

Effect of different concentrations of green tea extract solutions on bonding durability of etch-and-rinse adhesive system to caries affected dentin

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Aim: The *in vitro* study evaluated the effect of different concentrations of green tea extract solution (GT) on the bonding durability of etch-and-rinse adhesive system to caries dentin affected (CAD). **Methods:** Dentinal surfaces of human third molars were polished and submitted to a microbiological caries induction protocol for 14 days. After removal of the infected dentin layer, the samples were randomly divided into 4 groups (n= 10), according to the concentration of GT solution applied in CAD, after acid etching: 0.05%; 0.2%; 2% and NT (no treatment – control). After application of an etch-and-rinse adhesive system (Adper Single Bond 2, 3M ESPE), composite resin restorations were performed on the dentin. After 24 hours, the resin-dentin blocks were sectioned 1mm² specimens, which were subjected to the microtensile test immediately or after 6 months of storage in water. Data were submitted to two-way ANOVA for randomized blocks and Tukey test ($\alpha= 5\%$). **Results:** There was no effect of double interaction ($p= 0.934$). The application of 0.2% GT promoted a statistically significant increase in dentin bond strength values in comparison to the condition where GT was not used ($p=0.012$). There was a significant decrease of bond strength after 6 months of storage, regardless of dentin pretreatment ($p = 0.007$). The G test identified that there was no statistical difference regarding failure mode ($p= 0.326$). **Conclusion:** The concentration of 0.2% improved the bond strength of an etch-and-rinse adhesive system to caries affected dentin, however, none of dentin pretreatments could prevent the decrease in bond strength over time.

Keywords: Dentin. *Camellia sinensis*. Tensile strength. Dental caries.



Introduction

The longevity of adhesive restorations is directly related to the durability of the bond between adhesive systems and dental substrate. Unlike dental enamel, which is highly inorganic, dentin has an organic content and is essentially moist¹. During hybridization, it is important that the collagen exposed during the demineralization stage becomes fully encapsulated and protected by the polymerized composite monomer², a more difficult condition with etch-and-rinse adhesive systems, for which the acid conditioning of the dental structure and subsequent application of the primer/adhesive are done in separate steps³. Exposure and degradation of collagen can also occur over time, if hydrolytic degradation of the resinous components of the hybrid layer occurs, leaving the collagen exposed and unorganized⁴. This situation is more aggravated in adhesive interfaces produced in caries affected dentine⁵, a common and challenging dentin substrate that is available for adhesion in cavity preparations.

Collagen eventually exposed can be degraded by enzymes with collagenolytic activity present in the dentin matrix, such as matrix metalloproteinases (MMPs)⁶ and cysteine cathepsins (CTs)^{7,8}. Such degradation, along with the hydrolytic degradation of resin components, negatively affect the hybrid layer structure and lead to a loss of dentin bond strength over time², and this can directly affect the durability of adhesive restorations. MMPs activation occurs in moments when pH falls (denaturation) and subsequent dentin buffering (activation), as is the case due to dentin acid conditioning and subsequent application of the adhesive system^{9,10} or, yet, in the context of pathogenesis of dental caries, due to the drop in pH due to lactic acid fermentation by bacteria and subsequent neutralization by buffer systems¹¹⁻¹². In addition, CTs, normally present in the dentin matrix, are also activated at low pH, and once activated, participate in the activation of MMPs^{7,8}. Specially in caries-affected dentin, there is an abundance of MMPs and CTs¹³, what highlights the importance of studying this type of dentin substrate in the context of strategies that can prevent bond strength decrease over time and increase the stability of adhesive interface.

Green tea has been described as a natural inhibitor of MMPs, due to the presence of a catechin, epigallocatechin gallate (EGCG)¹⁴. It is a flavonoid with antioxidant properties¹⁴, without any reports of side effects¹⁵. Another property is related to the ability of EGCG of interacting with collagen fibers through hydrogen bonds and hydrophobic interactions and, thus, also considered a cross-linking agent, which improves the mechanical properties of collagen¹⁶. Regarding the stability of the bond with regards to dentin pretreatments with green tea extract, the results appear to have been contradictory. On one hand, the application of diluted green tea from commercially available tea (approximately 2% concentration) to healthy dentin¹⁷ or even the application of green tea extract in low concentrations (0.05%-0.2%) promoted the maintenance or even increased bond strength of adhesive systems to healthy dentin¹⁸ or caries affected dentin¹⁹. However, opposite results have also been found with the application of 0.2% green tea solution²⁰. Although the green tea application may be a promising strategy of dentin pre-treatment, it is evident that there is still no defined protocol regarding the concentration of green tea extract solution in relation to the bond strength, mainly in caries affected dentin substrate.

