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Research Article

Effect of Different Anti-Oxidants on the Shear Bond Strength of a Universal Adhesive to Smear Layer-Deproteinized Dentin

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Received: February 04, 2020; **Published:** March 21, 2020

Abstract

Objective: To evaluate the effect of different anti-oxidants on the shear bond strength of a universal adhesive to NaOCl-treated dentin.

Materials and Methods: Flat coronal dentin surfaces were prepared in sixty-six extracted human molars. Teeth were randomly divided into six groups (n = 10) according to different surface pre-treatments: no surface pre-treatment (Group A); sodium hypochlorite pre-treatment (Group B); sodium hypochlorite pre-treatment followed by anti-oxidant application either, cashew nutshell liquid (Group C), proanthocyanidin (Group D), rosmarinic acid (Group E), sodium ascorbate (Group F). Subsequently, Prime and Bond Universal adhesive was applied and composite resin cylinder build ups were done. Ten samples from each group were prepared for shear bond strength testing. One sample per group was additionally prepared to observe the resin - dentin interface under scanning electron microscope. Data obtained was subjected to statistical analysis using one-way ANOVA and post-hoc Tukey's test at a significance level of $p < 0.05$.

Results: Sodium hypochlorite application decreased the bond strength of universal adhesive as compared to the control group ($p = 0.125$). Application of all antioxidants including cashew nutshell liquid proanthocyanidin, rosmarinic acid and sodium ascorbate as dentin pre-treatment lead to reversal of bond strength with a significant increase compared to sodium hypochlorite treated group (($p = 0.0001$; $p = 0.002$; $p = 0.002$; $p = 0.001$ respectively).Cashew nutshell liquid significantly increased the bond strength as compared to the control group ($p = 0.012$) as well.

Conclusion: Subsequent application of anti-oxidants reversed the compromised shear bond strength of universal adhesive to sodium hypochlorite pre-treated dentin.

Keywords: *Anti-Oxidants; Cashew Nutshell Liquid; Proanthocyanidin; Rosmarinic Acid; Sodium Ascorbate*

Introduction

'Smear layer' plays a significant role in the bonding of resin-based adhesives to dentin [1]. It covers the superficial dentin surface and may extend into dentinal tubules, forming smear 'plugs' [2]. This layer consists of depleted hydroxyapatite crystals, denatured collagen fibrils, saliva, blood and food debris. The apparent denatured and gel-like collagen in the outermost part of smear layer is likely to act as a selective barrier for monomer infiltration during dentin bonding [3].

Dental adhesives can be classified into four categories according to how they deal with the smear layer: etch-and-rinse (E and R), self-etch (SE), multi-mode 'universal,' and resin-modified glass ionomer adhesives.

In E&R adhesive systems, dentin surface treatment is performed using phosphoric acid etching gels to totally remove the smear layer and open the dentinal tubules. Theoretically, this technique can enhance resin infiltration into the partially demineralized collagen network. However, E and R adhesives are reported to be technique sensitive.

SE adhesives were introduced to overcome the problems of E and R adhesives. SE adhesives depend on a smear layer-modifying (dissolving) bonding strategy. SE adhesives are more user-friendly, have fewer application steps and a shorter application time and do not require complicated technique-sensitive procedures [4]. Due to the incomplete removal of the smear layer, SE adhesives exhibit a marked reduction in postoperative sensitivity [5].

Self-etch adhesive can partially demineralize the smear-layer-covered dentin subsurface but cannot completely remove smear layer due to its mild acidity. They are not able to dissolve the organic phase of the smear layer. Therefore, the remaining organic phase of the smear layer can give rise to the resin-impregnated smear layer (so-called hybridized smear layer), which is formed on the hybrid layer by incorporating remnant of smear layer [6]. Smear layer does not have physiological and morphological continuous connection to the underlying dentin; therefore, hybridized smear layer is regarded as a weak link at the interface [7]. Moreover, this hybridized smear layer is likely to act as a selective barrier for monomer penetration into dentin substrate [3]. Therefore, eliminating the smear layer might be required to improve the quality of the adhesive interface of universal adhesive.

An important technique aiming to enhance resin/dentin hybridization involves pre-treatment of the dentin surface with a deproteinizing agent [8-10]. This dentin surface pre-treatment method is referred to as the smear layer deproteinizing process. Some researchers have demonstrated that sodium hypochlorite (NaOCl) solution can thin the smear layer by dissolving the collagen fibrils [11].

When NaOCl solution is applied to smear-layer-covered dentin, the smear layer is deproteinized and thinned by dissolution of the organic phase. Smear layer deproteinization can increase the mineral: organic ratio on the dentin surface [12] and improve the affinity of hydrophobic materials to dentin by removing hydrated collagen debris in the smear layer [13].

Therefore, it can be anticipated that deproteinizing the smear layer could improve the quality of the resin-dentin interface of self-etch adhesives by facilitating the penetration of self-etch adhesive into the dentin subsurface and eliminating the hybridized smear layer [12].

Nevertheless, NaOCl exhibits a strong non-specific proteolytic response and it has been reported that it may adversely affect the intact 'sound' collagen [14]. Also, the residual oxidization effect within NaOCl-treated dentine reversely affected the adhesive performance by impairing resin polymerization, leading to compromised dentine bonding [14] and an increase in nano leakage [15]. These negative effects on oxidized dentine can be reversed by the subsequent application of a reducing agent [14-16].

Proanthocyanidin (PAC) is a potent antioxidant and cross-linking agent with low toxicity. Several studies have shown that the use of grape seed extracts mainly composed of PA, improved the ultimate tensile strength and stiffness of dentin collagen [17] and long-term stability of dentin collagen matrices [18].

Plant extracts from *Anacardiaceae* such as *Anacardium occidentale* (cashew) [19] have potential significant capabilities for application in dentin biomodification. Cardol and cardanol are long carbon-chain phenols obtained from the industrial extraction of cashew nutshell liquid (CNSL) [20] during production of nuts. They have antioxidant capacity [21], enzyme inhibitory potential [22] and two hydroxyls in p-position (in the case of cardol) like those of PACs.

Rosmarinic acid (ROS) is a polyphenolic flavonoid extracted from rosemary, which has cross-linking and MMP-inhibitory abilities [23], as well as a high antioxidizing capacity [24]. The application of rosmarinic acid solution can improve the compromised initial bond strength of universal adhesives to NaOCl-deproteinized smear-layer-covered dentin [16].

The sodium ascorbate (SA) is a reducing agent that interacts with oxygen by product of NaOCl by redox reaction [25]. Treatment with ascorbic acid or sodium ascorbate for 10 minutes has been recommended for reversing the lowered bond strength of resin cements by NaOCl [26]. Also, 10% sodium ascorbate when applied on the NaOCl-treated dentin regains the bond strength to normal [25].

Some studies have investigated the effect of collagen deproteinizing on acid-etched dentin, however, little information is available for the effect of smear layer deproteinizing on bonding of universal adhesive to dentin [8]. Therefore, the aim of this study was to evaluate the bond strength of universal adhesive bonded to the NaOCl pre-treated dentin surface with or without subsequent application of reducing agents and also to observe micromorphology of the bonded interface under SEM.

The null hypothesis tested was that application of these reducing agents/antioxidants does not improve the bond strength of a universal adhesive to NaOCl-deproteinized smear layer-covered dentin.

Materials and Methods

Study was performed in sixty-six freshly extracted human molars

The teeth were examined under stereomicroscope (SZX10, Olympus, Tokyo, Japan) and teeth free of caries, cracks, or any developmental defects were included. Teeth were cleaned and stored in 0.5% chloramine T trihydrate (Sigma Aldrich, Bangalore, KA, India) for no more than 3 months. Sixty tooth crowns were flattened occlusally using a low-speed diamond saw (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water irrigation to expose superficial dentin. A standardized smear layer was created with 600 grit silicon-carbide (SiC) paper. The samples were embedded in an auto polymerizing resin at the level of cementoenamel junction with long axis perpendicular to the acrylic resin surface for shear bond strength testing. The remaining six samples were prepared for SEM evaluation.

Preparation of experimental solutions

All chemicals were of analytical grade and were used without further purification. All the required concentrations were prepared by diluting the extracts to attain a neutral pH:

- 2% Cashew nutshell liquid was prepared by diluting 2 ml of cashew nutshell liquid (Allen Petrochemicals pvt. Ltd., Meerut, India) in 98ml ethanol/water mixture (1:1 volume ratio). The solution was buffered to pH 7.2.
- 6.5% proanthocyanidin was prepared by dissolving 6.5% grape seed extract (Best source nutrition, Shop Clues) in ethanol/water (1:1 volume ratio) with 5 minutes agitation at 25°C and final double filtration. The solution was buffered to pH 7.2.
- 100 µM rosmarinic acid was prepared by dissolving 10 mg rosmarinic acid (Sigma-Aldrich, Bangalore, India) powder in 270 ml 5% ethanol solution. The solution was buffered to pH 7.
- 10% sodium ascorbate was prepared by dissolving 10 mg sodium ascorbate powder in 100 ml distilled water. The solution was buffered to pH 7.

Grouping and treatments

Teeth were randomly divided into six experimental groups according to the dentin pretreatments, i.e., sodium hypochlorite alone or sodium hypochlorite followed by application of anti-oxidant agents.

- Group (A): no dentin pre-treatment and direct application of Prime and Bond universal adhesive (Dentsply Sirona, Bliss International, Jaipur) according to the manufacturer's instructions.

- Group (B): smear layer covered dentin treated with 5.25% sodium hypochlorite for 30 seconds, rinsed with water for 15 seconds and blot dried, followed by application of Prime and Bond Universal adhesive.
- Group (C): sodium hypochlorite pre-treatment carried out as in group 2 followed by 2% cashew nutshell liquid application for 1 minute, blot dried and Prime and Bond Universal adhesive application.
- Group (D): sodium hypochlorite pre-treatment carried out as in group 2 followed by6.5% proanthrocyanidin application for 1 minute, blot dried and Prime and Bond Universal adhesive application.
- Group (E): sodium hypochlorite pre-treatment carried out as in group 2 followed by 100μM rosmarinic acid application for 1 minute, blot dried and Prime and Bod Universal adhesive application.
- Group (F): sodium hypochlorite pre-treatment carried out as in group 2 followed by 10% sodium ascorbate application for 1 minute, blot dried and Prime and Bond Universal adhesive application.

Composite cylinder build-up

Transparent plastic tubes (TYGON laboratory tubing, Saint Gobain, Akron, OH, USA) with 3 mm internal diameter and 2 mm in height and thickness of 0.5 mm were precut and placed perpendicular to the previously bonded dentin surfaces. A nanohybrid resin composite (Filtek Z350 XT, Body shade A2; 3M ESPE Dental Products) was filled into the pre-cut tubes. Each bonded specimen was light-cured for 20 seconds using quartz-tungsten-halogen (QTH) light curing unit (Spectrum 800, Dentsply, Caulk, Miliford, DE, USA) at a light intensity of 600 mW/cm^2. The plastic tubes were gently cut and carefully removed with a number 11 surgical blade after polymerization.

Storage of samples before testing:

All the samples were stored in distilled water at 37°C for 24 hours for completion of polymerization before testing and scanning electron microscope analysis.

Shear bond strength testing

Ten samples from each group were subjected to shear bond strength testing in a universal testing machine (Instron, ADMET, Enkay Enterprises, New Delhi) using the corresponding computer software. The specimens were placed and stabilized by the jig, while a straight knife-edged rod (2.0 mm) was applied at the tooth restoration interface at a cross-head speed of 1mm/minute. Load was applied until restoration failure. The load at the failure was converted to shear bond strength evaluation (MPa) by dividing the load by surface area of the specimen.

Fractographical analysis

Failure mode of ten samples from each group was determined by observation under stereomicroscope (SZX10, Olympus) at 10×magnification and classified into adhesive (A), mixed (M), or cohesive (C) failures in either dentin or resin.

