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Evaluation of Nanoleakage following deproteinization of dentin using varying concentrations and application times of Sodium Hypochlorite Solution and Gel-an in Vitro Confocal Laser Scanning Microscope study

Dr. C. Ganesh Dr. V. Gopikrishna****
Dr. R. Prakash* Dr. D. Kandaswamy***
Dr. A. Parameswaran ****

Abstract

The aim of the study was to test the efficacy of 3% & 5% sodium Hypochlorite gel as compared to 3% and 5% sodium hypochlorite solution for deproteinization and also to assess the nanoleakage following deproteinization of dentin by an indigenous sodium hypochlorite gel, of varying concentrations with different timings, using a confocal laser scanning microscope. Seventy non-carious maxillary premolars were taken and Class V cavities were prepared on the cervical aspect with the cervical margin in dentin. The cavities were etched, rinsed with water and blot dried. Sodium hypochlorite was used at a concentration of 3 % and 5% both in the solution form and gel form for the study. Prime & bond NT was applied as per manufacturer's instructions and the cavities were restored with TPH spectrum composite resin. Teeth were immersed in 50% alcoholic solution of 1% wt Rhodamine B fluorescent dye for 24hrs. After rinsing they were sectioned longitudinally and dentin / adhesive interface were examined under confocal laser scanning microscope. Dye leakage was measured in micrometers. Results suggest, Sodium hypochlorite gel is as effective as the solution form for deproteinization procedures and provides better handling and control. Efficacy of sodium hypochlorite in reducing nanoleakage increases with increase in concentration and time.

* P.G Student
** Reader
*** Professor and Head of the Department
**** Former Professor and Head of the Department

Introduction

Hybrid layer, a structure formed in dental hard tissues by demineralization of the surface and subsurface, followed by infiltration of monomers and subsequent polymerization was reported by Nakabayashi[3] in1982. The chemical and physical properties of these zones are very different from that of intact tooth structure because it is partially de-mineralized and then infiltrated with resin. This leads to the formation of a structure which is neither resin not tooth but a combination (hybrid) of the two which is situated within the substrate and not on the surface. This hybrid or resin impregnated layer is believed to play a major role in dentin adhesion[3].

Hybrid layer is composed of two inter phases. The layer in the outermost region is commonly of resin impregnated collagen and below there is a narrow partially de-

mineralized bond of dentin mainly composed of resin encapsulated hydroxyapatite crystals[4]. Ideally optimal bond strength is achieved from complete resin diffusion into the acid etched dentin. Recent studies have suggested that Dentin bonding agents do not fully diffuse through the collagen network remaining after acid conditioning[9]. This would be either due to non efficient penetration of the Dentin bonding agents into the substrate or due to over zealous acid conditioning. This would lead to a weak porous layer of collagen not encapsulated by resin or protected by hydroxyapatite[10].

Microleakage has been described as clinically undetectable passage of bacteria and bacterial products, fluids, molecules of loss from the oral environment along the *various gaps* present in the interface between the cavity and the restoration. Adhesive restorations have to a large extent minimized the microleakage at this interface.

Recently, Sano et al (1994) suggested the existence of a leakage pathway along the porous zone at the hybrid layer adhesive interface even in the *absence of gap formation.* This penetration reveals the lack of a perfect seal, but is not microleakage in the classical sense. Sano et al termed this leakage as *nanoleakage*[7]. They described this phenomenon as permeation laterally through micron to sub micron spaces in the base of the hybrid layer that have not been filled With adhesive resin or which were left when poorly polymerized resin was extracted in oral or dentinal fluids.

Dissolution and removal of the organic layer after acid conditioning and subsequent bonding directly to partially demineralized dentin layer can produce a more durable adhesion to the hydroxyapatite component of the dentin substrate. Sodium hypochlorite (NaOCI), a well known proteolytic agent has been employed on dentin as a deproteinizing agent to dissolve and remove the collagen layer after acid etching. Recent studies have suggested minimum of 3%to5% NaOCI solution for effective deproteinization.