Thus, considering the relevance of studying adhesion to dentin affected by caries and in an attempt to establish an efficient protocol for a therapeutic agent that can inhibit the degradation of the adhesive interface in this type of substrate, this *in vitro* study evaluated the effect of different concentrations of green tea extract solution on the bond strength immediately and after 6 months of storage in water, of an etch-and-rinse adhesive system to dentine affected by caries.

Materials and Methods

Ethical Aspects

The present study was approved by the Local Research Ethics Committee (CAAE: 49027515.1.0000.5374).

Experimental Design and sample size calculation

The study involved a microbiological model of caries lesion induction, carried out according to the protocol of Sanabe et al.²¹. The factors under study were: 1. Type of solution/concentration used on dentine affected by caries after acid etching and before applying the etch-and-rinse adhesive system (Adper Single Bond 2 - 3M ESPE, St. Paul, MN, USA), at three levels: CV 0.05. 0.05% green tea extract solution; CV 0.2. 0.2% green tea extract solution; CV 2. 2% green tea extract solution. As a control level, an untreated group was added (NT). 2. Moment of the bond strength test, in two levels: 24 hours (immediate) and 6 months (long-term).

The experimental units were composed of 32 human third molars, restored with composite resin, according to the treatment assigned to each group (n=8). From a pilot study carried out with three specimens, means and standard deviations were used to calculate the effect size ($f = 0.349$). The software G*Power 3.1.9.4 (Heinrich-Heine Universität, Dusseldorf, Germany) retrieved 7 specimens per group to detect difference among groups at a 0.05 alpha level and 80% power. The final sample size per group was fixed at 8 specimens to account for potential losses during the study.

From each tooth-restoration set, eight longitudinal sections were obtained, which were divided according to the storage time, therefore replicates were formed. The variable of continuous quantitative response was obtained through microtensile bond strength tests (MPa).

The materials cited in the experimental design, as well as their composition and mode of use, are described in Table 1.

Table 1. Materials, composition, application method and pH.

Material Batch number#	Composition	Application method	pH*
Condac 37 (FGM, Joinville, SC, Brasil) #230915	37% phosphoric acid	Applied to the dentin surface for 15 s, rinsed for 30 s and dried gently with absorbent paper.	--
Green tea extract (Farmácia Natal, Águas Frias, SC, Brasil) #185841	<i>Camellia sinensis</i>	Twenty microliters of the solution were passively applied to the dentin for 60 s. The dentin was dried gently with absorbent paper.	0,05% - 4.80 0,2% - 4.50 2% - 4.28

Continue

Continuation

Etch-and-rinse adhesive system Adper Single Bond 2 (3M ESPE, St. Paul, MN, EUA) #612979	BIS-GMA, HEMA, copolymer of acrylic and itaconic acids, water, ethyl alcohol, glycerol 1,3-dimethacrylate, UDMA, silane treated silica.	The adhesive system was applied in two consecutive layers; the remaining solvent was evaporated with a brief, gentle, dry air jet for 10 s and light cured for 20 s.	4.7
Composite resin Filtek™ Z250 XT (3M ESPE, St. Paul, MN, EUA) #452516	Aluminum oxide, silica, zirconium oxide, BIS-GMA, BIS-EMA, UDMA	Each 1-mm increment was light cured for 20 s.	--

HEMA (Hydroxyethyl methacrylate); BIS-GMA (Bisphenol A-Glycidyl Methacrylate), UDMA (Urethane Dimethacrylate); BIS-EMA (2,2-bis-4-2-(hydroxi-3- methylacriloxietoxi)-phenylpropane).

*pH measured in triplicate

Tooth selection and fragments preparation

Thirty-two human third molars were cleaned with a periodontal curette and kept in 0.1% aqueous thymol solution, at 4 °C, until the study was conducted. Occlusal portions were removed with a high concentration diamond cutting disc (Series 15 HC - Buehler Ltd, Lake Bluff, Illinois, USA) mounted on a precision electric cutter (Isomet 1000 Precision Diamond Saw (Buehler Ltd, Lake Bluff, Illinois, USA) under constant cooling and speed.

