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Antibacterial Activity and Bond Strength to Enamel of Catechin-Incorporated 4-META/MMA-TBB Resin as an Orthodontic Adhesive Resin

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Abstract: The purpose of this study was to assess the antibacterial efficacy of incorporating catechins into 4-META/MMA-TBB resin against Streptococcus mutans and to measure the shear bond strength of a metal bracket bonded to enamel using catechin-incorporated with 5% 4-methacryloyloxyethyl trimellitate anhydride (4-META)/methyl methacrylate (MMA)-tri-n-butyl borane (TBB) resin (catechin-resin). Catechin-resin disks were incubated with the bacterial suspension at 37 °C for 24 h. The bacterial counts (colony forming units) of the disk and the suspension were determined separately. Catechin release from catechin-resin was monitored. The catechin-containing composites were used to bond metal brackets to human premolars pretreated with self-etching primer. After the bonded samples were immersed in water at 37 °C for 24 h, shear bond strength was measured.

The most significant antibacterial activity was observed in (-)-epigallocatechin gallate-resin disk and (-)-epigallocatechin gallate-resin-treated bacterial suspension when compared with the control. Catechin was released from catechin-resin during 6 week immersion in deionized water. (-)-Epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate showed a large amount of catechin release. Only significant differences was detected in bond strength between the control and (-)-epigallocatechin gallate. The addition of (-)-epigallocatechin gallate to 4-META/MMA-TBB resin confers an antibacterial effect while retaining sufficient bond strength.

Key words: 4-META/MMA-TBB resin, Green tea catechin, Antibacterial activity, Shear bond strength, Orthodontic adhesive resin

Introduction

Orthodontic patients are known to have a higher incidence of white spots on the enamel around the bonded bracket than general patients1,2. Some enamel damage at debonding has also been reported when adhesive resin cement is used3,4. It was suggested that a high prevalence of caries may be caused by high cariogenic challenge prevailing in the plaque around orthodontic appliances5. Matasa6 reported that orthodontic composite adhesives may host and nurture a variety of microorganisms and their accumulation may lead to weakening of the bond and tooth attack. Oral hygiene practice is important during orthodontic treatment when the teeth are fitted with bonded orthodontic brackets. In the clinical setting, sustained release of antibacterial agents into the oral cavity is useful.

4-Methacryloyloxyethyl trimellitate anhydride (4-META)/methyl methacrylate (MMA)-tri-n-butyl borane (TBB) resin is widely known as an effective orthodontic resin. Yamauchi et al.7 reported that cured 4-META/MMA-TBB resin has a slight antibacterial effect. Kudou et al.8 attempted to incorporate vancomycin and metronidazol into 4-META/MMA-TBB resin to obtain an antibacterial effect. Resin disks containing vancomycin showed a higher antibacterial effect against Streptococcus mutans (S. mutans) than those containing metronidazol without loss of bond strength when bonded to dentin; however, their report provided no data on adhesion to enamel. Recently, Kazuno et al.9 reported that MMA/TBB resin containing newly developed amphiphilic lipids showed a strong antibacterial effect against S. mutans or Streptococcus sobrinus (S. sobrinus).

In a previous study, we added benzalkonium chloride (BAC) to 4-META/MMA-TBB resin and evaluated its antibacterial properties and cytototoxicity10,11. The addition of BAC to 4-META/MMA-TBB resin conferred antibacterial properties due to release...
of the antibacterial agent against *S. mutans* and *S. sobrinus*. In cytotoxicity tests, no marked cell death was observed for resins containing 0.25 or 0.75 % BAC; however, after 6 days of incubation, most cells did not survive when exposed to resins with 1.75, 2.5 and 5 % BAC. Our results thus indicate that higher antibacterial activity is accompanied by a higher degree of cytotoxicity, highlighting the importance of oral safety in the development of antibacterial adhesive resins.

Green tea is the second most consumed drink in the world. It is a non-fermented tea, and contains more catechins than black tea or oolong tea. The green tea catechins used in this study were originally isolated from the leaves of *Camellia sinensis*, used for Japanese green tea, which is a popular and safe drink found to contain major catechins such as (-)-epigallocatechin gallate (EGCg), (-)-epicatechin gallate (ECg), (−)-epigallocatechin (EGC), (−)-epicatechin (EC), and (−)+catechin (C) (Fig. 1). C and EC are epimers, which are diastereomers differing in configuration at only one stereogenic center. EC is structurally modified to EGC, ECg and EGCg by 5-hydroxylation and 3-gallate esterification. Catechins from green tea have been reported to have antibacterial12-15), antifungal16-18), antiviral19-22) and protein- denaturing13, 14, 23, 24) properties. The biological activities of these catechins are significantly influenced by such structural difference.12, 25, 26).

The purpose of the present study was to assess the antibacterial efficacy of incorporating catechins into 4-META/MMA-TBB resin and to measure the shear bond strength of a metal bracket bonded to enamel using catechin-incorporated 4-META/MMA-TBB resin.

**Materials and Methods**

**Materials**

Table 1 lists the materials used in the present study. Superbond C&B resin cement (Sunmedical Co. Ltd., Shiga, Japan) was used in this study. Commercially available catechins (KRT Biomedicals Inc., Shizuoka, Japan) in powder form were incorporated into the resin.

Five catechins were assessed in this study: EGCg, ECg, EGC, EC, and C. Figure 1 shows the chemical structures of the five catechin compounds. Each catechin was added to the polymer powder of Superbond C&B at a concentration of 10.0 % (wt/wt) (catechin-resin). The original Superbond C&B resin was used as a control (control resin).

**Antibacterial activity of catechin samples**
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Streptococcus mutans NCTC10449, an oral cariogenic species, was used as the test bacteria. The bacteria were cultured at 37 °C for 24 h in brain-heart infusion broth (BHI; Difco Laboratories, Detroit, Mich., USA). The bacterial suspension was adjusted to an optical density of 1.0 (550 nm), and there were approximately $1 \times 10^6$ CFU/ml.

Resin disks of uniform size (8.0 mm diameter × 2.0 mm thick) were made from the five kinds of catechin-resins using custom-made molds with brush-dip technique. Namely, the catalyst, a partly oxidized TBB initiator, was added to the monomer mixture of 4-META and MMA to prepare an activated polymerized monomer liquid. The polymer powder containing catechin and activated monomer liquid were mixed and then were put into the mold. After curing of the resin, the mold was removed.

Two methods were employed for the evaluation of antibacterial activity of catechin-resins. First, a catechin-resin disk was placed in the bacterial suspension and incubated at 37 °C for 24 h. The disk was then removed, washed, and sonicated in 2 ml of 50 mM Tris-HCl buffer at 50 W for 20 s. The suspension was serially diluted and plated on mitis salivarius (MS) agar, and the plates were incubated at 37 °C for 48 h. The numbers of colony forming units (CFU) were counted. This was the disk method.

Another method, cell suspension method, is as following. Secondly, after removal of the disk, 100 µl of the remaining bacterial suspension was sampled, serially diluted with Tris-HCl buffer and plated on MS agar. The MS agar plates were cultured anaerobically at 37 °C for 48 h, and the numbers of CFU were counted.

**Analysis of catechin release**

Each catechin solution containing 0.03, 0.06, 0.12, 0.25, 0.5 and 1.0 mg/ml catechin was used to obtain a calibration curve for monitoring the release of catechin from cured catechin-resins. Analysis of the released amount of catechin was determined using a spectrophotometer (ND-1000; Thermo Scientific, MA, USA). The maximum absorbance peak of catechin was confirmed at 277 nm.

Each disk was individually immersed at 37 ºC in 2 ml deionized water inside a sealed container. Then, 5 µl each of solution samples were collected 0, 1, 2, 3, 4, 5 and 6 weeks later. At appropriate time intervals, the absorbance peak height of the storage solutions at 277 nm were obtained and converted to the quantities of catechin released based on the established calibration curve.

**Bonding procedures**

Thirty extracted human premolars, which were stored in distilled water in refrigerator, were used in this study and were randomly divided into 6 groups. The teeth were embedded in acrylic resin with the buccal surfaces available for bonding. After curing the acrylic resin, the tooth surfaces to be bonded were cleansed and then polished with pumice and rubber prophylactic cups for 10 seconds in order to simulate routine clinical procedures.

Orthodontic metal brackets (Super mesh STD Edgewise 131-45B; Tomy International Inc., Tokyo, Japan) were used in this study. The average bracket surface area was determined to be 11.188 mm². A tooth was primed with Clearfil SE bond primer (Kuraray Medical Inc., Tokyo, Japan) for 30 s. Excessive primer solution was evaporated using compressed air. A metal orthodontic bracket was bonded to the etched enamel surface using 10 % catechin-containing composite resin cement. The catalyst was added to the monomer mixture of 4-META and MMA to prepare an activated polymerized monomer liquid. The polymer powder and activated monomer liquid were mixed and used to bond metal brackets to the treated enamel surface using the brush-dip technique. Preliminary experiments revealed that it was impossible to prepare the bonded specimens for catechin-resins with more than 10 % of catechin.

Each bracket was subjected to a 300 g force, according to the report of Bishara et al. (27), and excess bonding resin was removed with a small scaler. The bonded specimens were immersed in water at 37 °C for 24 h. Shear bond strength was measured according to the methods recommended by the International Organization for Standardization (28), using a testing machine (TCM-500CR; Shinkoh, Tokyo, