Epigallocatechin-3-gallate (EGCG) enhances the therapeutic activity of a dental adhesive

Xijin Du a,1, Xueqing Huang a,b,1, Cui Huang a,* Yake Wang a, Yufeng Zhang a

a The State Key Laboratory Breeding Base of Basic Science of Stomatology (Hubei-MOST) & Key Laboratory of Oral Biomedicine Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, People’s Republic of China
b Department of Prosthodontics, Guanghua School and Hospital of Stomatology and Institute of Stomatological Research, Sun Yat-sen University, Guangzhou, People’s Republic of China

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A B S T R A C T
Objectives: The purpose of this study was to evaluate the antibacterial potential and physicochemical properties of a dental adhesive incorporated with epigallocatechin-3-gallate (EGCG) in different concentration over time.

Methods: EGCG was incorporated at a ratio of 100, 200, and 300 μg/ml into a dental adhesive. The effects of the cured adhesives on the growth of Streptococcus mutans were determined by direct contact test immediately or one month later and by scanning electron microscopy (SEM), respectively. Microtensile bond strength (μTBS) test was used to test the mechanical property of the adhesives immediately or six months later. The degree of conversion (DC) of the adhesives was evaluated by Fourier Transform Infrared Spectroscopy (FTIR).

Results: Compared with negative control, the 200 μg/ml and 300 μg/ml EGCG-incorporated dental adhesive were found to exhibit inhibitory effect on the growth of S. mutans. The μTBS of the EGCG-incorporated dental adhesive was higher than the control. The DC of the adhesive system was not affected by the addition of EGCG.

Conclusions: 200 μg/ml EGCG incorporated dental adhesives could accomplish therapeutic goals that play in antimicrobial function whilst keeping the durability of resin–dentine bond.

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1. Introduction

Since Buonocore’s first introduction of the acid-etch technique, resin-based dental adhesive materials have demonstrated good performance in daily oral clinical practice.1 However, contemporary adhesive systems have some limitations that influence their long-term clinical application.2–4 Secondary caries and dental adhesives’ poor durability are the two main reasons for the replacement of the resin-based restorations.5,5 Therefore, the dental adhesives are expected to accomplish multiple therapeutic goals that play in antimicrobial function whilst keeping the durability of the materials.6

Resin-based restoration materials have been shown to accumulate significantly more dental plaque than glass ionomer cements and amalgam.7 Therefore, residual bacteria or invading bacteria along the tooth/restoration interface might be the possible reason for the development of secondary caries and that of pulp irritation after treatment. To avoid cariogenic bacterial colonization on the tooth/restoration interface and to avoid growth of remaining bacteria in the cavity after preparation, an antimicrobial effect of the adhesive systems and restorative composites is desirable.9 This effect can be achieved by incorporation of antimicrobial agents or that of antibacterial monomers in the adhesive systems’ formulation.9,10 However, when these antibacterial...
agents were incorporated into the adhesives, the physicochemical properties of the adhesives might be compromised.\textsuperscript{11,12} Several studies have shown that the hybrid layer created by current adhesive systems is imperfect and susceptible to enzymatic degradation.\textsuperscript{13,14} Endogenous dentine matrix metalloproteinase (MMPs) can be activated by adhesives through different mechanisms. Degradation of collagen fibrils by MMPs in hybrid layers of resin-bonded interface over time remains a problem.\textsuperscript{15} This probably accounts for the reduced longevity of clinically applied resin-based restorations.

Nowadays, many studies focus on the modification of dental adhesives to improve the clinical performance of the resin-based restorations. In these studies some chemical synthetics such as chlorhexidine were used.\textsuperscript{16} However, the drug resistance of the chemical synthetics is concerned increasingly.\textsuperscript{17} Epigallocatechin-3-gallate (EGCG) (Fig. 1), the major polyphenol present in green tea, has been known to contain potential health ingredients, including antioxidant, antimicrobial, antidiabetic, anti-inflammatory, and cancer-preventive properties.\textsuperscript{18} Significant antimicrobial activity in vitro has been demonstrated against a variety of Gram-positive, Gram-negative and fungal pathogens.\textsuperscript{19} EGCG was also found to have distinct inhibitory activity against MMPs.\textsuperscript{20,21} However, the use of EGCG in dental adhesives as a kind of modification has not been investigated.

Thus, the purpose of this study was to evaluate the antibacterial potential and physicochemical properties of a dental adhesive incorporated with EGCG in different concentration over time. The null hypothesis was that there was no difference in the antibacterial and physicochemical effect of a dental adhesive containing different concentration of EGCG.

2. Materials and methods

2.1. Adhesives preparation

A commercially available dental adhesive, Adper\textsuperscript{TM} Single Bond 2 (SB) (3M ESPE, St. Paul, MN, USA), was used as control. EGCG (Sigma–Aldrich, St. Louis, MO, USA) was dissolve into absolute ethyl alcohol at the concentration of 5 mg/ml, 10 mg/ml and 15 mg/ml. Then the EGCG/ethanol was added into SB at the ratio of 2% to get the final concentration of 100 µg/ml (EGCG100), 200 µg/ml (EGCG200) and 300 µg/ml (EGCG300) EGCG in the dental adhesive, respectively.

2.2. Antibacterial effect test

Streptococcus mutans UA 159 (ATCC #700610), a well-described cariogenic pathogen, was cultured overnight at 37°C in Brain Heart Infusion broth (BHI) (BD, Sparks, MD, USA) in an anaerobic atmosphere. The bacterial suspension obtained was adjusted to an optical density (OD) of 0.3 at 600 nm (about 3 × 10\textsuperscript{8} CFU/ml) for further usage.

The cover of a sterile 96-well plate (Corning Inc., Corning, NY, USA), having 96 round indentations, was used for specimen preparation. An adhesive layer of each group was prepared on the bottom of the indentations. Composite resin (Charisma; Heraeus Kulzer, Hanau, Germany) was applied to the adhesive layer and photo-cured covered with a glass plate to obtain specimens of 8 mm in diameter and 1 mm in thickness.

All specimens were immersed in 10 ml sterile distilled water at 37°C, agitated for 2 h to remove unpolymerized monomers and dried at room temperature before they were sterilized by ultraviolet light.

Ten specimens were placed in the wells of a 24-well plate, with the adhesive layers facing upward. A 20-µl of bacterial suspension was applied to the adhesive layer of each specimen. After incubation at 37°C for 1 h, a 2-ml quantity of BHI supplemented with 1% sucrose was added to each well, and the specimen was incubated for a further 24 h. Each specimen was sonicated in 1 ml of distilled water to detach adherent bacteria and disperse congregated cells. Then, the OD600 value of the bacterial suspension was measured with a spectrophotometer (Powerwave 340, Bio-tek Instruments, Winooski, VT, USA).

Another ten specimens of each group were aged for 1 month. During this time, 1 ml of distilled water was added to each well containing the specimen and replenished every 48 h. Then, aged specimens were subjected to a biofilm accumulation test as mentioned above.

For the scanning electron microscopy (SEM) evaluation, biofilms were generated as mentioned above on two specimens of each group. After 24 h incubation, the specimen with biofilm was then gently rinsed with distilled water and fixed in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer at pH 7.2 for 4 h at room temperature. Specimens were then dehydrated in ascending ethanol series and dried with hexamethyldisilazane (HMDS). Finally they were mounted on the microscope stubs and coated with gold. Spatial distribution and architecture of biofilm were observed under SEM (Quanta 450 FEG, FEI, Eindhoven, The Netherlands).

2.3. Microtensile bond strength test

Human third molars were collected after obtaining the donors’ informed consent for the teeth to be used in this study. The study protocol was approved by the Ethics Committee for
Human Studies of the School & Hospital of Stomatology, Wuhan University [2011(067)].

The extracted teeth were stored in 0.9% (w/v) NaCl containing 0.002% sodium azide at 4 °C for no longer than 1 month. Twenty teeth were used in this study. The roots of the teeth were removed by a water-cooled low-speed cutting saw. Then, a flat surface was prepared by removing the occlusal one-third of the tooth crowns to expose midcoronal dentine. The dentine surface was polished with 600-grit SiC paper to create a standardized smear layer. The crown segments were randomly allocated to four groups, according to the adhesives tested. Each group had five teeth.

After etching and rinsing, two applications of adhesive were made on the blotted water-moist dentine surface and gently agitated with a microbrush for 10 s. Then, after excess solvent was evaporated with a gentle air stream for 10 s, the adhesive was light-cured for 10 s and resin composite build-ups were constructed with 2-mm increments of resin composite (Charisma; Heraeus Kulzer, Hanau, Germany). After storage in deionized water at 37 °C for 24 h, each tooth was vertically sectioned into 0.9 mm × 0.9 mm composite-dentine beams by the cutting saw with water-cooling, excluding those situated peripherally that showed presence of enamel. The bonded sticks that originated from the same teeth were randomly divided and assigned to be tested immediately, or after 6 months of storage in distilled water at 37 °C. The storage solution was changed monthly and its pH was monitored.

Fifteen bonded beams from each group were then pulled apart through a microtensile bond strength (μTBS) testing protocol. The exact dimensions of each beam were measured with a digital calliper. Each beam was attached to the testing apparatus with a cyanoacrylate adhesive (Zapit, Dental Ventures of America, Corona, CA, USA) and loaded until failure under tension by a μTBS tester (Microtensile Tester, Bisco Inc., Schaumburg, IL, USA) at a cross-head speed of 1 mm/min. The μTBS was calculated as the maximum load at failure, divided by the cross-sectional area and was expressed in MPa.

2.4. Degree of conversion

The degree of conversion (DC) of the dental adhesives was analysis by Fourier Transform Infrared Spectroscopy (FTIR). For FTIR analysis, one drop of adhesive resin was dispensed into a well immediately prior to application. An applicator tip was dipped into the adhesive solution and the adhesive was applied to the surface of a potassium bromide (KBr) pellet. An FTIR spectrum of unpolymerized adhesive solution was obtained from each sample using 20 scans at 4 cm⁻¹ in the transmittance mode (Nicolet 6700, ThermoFisher, MA, USA).

The adhesive systems were light cured for 10 s using a conventional quartz-tungsten-halogen light source (XL 3000, 3M ESPE). The tip of the curing unit was positioned 3 mm from the pellet surface. Additional FTIR spectra were obtained immediately after light curing. The analyses were performed at 24 °C, under 64% relative humidity. Ten specimens of each group were tested by FTIR (n = 10).

For analysis of the DC, the aliphatic carbon-to-carbon double bond absorbance peak intensity, located at 1638 cm⁻¹, and that for the aromatic component, located at 1608 cm⁻¹, were compared in each spectrum before and after the polymerization reaction, and monomer conversion was determined using the following equation

\[
\text{DC} = \frac{\text{abs(likely C=C)}}{\text{abs(likely C=C) + abs(aromatic C=C) + polymer}} 
\]

where (%) C=C is the percentage of remaining carbon double bonds and abs is the absorbance.

The DC was obtained by subtracting the percentage of the remaining carbon double bonds (%) C=C from 100%.

2.5. Statistical analysis

OD600 value, μTBS and DC, was analyzed using a statistical analysis of variance (ANOVA) with Tukey’s test or Tamhane’s T2 post hoc test for pairwise comparisons between the means. SPSS software, version 16.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. The significance level was set at α = 0.05 for all tests.