Scanning electron microscope study

Composite build-ups were done and one sample from each group was sectioned to obtain a flat surface in order to observe the interface.

The specimens were fixed in 10% formalin for 24 hours and decalcified in 6N HCl for 30 seconds, rinsed in distilled water and deproteinized by 10 minute immersion in 1% NaOCl and rinsed in distilled water. After acid-base treatment, the specimens were subjected to dehydration in ascending grades of ethanol upto 100% (25% for 20 minutes, 50% for 20 minutes, 75% for 20 minutes, 95% for 30

minutes and 100% for 60 minutes), then transferred to a critical point dryer for 30 minutes. The specimens were then gold sputtered and resin-dentin interfacial adaptation was observed under a SEM (LEO 430, England).

Statistical analysis

Values obtained from the shear bond strength were subjected to statistical analysis using parametric tests at a significance level of $p \leq 0.05$. Mean and standard deviations were calculated for each group. The statistical analysis on the shear bond strengths was done using SPSS (Statistical Package for Social Sciences) Version 21.0 statistical analysis software. The statistical tools used were Tukey's HSD test, one-way ANOVA (multivariate assessment), Independent-t test and chi^2 test.

Results

Shear bond strength

Mean shear bond strength values are presented in table 1. Sodium hypochlorite application decreased the bond strength of universal adhesive as compared to the control group. This group depicted the lowest bond strength amongst all groups.

Groups		Mean (MPa ± SD)
Group A	(C)	$36.97 \pm 5.65^{a,d}$
Group B	(C+SH)	$24.74 \pm 3.07^{b,d}$
Group C	(SH+CNSL)	53.63 ± 11.77^{c}
Group D	(SH+PAC)	$43.93 \pm 13.10^{a,c}$
Group E	(SH+ROS)	$44.35 \pm 14.43^{a,c}$
Group F	(SH+SA)	$45.26 \pm 11.16^{a,c}$

Table 1: *Mean shear bond strength of six groups.*
Different lowercase superscript letters indicate statistically significant difference within the column ($p < 0.05$)
C: control; SH: Sodium Hypochlorite; CNSL: Cashew Nutshell Liquid; PAC: Proanthocyanidine, ROS: Rosemarinic acid; SA: Sodium Ascorbate.

Cashew nutshell liquid significantly increased the bond strength of the universal adhesive as compared to both the sodium hypochlorite treated group ($p = 0.0001$) as well as the control group ($p = 0.012$).

Application of proanthocyanidin, rosmarinic acid and sodium ascorbate as dentin pre-treatment lead to reversal of bond strength with a significant increase compared to sodium hypochlorite treated group ($p = 0.002$; $p = 0.002$; $p = 0.001$ respectively).

Bond strength of all anti-oxidant treated groups were higher than both the sodium hypochlorite treated group and the control although the difference was significantly higher with sodium hypochlorite treated group, except for cashew nutshell liquid which showed significantly higher bond strength than control group also.

Failure mode analysis

Sodium hypochlorite treated group showed more adhesive failures, while cashew nutshell liquid pre-treated group showed the least number of adhesive failures. The observed increase in adhesive failure after treatment with 5.25% sodium hypochlorite indicates the presence of a "weakened area" in the bonding interface that reflected a reduction in bond strength.