Artificial caries induction

For the induction of carious lesions, the protocol obtained in the study by Sanabe et al.²¹ was used. The roots were sealed with composite resin and then waterproofed with a layer of epoxy adhesive (Araldite, Ciba Especialidades Quimicas Ltda., São Paulo, SP, Brasil) and another one of nail polish (Colorama, Ltda CEIL Com. Exp. Ind. Ltda, São Paulo, Brasil), leaving only the dentin surface exposed. All teeth were autoclaved for 20 minutes at 121 °C. The cariogenic solution consisted of 3.7 g of BHI broth (Brain Heart Infusion Broth, Becton Dickinson and Company, Sparks, MD, EUA), 2 g of saccharose (Synth; LabSynth, São Paulo, SP, Brasil), 1 g glucose and 0.5 g yeast extract (Becton Dickinson and Company, Sparks, MD, EUA) for every 100 ml of distilled water. This solution was autoclaved for 20 minutes at 121 °C prior to inoculation of 2% strains of *Streptococcus mutans* ATCC25175 (Andre Tosello Foundation Tropical Cultures Collection) (10⁸ UFC/ml). The teeth were suspended in the cariogenic environment and the set was taken to microaerophilia (CO₂ incubator, TE 399, Tecnal, Piracicaba, SP, Brazil) for 14 days. During this period, the cariogenic solution was replaced every 48 hours, but without the inoculation of new microorganisms. After the incubation period, the teeth were again autoclaved. The biofilm was removed with gauze and the insulating materials (epoxy adhesive and nail polish) were removed manually with scalpel blades and the teeth were then washed with deionized water. Dentin surface was slightly darkened and softened, a condition that was measured with a non-cutting manual instrument.

Adhesive procedures

The infected dentin was removed with an excavator until a more hardened and touch-resistant dentin to exploratory probe was obtained, without pressure. This

procedure was performed by a single, previously trained, operator. After that, the fragments were conditioned for 15 seconds with 37% phosphoric acid, washed and rinsed for 10 seconds and dried with absorbent paper. The dentin fragments were randomly divided into 4 groups, according to the treatment performed on the dentin surface: 0.05%, 0.2% and 2% green tea extract solution (Groups CV 0.05; CV 0.2 and CV 2 respectively); and no treatment (NT).

The green tea extract solution was diluted to concentrations of 0.05%, 0.2% and 2%. The initial concentration was based on the study by Zheng et al.¹⁸ and the final concentration on the study by Carvalho et al.¹⁹. The volume of the solution (CV) was 20µl per fragment¹⁷, that were passively applied for 60 seconds on the conditioned dentin and the excess removed with absorbent paper, keeping the tissue moist always. Then, the adhesive system (Adper Single Bond 2, 3M ESPE, St. Paul, USA) was applied according to the manufacturer's recommendations. The dentin surface was restored with a composite resin (Filtek™ Z250 XT, 3M ESPE, Irvine, CA, USA - color A2). Each 1-mm resin composite increment was light cured for 20 seconds with a with LED light device (Sdi Radian Cal light curing unit, SDI Limited Bayswater, Victoria 3153 Australia), positioned as close as possible to the equipment. The final resin block was 4-mm high.

Microtensile test preparation and water storage

The restorations were stored in distilled water, at 37°C, for 24 hours and then sectioned perpendicularly to the adhesive interface with a flexible diamond disk mounted on a precision electric cutter, obtaining stick-shaped specimens, with dimensions of 0.8 mm². Each tooth-restoration block provided, on average, 8 stick-shaped specimens that were randomly divided into two groups, according to the storage time in distilled and deionized water, 24 hours and 6 months. The specimens were kept in a bacteriological incubator (Odontobrás, Ribeirão Preto, SP, Brazil) at 37°C and the water changed every 2 days.

Microtensile bond strength testing

Each stick-shaped sample was fixed in a microtensile test device with the aid of instant adhesive (Super Bonder gel, Henkel Loctite Ltda, São Paulo, SP, Brazil) and subsequently subjected to the microtensile test in a Universal Testing Machine (MEM-2000 model, EMIC, São José dos Pinhais, PR, Brazil) with a speed of 0.5 mm/min. The load at the time of the fracture of each specimen was recorded in Kgf and divided by the cross-sectional area of the stick (mm²), in order to obtain the microtensile bond strength values expressed in MPa. The comparison was made by the average of the sticks in each tooth, each tooth being considered an experimental unit. The surfaces of the fractured specimens were examined using a stereoscopic magnifying glass (Eikonol Equip. Optical and Analytical, model EK3ST, São Paulo, SP, Brazil), with a 30-fold magnification, to classify the type of fracture that occurred. The fractures were classified as: adhesive (failure of adhesion), cohesive in dentin (failure of the dental substrate), cohesive in composite resin (failure of composite resin) or mixed (adhesive failure and cohesive in composite resin).