3. Results

3.1. Antibacterial effect

Mean OD600 values and standard deviations are displayed in Fig. 2. Before ageing, SB and EGCG100 yielded higher OD600 value than other groups (P < 0.05); no statistically significant difference was found between the values of SB and EGCG100 (P > 0.05). The OD600 value of EGCG300 was significantly lower than EGCG200 (P < 0.05). Additionally, no significant elevation...
of OD600 value was displayed for each group after the one-month ageing process (P > 0.05) (Fig. 2).

SEM images show that after 24 h incubation, bacteria were accumulated on specimens of all 4 groups. Bacteria were densely packed within extracellular matrix on SB and EGCG100 group. However, biofilms accumulated on the EGCG200 and EGCG300 group were not compact (Fig. 3).

3.2. Microtensile bond strength

Mean μTBS values were calculated from all experimental groups and are displayed in Table 1. The variables dental adhesive (F = 19.502, P = 0.000) and time (F = 6.626, P = 0.011) significantly influenced bond strength.

After 100 μg/ml and 200 μg/ml EGCG incorporated, the immediate μTBS value of the dental adhesive was significantly higher than the control group (P < 0.05). There was no difference between the 300 μg/ml EGCG group and the control (P > 0.05). After 6 months of in vitro water storage, the μTBS of the three EGCG incorporated groups were significantly higher than the control group (P < 0.05). Significant reduction of μTBS was observed for the control group (P < 0.05), whilst no significant reduction was observed when EGCG was incorporated (P > 0.05).

<table>
<thead>
<tr>
<th></th>
<th>SB</th>
<th>EGCG100</th>
<th>EGCG200</th>
<th>EGCG300</th>
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<tr>
<td>Immediate</td>
<td>36.80 ± 6.29&lt;sup&gt;a,1&lt;/sup&gt;</td>
<td>43.58 ± 6.51&lt;sup&gt;a,2&lt;/sup&gt;</td>
<td>48.21 ± 6.60&lt;sup&gt;a,2&lt;/sup&gt;</td>
<td>41.83 ± 8.00&lt;sup&gt;a,1,2&lt;/sup&gt;</td>
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<tr>
<td>6 months</td>
<td>28.07 ± 6.73&lt;sup&gt;b,1&lt;/sup&gt;</td>
<td>42.20 ± 8.97&lt;sup&gt;b,2&lt;/sup&gt;</td>
<td>46.49 ± 8.53&lt;sup&gt;b,2&lt;/sup&gt;</td>
<td>39.08 ± 9.68&lt;sup&gt;b,2&lt;/sup&gt;</td>
</tr>
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For each column, groups labelled with the same letter superscripts are similar (P > 0.05). For each row, groups with the same number are significantly similar (P > 0.05).

Fig. 3 – SEM micrographs (magnification 10,000×) of S. mutans biofilms on adhesives of 4 groups. (A) Single bond 2, (B) EGCG100, (C) EGCG200, and (D) EGCG300. Bacteria were densely packed within extracellular matrix on SB and EGCG100 group. However, biofilms accumulated on the EGCG200 and EGCG300 group were not compact.
3.3. **Degree of conversion**

Fig. 4 showed the DC means and standard deviations for dental adhesives. After EGCG incorporated, the DC of dental adhesives was decreased slightly. But the difference was not statistically significant (P > 0.05).

Fig. 5 showed the representative spectrum sites obtained from the adhesive systems before (uncured state) and immediately after light curing. These sites were used to calculate the DC. Fig. 5A–D exhibit the spectrum sites obtained from SB, EGCG100, EGCG200 and EGCG300 group light cured by halogen light, respectively. There was little reduction in the peak located at 1638 cm⁻¹ after 10 s of light curing for adhesive systems.

4. **Discussion**

The addition of EGCG significantly altered antibacterial and mechanical properties evaluated in this study. The direct contact test and SEM evaluation showed that antibacterial activity of the dental adhesives was increased after 200 μg/ml or higher concentration EGCG incorporated. The bond strength test result revealed that after EGCG incorporated, the immediate bond strength of the dental adhesives was higher than that of SB. And the durability of the adhesives was improved when EGCG was incorporated. Result of FTIR analysis showed that the DC of dental adhesive was not influenced after EGCG incorporated. Thus, the null hypothesis was rejected.