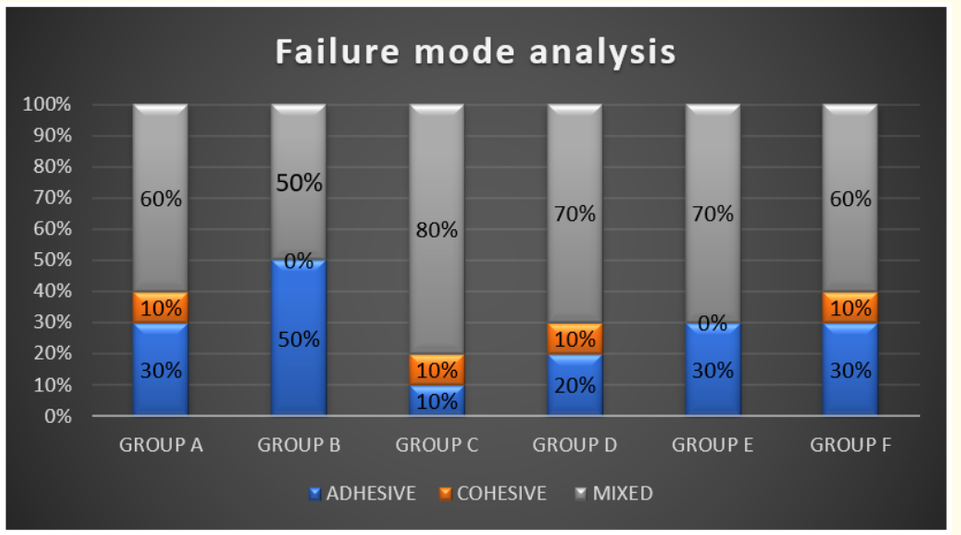

Figure 1: *Failure patterns of experimental groups according to the dentin pre-treatments (represented in percentage).*

SEM analysis

SEM images are presented in (Figure 2-4). The bonded interface in group B clearly shows gap with poor interfacial seal between the resin and dentin surface, depicting the compromised bonding. Groups A, C, D, E and F showed no gap and good interfacial adaptation at resin-dentin interface.

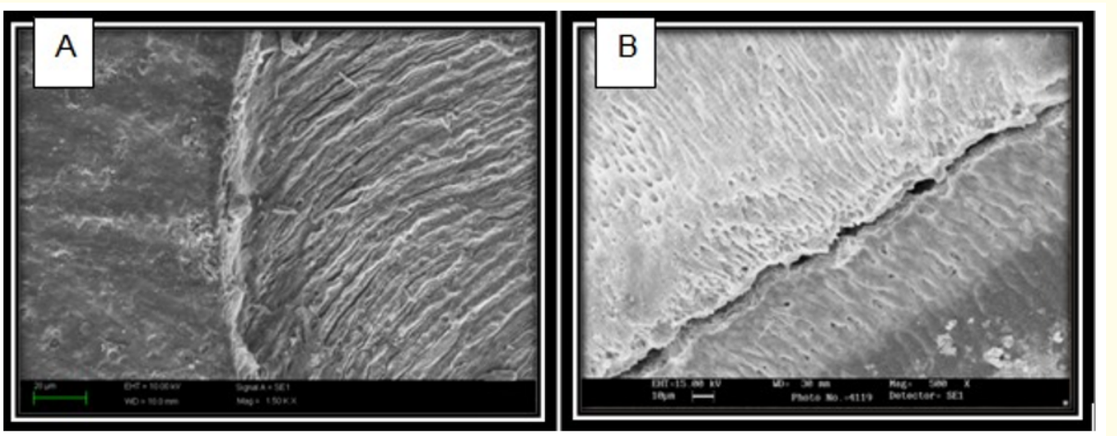

Figure 2: *SEM photomicrograph of resin-dentin interface obtained after (A) no dentin pretreatment (B) NaOCl pre-treatment.*