Statistical analysis

The compliance with the presuppositions of normality and homoscedasticity was verified by the Shapiro-Wilk and Levene tests, respectively, to compare the effect of applying green tea extract solution in different concentrations and the storage time, as well as the interaction of these two study factors, the bond strength data were subjected to analysis of variance at two criteria for randomized blocks. Tukey's test was used for multiple comparisons. The failure modes observed were subjected to descriptive approaches and G test. Statistical calculations were performed using the SPSS 23 program (SPSS Inc., Chicago, IL, USA), adopting a significance level of 5%.

Results

The two-way analysis of variance for randomized blocks demonstrated that there was no statistically significant interaction between the concentration of the green tea extract solution and the storage time ($p = 0.934$).

The bond strength was significantly influenced by the concentration of green tea extract from the solution that was applied to the dentin ($p = 0.012$). Regardless of dentin pretreatment, it was observed a significant decrease in bond strength values after 6 months of water storage ($p = 0.007$) (Table 2).

Table 2. Average values and standard deviation of the bond strength (in MPa), according to the concentration of the green tea extract solution applied to the dentin and the storage time.

Green tea extract	Storage time		Grand mean
	24 hours	6 months	
0.05%	14.42 (6.20)	9.53 (4.83)	11.97 (5.91) AB
0.2%	17.80 (6.49)	13.25 (5.82)	15.52 (6.37) A
2%	11.04 (2.94)	7.09 (4.14)	9.07 (4.01) B
Absent (control)	11.29 (4.78)	8.82 (6.23)	10.05 (5.48) B
Grand mean	13.64 (5.72) a	9.67 (5.51) b	–

Grand means followed by distinct uppercase letters indicate a significant difference between dentin pretreatments, regardless of storage period ($p < 0.05$). Grand means followed by distinct lowercase letters indicate a significant difference between dentin storage periods, regardless of dentin pretreatment ($p < 0.05$).

The G test identified that there was no statistically significant difference between the groups regarding the failure mode ($p = 0.326$). In all groups, there was a predominance of adhesive failures (Figure 1).

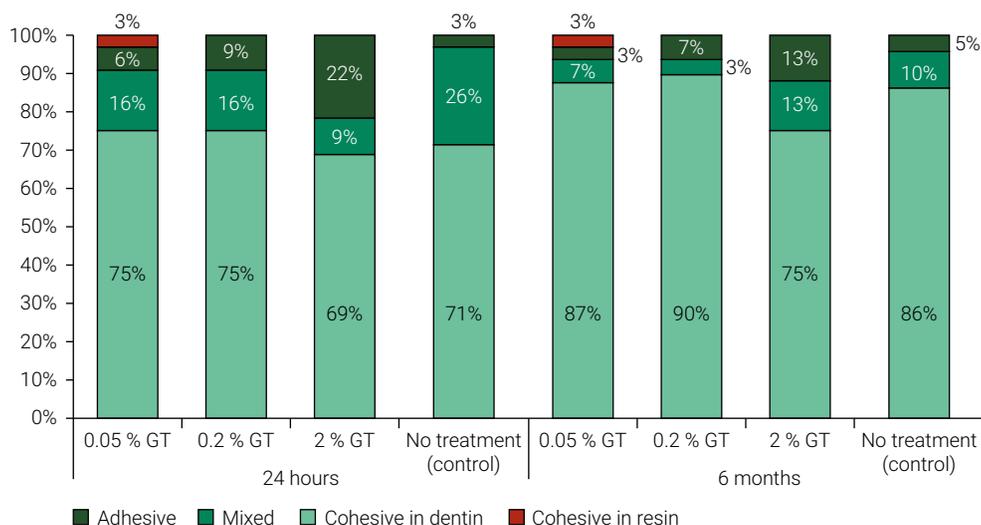


Figure 1. Bar diagram of the relative frequency (%) of failure modes, according to the concentration of the solution containing green tea extract applied to the dentin and the storage time.

Discussion

Considering knowledge of the degradative processes of the hybrid layer, it is important to search for and evaluate compounds that can delay the decrease in bond strength over time. In this study, we sought to evaluate the effect of pretreatment on dentine affected by caries with a solution containing green tea extract, in different concentrations, since this polyphenol contains catechins, such as EGCG, with proven inhibitory activity against MMPs²² and it is a crosslinking agent¹⁶, which improve the mechanical properties of collagen.

It was found that, regardless of the storage time, the application of green tea extract at 0.2% concentration provided bond strength values higher than the other groups, except with respect to the 0.05% concentration, this one with intermediate bond strength values. The concentration of 2% obtained the lowest values of bond strength, compared to other treatments both in 24 hours and in 6 months of storage. Therefore, the null hypothesis failed to be rejected.

Lower bond strength findings for green tea extract at a concentration of 2% may be related to the saturation of the extract in solution, which may have interfered negatively in the bond strength, similar to the tests in the immediate time of Carvalho et al.¹⁹, who also observed bond strength to dentin affected by caries to be inferior when using a solution containing 2% green tea extract. Gerhardt et al.²³, using the same concentration of EGCG (2%) for dental pre-treatment before applying a two-step self-etching adhesive system (Clearfil SE Bond), reached results similar to the present study. Although the solution containing green tea extract at a concentration of 0.05% did not differ from the 0.2% group, it also did not differ significantly from the group without dentin pre-treatment (NT). It is speculated that the 0.05% concentration may not have been sufficient, that is, the concentration of green tea extract was too low to produce any significant effect on bond strength in caries affected dentin.

The concentration of solution containing 0.2% tea extract seems to be, among all the studied, the best concentration to be applied to caries affected dentin, as it obtained the highest average bond strength, higher than the control group (NT), regardless of storage time. This result can be attributed to the concentration of catechins in the green tea extract, such as EGCG, which is a cross-linking agent¹⁶ and may have improved the mechanical properties of collagen, besides of not impairing hybrid layer formation when the adhesive interface is evaluated under scanning electronic microscopy analysis²⁰.

However, it is worth noting that none of the dentin pretreatments prevented the decrease in bond strength over 6 months, similar to bond strength studies on sound dentin²⁰. In fact, the dentin pretreatment with 0.2% EGCG solution did not prevent the increase of nanoinfiltration in the hybrid layer formed in caries affected dentin, over a year of storage in water²⁴. One explanation for this result may be related to the time that solutions were applied to dentin, which in the present study was 60 seconds as done in previous studies^{17,20,24}. It has been observed that the increase in dentin elasticity modulus, interpreted as an increase in dentin mechanical strength, was achieved only after 60 minutes of application of green tea extract²⁵. In fact, studies that applied other natural extracts on dentin, such as 6.5% grape seed extract have found a positive effect on the bond strength stability of the pre-treated dentin with the application time of 10 minutes²⁶. It is hypothesized that the effect of pre-treatments with polyphenols, such as green tea, is time and concentration dependent. Thus, future studies should assess whether longer application times of the green tea extract solution can increase the modulus of dentin elasticity (proving its cross-linking effect) and at the same time stabilize the bond strength over time.

Also, the decline in bond strength after 6 months of water storage can be explained by the hydrolytic degradation of the resin-dentin adhesive interface after immersion in water, that promotes loss of resinous material throughout the hybrid layer and micromorphological changes in the collagen fibrils⁴. These micromorphological changes seem to be responsible for the degradation of the hybrid layer and reduction of the bond strength. Specially in caries affected dentin, the hybrid layer presents an increase in the exposed collagen zone and a decrease in the quality of the adhesive infiltration, and consequently, the adhesive interface is more prone to hydrolytic degradation⁸. In fact, the percentage of adhesive failures increased over time, which can confirm the degradation that occurred at the adhesive interface.

An important point is in regard to the use of water as a storage medium. Based on the fact that MMPs require zinc and calcium to be active²⁷. It has been reported that the use of water underestimates the activity of dentin MMPs, thereby promoting loss of calcium and zinc from dental matrices instead of restoring these ions²⁵. In this sense, the use of solutions that simulate body fluids as a means of storing samples is of interest in future evaluations involving MMP inhibitors as dental pre-treatments.

In terms of clinical application, the results found here allow for reflection and the need for a greater understanding of the action of the activity of collagen-degrading enzymes, and it is important to conduct further studies in order to assess the ideal concentration as well as time and form of application of green tea extract to dentin. Also, clinically, the caries affected dentin is considered a challenging substrate, which can outweigh

the benefits of pretreatments applied to it. So, this study revealed that, although the application of 0.2% green tea extract solution promoted greater bond strength than the other studied concentrations, none of the dentin pre-treatment solutions were sufficient to prevent the decrease in bond strength to caries affected dentin over time.

In conclusion, dentin pre-treatment with aqueous solution containing 0.2% green tea extract promoted higher bond strength of etch-and-rinse adhesive system to caries-affected dentin than the concentrations of 0.05% and 2%. However, bond strength decreased 6 months of water storage, regardless of whether or not the dentin received pre-treatment solutions.

Disclosure Statement

No potential conflict of interest was reported by the authors.

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