20]

The dentin is very porous and rough with many lateral branches of tubules are detectable in main tubules, which may contribute to the increase in the spreading of adhesive monomers through dentin, this complies with Inai *et al*., in 1998[21] and Ferrari *et al*., in 2000[16] who showed the same explanation.

Conclusion

We concluded from this study that treatment of the acid etched dentin with bromelain enzyme for 1 min can lead to removing collagen network and significantly decrease the global leakage scores of the adhesive system. The step of deproteinization with bromelain enzyme is very important to get high adhesive quality and should be taken in consideration before applying bonding agent.

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