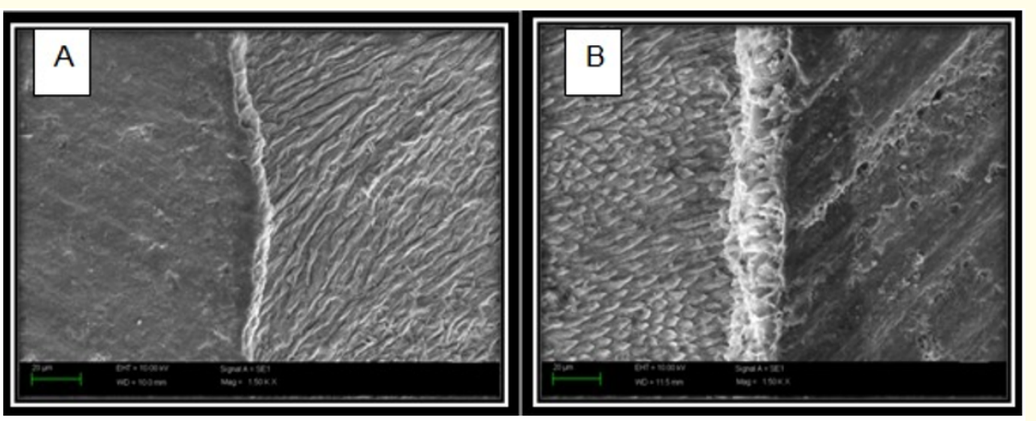

Figure 3: *SEM photomicrograph of resin-dentin interface obtained after (A) NaOCl and CNSL pretreatment and (B) NaOCl and proanthocyanidin pre-treatment.*

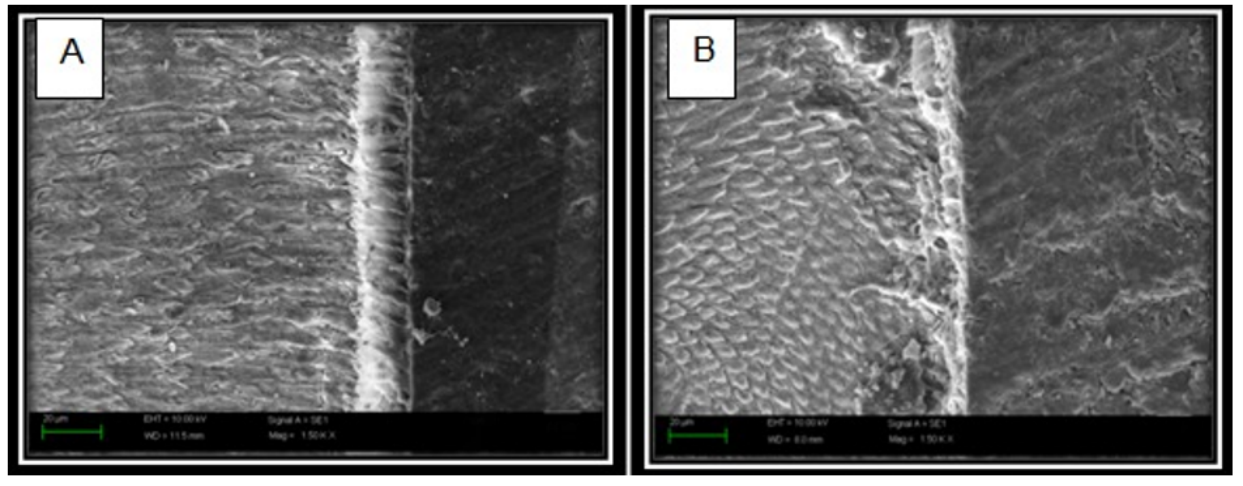

Figure 4: *SEM photomicrograph of resin-dentin interface obtained after (A) NaOCl and rosmarinic acid pre-treatment and (B) NaOCl and sodium ascorbate pre-treatment.*

Discussion

It is well known that a micromechanical adhesion mechanism plays an essential role in the adhesion of resin-based bonding agents to dentin, in which adhesive primers infiltrate into the superficial demineralized collagen fibers of 'hybridized' dentin. However, universal adhesives can dissolve only the mineral phase, but not the organic phase of the smear layer. The undissolved remaining organic phase, being incorporated to form a hybridized smear layer, might in turn hamper resin monomer infiltration into the underlying dentin. Therefore, it is anticipated that the use of deproteinizing agent before universal adhesive application may improve resin infiltration.

In this study, a 30 second pre-treatment of 5.25% NaOCl solution was done to remove the organic phase of the smear layer covering the dentin surface. Removal of the organic phase in the smear layer is expected to facilitate resin monomer infiltration into the underlying dentin, leading to an increased bond strength. However, in the current study no increase in the bond strength could be observed in the NaOCl-treated dentin. Rather, the bond strength of the Prime and Bond Universal adhesive to 30second NaOCl-treated dentin was lower than to that of normal dentin. Many researchers have also reported that NaOCl pre-treatment compromises the bond strength to dentin [11,14]. As NaOCl is a potent biological oxidant [27], the breakdown of this molecule yields reactive, residual free radicals, which could compete with the propagating vinyl free radicals generated during light activation of the resin adhesive. Presumably, this impaired polymerization of resin monomer resulted in decreased bond strength in NaOCl-treated dentin.

NaOCl solution has a low surface tension and a high potential to disrupt both sound and denatured collagen. It may deteriorate the mechanical properties of dentin via the degradation of the sound collagen fibers [14]. NaOCl solutions may degrade the collagen scaffolds of dentin, consequently reducing the number of bonding sites for adhesive primers. This impairs resin hybridization with dentin, leading to a marked reduction in the bond strength [10].

Also, Taniguchi*., et al.* [11] investigated the surface pH of NaOCl-treated dentin and reported that these surfaces exhibited significantly higher pH values than non-treated dentin surfaces, even after copious rinsing with water for enough time periods. The high alkalinity of NaOCl-treated surfaces could be explained by the high concentration of hydroxyl (OH) groups on the dentin surface [28]. The alkalinity of NaOCl might buffer the acidity of universal adhesives and thus reduce their hybridization with the underlying dentin.

Applying an anti-oxidant/reducing agent (e.g. cashew nutshell liquid, rosmarinic acid, proanthocyanidin, sodium ascorbate solution) reversed the negative effect on dentin bonding by NaOCl. Antioxidants possess three main mechanisms to control oxidation: free-radical

chain breaking, metal chelating and free-radical quenching. In the latter, antioxidants react with oxidants to neutralize unpaired electrons and form stable products, which limits the activity of oxidants [29]. In other words, treatment with an antioxidant agent can restore the redox potential of the oxidized dentin substrate, leading to optimal polymerization of the resin composite [26].

In our study, cashew nutshell liquid not only reversed the decreased bond strength of NaOCl treated dentin, but also significantly increased the bond strength. This may be attributed to a lower molecular weight of cashew nutshell liquid to attain greater and faster penetration in dentin collagen matrix [30], thus explaining the highest increase in the bond strength. Cardol and cardanol correspond to more than 95% of the composition of technical cashew nutshell liquid produced in the industries [31]. The presence of a long 15 carbon alkyl side chain provides additional hydrophobic interaction [32] with dentin collagen fibrils which may contribute to dentin biomodification. Cardol and cardanol are non-cytotoxic [20] compounds in low concentrations possessing antioxidant effects [21] and like PACs, they present inhibition of matrix metalloproteinase-2 and matrix metalloproteinase-9 [33]. Moreira., *et al*. [34] reported that CNSL yield best biomodification outcomes when applied for one minute without staining the dentin collagen. Lomonco., *et al*. [31] also concluded that technical CNSL and its main components have a good antioxidant activity in concentration as low as 1%.

Proanthocyanidin from grape seed extract has been shown to be an effective non-toxic cross-linking agent *in vitro* and *in vivo* [35]. Proanthocyanidin or condensed tannins is composed of condensed flavon-3-ol subunits, catechin, epicatechin and epicatechin-3-O-gallate and linked mainly through C4 - C8 [36]. These components are responsible for their various biological properties such as free-radical scavenging and antioxidant activities. Compared with sodium ascorbate, proanthocyanidin has twenty times the antioxidant capacity [37]. PAC also possesses collagen cross-linking and matrix metalloproteinases inhibition properties [33]. The reverse to the NaOCl-treated dentin bond strength after PA treatment is both concentration and time dependent. No recovery of bond strength has been detected after the application of 5% PAC for 1 minute. However, the decreased bond strength of NaOCl-treated dentine could be reversed by treatment with 5% PA for more than 5 minutes or with 10% or 15% PA for more than 1 minute [38]. In the present study, shear bond strength was reversed by application of 6.5% proanthocyanidins for 1 minute.