Adhesive restoration was used broadly because of its aesthetic performance. However, there were also many disadvantages, such as lack of antibacterial effect, poor bond durability, cytotoxicity of the unpolymerized monomer, that prejudice the use of dental adhesive. Nowadays, modification of dental adhesive was done by many researchers to get new products with better performance. But these studies only improve one kind of function, such as addition of fluoride to promote the antibacterial effect of the dental adhesives, application of MMPs inhibitor to protect the bond durability of the dental adhesives. Few studies are seeking for the modification to get multifunctional dental adhesives that can improve the various performances simultaneously.

EGCG is a flavonoid produced in large amounts as a secondary metabolite by the *Camellia sinensis* plant, “green tea”. The compound has been studied widely due to its excellent chemopreventive properties in the context of most
types of cancer, as well as chronic diseases such as pulmonary fibrosis. EGCG reduces cardiovascular risk factors by scavenging reactive oxygen species (ROS), limiting the proliferation of endothelial cells, inhibiting angiogenesis, and reducing inflammation.28

EGCG was added into the dental adhesive to improve its antibacterial and mechanical activity in this study. The incorporation of EGCG into the single bond 2 adhesive as described herein is an example of a so-called “therapeutic adhesive”. As the commercial adhesives have their unique formulation, other substances cannot be incorporated into them randomly without considering the possible negative effects on bonding efficiency and handling property.29 In the present study, EGCG was first dissolve into ethanol at different concentration, and then add to the dental adhesive at the same ethanol concentration. This guaranteed the volume of ethanol add into the adhesive was the same. Before the light curing of the dental adhesive, ethanol will volatilize along with the solvent of the adhesives. This procedure can minimize the influence of the change of the adhesive formulation. Result of FTIR analysis verified that the DC of dental adhesive was not reduced significantly after EGCG incorporated. However, a lower DC tendency was noted after higher concentration EGCG incorporation. During the polymerization of dental adhesive, linear polymer chains are formed.30 With the incorporation of 100-300 μg/ml EGCG into dental adhesive, EGCG could be trapped within the linear chains after curing. However, with higher concentrations of EGCG, the formation of linear polymer chains might be disturbed. This results in inadequate polymerization of the adhesive. Furthermore, EGCG has a free radical scavenging effect, which can disturb the free radical polymerization of the adhesive.31 The result in this study only suggest that the DC of adhesive would not affected by EGCG at the concentration we tested. It might be affected if more EGCG was added. So it is necessary to assess the DC of adhesives when an addition agent would be incorporated.

Most of the adhesive restorations have no antibacterial effect and the resin based materials are prone to accumulate bacterial.3 Therefore, it demonstrated that EGCG showed significant antimicrobial activity against a variety of Gram-positive, Gram-negative and fungal pathogens in vitro.32 In the present study, EGCG was incorporated into the dental adhesive to get the adhesive with antibacterial effect. As demonstrated by Xu et al., the MBIC50 (the lowest EGCG concentration that showed at least 50% inhibition of the formation of biofilms compared with control) was 312.5 μg/ ml.33 In our study, EGCG300 group showed about 50% inhibition of the biofilm formation. However, the 100 μg/ml concentration was not effective for the inhibition of S. mutans biofilm. The EGCG100 group showed no significant difference to the control. The present findings support the evidence that the presence of more than 200 μg/ml EGCG in the adhesive produced an antimicrobial activity.