One of the practical difficulties of using sodium hypochlorite solution is due to its caustic nature, which can cause damage to adjacent soft tissues. Difficulty in handling and flow properties of the solution are factors, which contribute to this problem. This potential problem can be overcome by employing sodium hypochlorite in the form of a gel, which will have better handling and control over area of application. As sodium hypochlorite gel was not commercially available, an indigenous gel form of sodium hypochlorite was prepared in varying concentrations for this study.

The aim of the study was to test the efficiency of 3% & 5% sodium hypochlorite gel as compared to 3% and 5% solution for deproteinization and also to assess the nanoleakage following deproteinization of dentin by an indigenous sodium hypochlorite gel, of varying concentrations with different timings, using a confocal laser scanning microscope.

Materials and Methods

70 non carious freshly extracted maxillary premolars were used in the study. The teeth were stored in de-ionized water with thymol at room temperature. The specimen teeth were utilized for the study within one month of extraction.

Preparation of samples

Standardized Class V cavities were prepared in all teeth (3X 3mm and 2mm deep). The cervical margin of the restoration was located in dentin or cementum. The prepared cavity surfaces were then acid etched with Detrey conditioner (Dentsply) for 15sec and rinsed with water for 20 sec. The cavities were then blot dried with absorbent paper and maintained in a moist condition.

Sodium hypochlorite was used at a concentration of 3 % and 5% both in the solution form and gel form for the study. A thickening agent, methylcellulose (Loba chem) was added to Sodium hypochlorite solution of 3% and 5% to prepare the sodium hypochlorite gel. The sodium hypochlorite solution was added drop by drop to methylcellulose powder and mixed in a Mortar and Pestle until uniform consistency of the gel was obtained.

The teeth were then divided randomly into 7 groups of 10 teeth each. The teeth were then treated as given below.

Group I Control

In this group no NaOCI was used and dentin bonding agent was applied and cured according to manufacture's instruction.

Group 11

3% of sodium hypochlorite solution was applied for 60 sec and rinsed with water for 20sec and blot dried.

Group III

5% solution of sodium hypochlorite was applied for 60 sec, rinsed with water for 20 sec and blot dried.

Group IV

In this group 3% sodium hypochlorite gel was applied for 30sec rinsed with water for 20 sec and blot dried.

Group V

For this group 3% NaOCI gel was used for 60 sec, rinsed with water for 20sec and blot dried

Group VI

In this group 5 % sodium hypochlorite gel was applied for 30 sec, Rinsed with water for 20sec and blot dried.

Group VII

5% sodium hypochlorite gel was used for 60sec, rinsed with water for 20sec and blot dried.

Following this pretreatment of the prepared cavities, dentin bonding agent was applied with applicator brush and light cured for 20sec for all the groups. The dentin bonding agent used for this study Prime & Bond NT (Dentsply).

The cavities were then restored using TPH spectrum composite resin (Dentsply).

The restoration surfaces were polished according to manufacturers instructions. For dye penetration the specimens were stored in 50% alcoholic solution of 1% wt Rhodamine B fluorescent dye[13].

After rinsing with water for 10sec the specimens were stored in water for 24hrs. The teeth were then sectioned parallel to long axis of the tooth, using slow speed diamond disk under copious water supply. The sectioned surface was polished with 600 grit silicon discs.

The dentin / adhesive interface region was examined using a confocal laser scanning microscope*. It uses illumination from helium neon laser. The 514nm excitation line was used and fluorescence emissions were detected using 580 / 30 Band Pass. The magnifications used were 10X objective lens. Images were analyzed using LSM 510 software.

The distance of dye penetration from outer tooth surface towards the bottom of cavities, representing the degree of nanoleakage was measured for each group.

The results were subjected to statistical analysis using One Way ANOVA to calculate P value and multiple range test by Tukey HSD procedure was employed to identify the significant groups.