Application of 100 µM rosmarinic acid for 1 minute to NaOCl-treated dentin significantly increased the bond strength when compared with the NaOCl-treated group. Rosmarinic acid consists mainly of rosemary extracts [24]. Among 72 species of herbs and their solvent extraction in oil systems or oil-in-water emulsions, rosemary possesses the best antioxidant activity. Rosmarinic acid (a-o-caffeoyl-3,4-dihydroxyphenyllactic acid) is a diphenolic compound [39] that contains two catechol (1,2-dyhydroxy- benzene) rings, contributing to rosmarinic acid's polarity. The antioxidant activity of rosmarinic acid can be attributed to the ability of catechol to form an intermolecular hydrogen bond between free hydrogen of its hydroxyl and phenoxyl radicals, improving its radical stability. Moreover, rosmarinic acid has a MMP-inhibitory effect and cross- linking effect in addition to its antioxidant activity [16].

The cross-linking effect of rosmarinic acid is attributed to its interaction with proline-rich proteins, such as collagen. Rosmarinic acid has four phenolic hydrogens (-OH) which contribute to controlling free radical oxidation [29]. The -OH group in phenol acts as a chain-breaking antioxidant because it scavenges reactive radicals. The resulting radicals tend to be poorly reactive because of electron delocalization into the aromatic ring, so that the reactive radical is replaced by one of limited reactivity [24].

Application of 10% sodium ascorbate on NaOCl-treated dentine significantly increased the bond strength of universal adhesive on dentin. This finding agrees with Lai., *et al*. [14]. Since ascorbic acid and its sodium salt are potent anti-oxidants it is possible that sodium ascorbate has some potential to alter the oxidizing agents via redox reaction on the treated substrate. This sodium ascorbate allows free-radical polymerization of the adhesive to proceed without premature termination and reverse the compromised bonding on NaOCl acid etched dentin [9,14,40].

Morris., *et al*. [25] conducted a study which indicated that 1 minute treatment with sodium ascorbate is just as effective as 10 minutes in the reversal of compromised bond strength of C and B Metabond resin cement. Treatment with sodium ascorbate changes the oxidized

substrate to a reduced substrate which restores the redox potential of dentin and is thought to aid the polymerization of the methyl methacrylate/polymethylmethacrylate resin. As vitamin C and its salts (e.g. sodium ascorbate) are non-toxic and are used in food industry, it seems that their use on dentin will create no adverse biological effect.

Dentin specimens treated with PACs presented brownish colour. Although CNSL is a dark oil, its solution is transparent and treated specimens did not depict noteworthy colour alteration. This may be explained by their lower molecular weight and lesser capacity to form polymeric structures. In fact, the colour stability of dentin after biomodification with CNSL might be considered another advantage for its clinical use and a drawback when using PACs solutions [31]. Cardol and cardanol present in CNSL are single molecules prone to chemical synthesis of dental monomers which would afford the designing of collagen-binding and collagen-crosslinking monomers with substantial perspective for dentin bond. Further studies are needed to understand long-term benefits of these Anacardiaceae derived crosslinkers as well as their biocompatibility with odontoblast-like cells.

Also, in restorative dentistry, rosmarinic acid with cross-linking and MMP-inhibitory effects would be a useful agent for improving the longevity of bonding of simplified adhesives to normal and smear layer-deproteinized dentin, in addition to its antioxidant ability. One limitation of this study was that it only evaluated the immediate recovery effect of antioxidants on bond strength to NaOCl-treated dentin. The durability of bonding improvement of antioxidants to NaOCl-treated dentin, over time needs further work.

Conclusion

NaOCl deproteinizes the smear layer which cannot be completely removed by universal adhesives. Although, NaOCl improves resin penetration, it has an oxidizing effect thus reducing the bond strength. A subsequent application of an anti-oxidant agent can revert the compromised bond strength. Further, long term *in vivo* studies are still warranted to evaluate the effect of antioxidants in order to develop biofunctional adhesives with integrated antioxidant, cross-linking and MMP inhibitory potential.

Conflict of Interest

The authors deny any conflicts of interest and no financial assistance was undertaken from any other source.

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