The antimicrobial mechanism of EGCG is mainly attributable to influence the initial bacterial adhere to the surface. Microbial biofilm commonly exhibits increasing levels of resistance to most antibiotics or therapeutic agents. EGCG represents a natural anti-cariogenic agent by exhibiting antimicrobial activity against S. mutans, and by suppressing the specific virulence factors associated with its cariogenicity.33 EGCG may be capable of suppressing gtf B, C, D gene expression leading to disruption of S. mutans biofilm formation at a lower concentration.34 In this research, we demonstrated that the growth of S. mutans was inhibited by the contact with the no less than 200 μg/ml EGCG-incorporated adhesive. Direct contact test reflects the antibacterial effect of agent stuck into the adhesive after polymerization.35 Adhesives with long-lasting antibacterial activity would be welcomed because the accumulating effects of long-term bacterial metabolism on failed bond interface are blamed for generating secondary caries.36 Pashley et al. stated that once polymerized, reagents with large molecular weight dissolved in adhesives cannot diffuse out of the polymerized resin.6 In the present study, no significant reduction of the antibacterial activity was observed after 1 month ageing process. However, the findings with the ageing process of only 1 month are inadequate to elicit a conclusion of a long-lasting antibacterial activity. Therefore, further investigations are required to evaluate the persistency of the antibacterial activity of the EGCG-incorporated adhesive.

There are many methods to improve dentine bond strength and bond durability of the adhesives, such as application of selective collagen cross-linkers during adhesive restorative procedures,36 ethanol-wet bonding technique,37 usage of MMP inhibitors.38 A previous study suggested that sodium ascorbate could increase the bond strength of dental adhesives because ascorbic acid and its sodium salt are potent antioxidants.39 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. EGCG is an anti-oxidant and may improve the bond strength through the same mechanism. Another reason for this might be that EGCG interact with dental adhesive and/or dentine. More research is needed for further evaluation.

Demineralized dentine contains bound matrix metalloproteinas-2, -3, -8, -9 and -20 (MMPs) and cathepsins that, once activated by acid-etching, can slowly degrade the collagen fibrils of resin-infiltrated hybrid layers.40,41 Many authors have shown the efficacy of incorporating chlorhexidine (CHX) in adhesive primers at preventing degradation of hybrid layers.33,38 However, the cytoxicity and drug resistance of CHX are concerned these years. EGCG is a natural plant extracts and the biocompatibility was verified in the previous studies.42 EGCG seems to exhibit hydrogen bonding and hydrophobic interactions with collagenases, which are responsible for the change in the secondary structure of collagenases and, consequently, for their inhibition of MMPs. The MMPs inhibit effect of EGCG had been confirmed by several studies.20,43 In the present study EGCG was mixed with the adhesives to a final concentration of 100, 200, and 300 μg/ ml. It will bond to the resin on the hybrid layer and inhibit the MMPs. Thus, the bonding durability was retained.

Mechanical disruption and the presence of free radicals may also augment the degradative process of the demineralized collagen matrix.44 These reactive free radical species are normally associated with several forms of tissue damage and the process of ageing. Oxidants such as the hydroxyl radical (formed from hydrogen peroxide), contain an unpaired electron and thus are highly reactive and damaging to the protein structures at the molecular level.45 Antioxidants, by
counteracting the harmful effects of free radicals, protect structural integrity of the protein. EGCG is an antioxidant that is capable of neutralizing potentially damaging free radicals. Reactive oxygen species can ‘abstract’ hydrogen atoms from EGCG. EGCG may suppress the denaturing effect of etching on dentine collagen, offering protection against the degradation of composite–dentine bonds.

The presence of salivary proteins, which could mediate in vivo bacterial adhesion to oral surfaces and reduce the antibiofilm effects of the materials, was not taken into account in the in vitro model for the antibacterial activity investigation. Therefore, it is impossible to evaluate the clinical relevance of the bonding system with EGCG from the results of the present study. Further studies are needed to clarify whether the likelihood of developing secondary caries could be reduced by this kind of adhesive materials.

5. Conclusion

EGCG incorporated dental adhesive at the concentration of 200 μg/ml could accomplish multiple therapeutic goals that play in antimicrobial function whilst keeping the durability of the material.

This research may help to provide a new understanding of the resin-based dental materials with therapeutic goals.

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