* Zeiss instruments, Germany

Results

The least leakage was shown by Group VII followed by Group III> VI> V>11>IV>I. (Table. 1)

Nanoleakage values for each group in Micrometers

No	Group I Control	Group II 3% Sol. 60 sec	Group III 5% Sol. 60 Sec	Group IV 3% Gel 30 Sec	Group V 3% Gel 60 Sec	Group VI 5% Gel 60 Sec	Group VII 5%Gel60 Sec
1.	530.50	320.84	191.35	390.97	300.33	263.43	151.33
2.	519.93	327.72	177.53	380.38	290.31	310.72	174.20
3.	485.75	285.15	187.63	360.46	285.66	310.57	150.31
4.	515.22	295.61	208.93	410.72	305.12	280.13	155.50
5.	460.16	319.14	190.60	379.43	288.11	275.97	167.30
6.	471.20	309.98	165.13	384.79	310.74	290.17	181.56
7.	510.56	308.07	187.39	415.45	295.67	273.47	135.93
8.	494.37	295.38	166.24	395.83	318.12	260.57	159.10
9.	515.73	315.80	179.55	404.57	280.16	290.72	177.35
10.	475.15	318.28	185.10	388.17	315.43	303.89	167.17

- Group I was control group showed most leakage and was statistically significant different with all groups.
- There was no statistically significant difference between group VII (5% gel for 60 sec) and Group III (5%solution for 60sec) and also between Group II (3% solution for 60 sec), Group V (3% gel for 60sec) and Group VI (5% gel for 30sec).
- Leakage values of Group VI (5% gel for 30sec) was statistically significant with that of Group VII (5% gel for 60 sec), Group IV (3 % gel for 30sec), Group III (5%solution for 60sec) and Group I (control), but not significant with Groups V (3% gel for 60sec) and Group II (3% solution for 60 sec).
- In Group V (3% gel for 60sec), there was a significant difference with Group VII (5% gel for 60 sec), Group IV (3 % gel for 30sec) and Group III (5%solution for 60sec) but no statistically difference with Group VI (5% gel for 30sec) and Group II (3% solution for 60 sec).
- Group IV (3 % gel for 30sec) showed statistically significant difference with all groups.
- Group III (5%solution for 60sec) showed statistically difference with all groups Group VII (5% gel for 60 sec).
- In Group II (3% solution for 60 sec) there was a statistically difference with Group III (5%solution for 60sec, Group IV (3 % gel for 30sec) and Group VII (5% gel for 60 sec) but no difference with Group V (3%gel for 60sec) and Group VI (5%gel for 30sec).

Discussion

Bonding to dentin has always been problematic because of its heterogeneous nature. Dentin is characterized by a combination of collagenous matrix, filled with hydroxyapatite crystals dispersed between dentinal tubules[3]. Dentinal tubules are filled with dentinal fluid, which also contributes to the difficulties of bonding. For restorative purpose, many methods have been tried to bond to dentin that have included both chemical and micro mechanical bonding.

The major breakthrough in achieving optimal dentin bonding came with the introduction of the total etch concept by Fusayama in 1979. The clinical success of resin bonding to acid etched enamel as introduced by Buonocore in 1955, probably led Fusayama to etching of the entire cavity with phosphoric acid to remove all smear layer. The use of acids not only removed the smear layer but also demineralized the underlying intact dentin. The use of total etch technique led to the formation of a structure in the dental hard tissue by the demineralization of the surface and sub surface followed by infiltration of monomer

and subsequent polymerization[3]. This layer was first reported by Nakabayashi in 1982 and was termed as a "hybrid" layer. This resin impregnated dentin or hybrid layer is a transitional zone of resin reinforced dentin sandwiched between cured resin and unaltered dentin substrate.

The eventual longevity of such bonded restorations and their bond strength depends upon the extent to which polymerized resin will penetrate into the demineralized dentin[2]. The currently employed dentin bonding agents may not completely penetrate into the demineralized dentin leaving behind an un infiltrated weak collagenous layer of dentin that is susceptible to long term degradation. In vitro tests have demonstrated the deterioration in bond strength after long term water immersion. This degradation occurs in the band of exposed collagen which is present beneath the resin impregnated demineralized dentin that has not been impregnated with the resin[11]. This susceptible layer is primarily composed of peptide molecules, which on long term water immersion undergo hydrolytic degradation[4].

Formation of this non hybridized band can be explained as follows. Acid etching results in the demineralization of superficial dentin to a depth X, monomer diffusion and penetration of this demineralized dentin and its polymerization therein occurs to a depth Y; depths X and Y may or may not coincide. The difference, if any, between depth X and depth Y is the collagenous band of demineralized dentin that has not been sufficiently impregnated to create a complete hybrid layer[4].

The susceptibility of this demineralized collagenous zone was proved by Sano et al[26] who showed the penetration of silver ions beneath the resin impregnated layer between resin and decalcified tooth structure even in the absence of any gap formation. This leakage in the absence of gaps was later termed "Nanoleakage" by Sano et al[7, 8] in 1995. They also stated that this might allow dentinal or oral fluids to slowly permeate through the hybrid layer into the non hybridized collagen zone causing its hydrolytic degradation even in the absence of microleakage.

This problem can be minimized or eliminated by the use of a proteolytic agent like sodium hypochlorite to remove the collagen fibers exposed by acid etching. This concept of collagen removal after acid etching was termed as deproteinization. Prati et al[6] reported the formation of a "reverse" hybrid layer by application of NaOCl after acid etching. This procedure not only removes the exposed collagen but also solubilizes the fibrils down into the underlying mineralized matrix to create sub micron porosities within the mineral phase. Cylindrical channels previously occupied by

collagen fibrils are now available for resin infiltration within the mineralized matrix. They also reported a complete difference in morphology of deproteinized dentin as compared with acid etched dentin. The diameter of tubule orifices increased after NaOCI treatment of acid etched and demineralized dentin due to loss of demineralized peri tubular dentin. This substrate is rich in hydroxyapatite crystals and may result in a more stable interface over time as it is essentially made of minerals[14]. The remaining surface is also very wettable with the bonding system and is similar to enamel because only insignificant amounts of protein are left. Other studies have also suggested that collagen removal and application of adhesive resin directly on exposed dentinal apatite significantly improves the bond strength and potentially overcomes the problem of long term hydrolytic degradation of non hybridized band of collagen[1, 6, 14].

Acetone based bonding agents[1] have shown to have a better effect on deproteinized dentin. The positive effects of acetone based adhesives are explained by the higher diffusability of acetone and higher capacity to displace water, which improves the contact of the monomer with the irregular inter-tubular dentin structure. For the purpose of this study Prime & Bond NT, an acetone based bonding agent was therefore selected.

This study was conducted to check the efficacy of an indigenous sodium hypo chlorite gel as compared to a sodium hypo chlorite solution for deproteinization and to assess the nanoleakage following deproteinization of dentin by an indigenous sodium hypochlorite gel of varying concentrations with different timings.

The various techniques advocated for assessing nanoleakage include Scanning electron microcopy (SEM), Transmission electron microscopy (TEM), Conventional microscopy and Confocal laser scanning microscopy[12, 13, 15]. The major advantage of CLSM is that it does not require special specimen processing. SEM requires the drying of specimen whereas TEM involves embedding procedures, all of which can lead to formation of artifacts that can affect the interpretation of the results. Another advantage of CLSM is that out of focus blur as well as light scattered within the optical instrument is absent resulting in increased contrast in the final image.

The confocal laser scanning microscope has been used in dental material research to examine the resin dentin interface. The confocal principle is based on the detection of florescence emission from the focal plane or well- defined optical section, while light from planes above and below is effectively suppressed by a pinhole that acts as a spatial filter. The detected light is converted to a video signal for display as a two dimensional image on the computer screen[15].

The scanning laser beam illuminates the surface and sub surface of the sample. The portion of the beam, which is reflected or scattered of the microscopic structures of the sample passes through the confocal pinhole and is detected electronically. The use of reflection mode enables the various structures of the sample (Dentin, Dental Material etc) to be distinguished by the individual reflection properties. In fluorescence mode the penetration pathways can be observed, as structures labeled with the fluorescent dye can be visualized. Overlay or superimposed images of the reflected and fluorescent mode can be reconstructed with the CLSM software[13]. A Confocal laser scanning microscope was used for the purpose of this study.An indigenous sodium hypochlorite gel was prepared by mixing sodium hypochlorite solution of 3% and 5% with methyl cellulose. Methylcellulose is a commonly used thickening agent in the food and beverage industry and forms a common ingredient of many food products and therefore a biocompatible material. Toxicology studies done on methylcellulose failed to demonstrate any carcinogenic properties[8]. Human studies have shown that methylcellulose passed through the digestive tract practically unaltered[8].

Group I which used no sodium hypochlorite in any form showed the maximum leakage (P value <. 0001) as compared to the groups which used sodium hypochlorite, confirming that the use of sodium hypo chlorite effectively reduced the nanoleakage at the resin tooth interface.

The least nanoleakage score was showed by group VII (5%gel for 60sec) and Group III (5%solution for 60 sec). The gel form showed less nanoleakage values but there was no statistically significant difference between their values and this showed that the methylcellulose did not have any adverse effect on the properties of the Sodium hypochlorite solution. The value of nanoleakage between 3% solution for 60 sec and 3% gel for 60sec, also showed no statistically significant difference and this further confirmed that the gel form was as effective as the solution for the deproteinizing procedure.

The disadvantage of using sodium hypochlorite solution clinically as a deproteinization agent is its caustic effects on the soft tissues[5]. Being in the solution form, it has less viscosity and therefore decreased control on application creating a tendency for it to flow into the adjacent areas. This additional step of surface conditioning has only been tried in in vitro tests, as its clinical use is problematic. Even though this adverse effect can be reduced by the use of a rubber dam it can be further minimized by using the sodium hypochlorite in the gel form. The gel provides better handling properties and control over the area of application. It allows for precise

placement of the agent in the area to be deproteinized with minimum flow into the adjacent tissues. Proper visualization of the agent is possible and rinsing of the gel is also more convenient.

When comparing the varying concentrations of the gel, and the timings, it was seen that 5% gel for 60 sec (group VII) was the most effective in reducing the nanoleakage. This was followed by 5% gel for 30 sec (group VI). However 5% gel for 30 sec did not show any difference statistically with the 3% gel for 60sec.Also it was seen that reducing the concentration to 3% and the timing to 30sec(group IV) showed more leakage.

A decrease in nanoleakage when the concentration of Sodium hypochlorite was increased to 5% and applied for 60sec was evident and the reason for this could be the following

- Higher concentration meaning higher proteolytic activity.
- More dwell time (60sec) for Sodium hypochlorite on the area to be deproteinized.
- Removal of collagen layer by Sodium hypochlorite can make the dentin more permeable to adhesive resin due to increase in sizes of dentinal tubules and increased porosity of dentin.
- Finally the higher diffusability and water chasing properties of Acetone based dentin bonding agents (Prime & Bond NT) can also contribute to a better bond leading to less nanoleakage.

An increase in concentration of the sodium hypochlorite gel from. 3% to 5% significantly reduced the nanoleakage value for both the 30sec and 60sec. groups. So 5% gel used for 60sec showed the least leakage for all the groups. 3% gel groups when used for 60sec showed statistically no significant difference with 5% gel used for 30sec (Gr. VI) and 5% solution for 60sec (Group III). This meant that with lower concentration, higher timings can reduce the leakage values, but clinically increasing the timing over 60sec is a hindrance and may not be practicable.

A complete elimination of nanoleakage was not possible with any groups used in this study. An increase in concentration brings its own risk, while increase in timing can increase the chair side timings At the cost of increasing either of the concentration or the timing of sodium hypochlorite, an optimal balance can be achieved for best results for use in clinical conditions.

Laser Scanning Microscopic View

Conclusion

From this study it can be concluded that

- Sodium hypochlorite gel is as effective as the solution form for deproteinization procedures and provides better handling and control.

  Efficacy of sodium hypochlorite is reducing nanoleakage increases with increased concentration and time.

  5% gel sodium hypochlorite for 60seconds was the most effective in reducing nanoleakage among the samples used in this study.

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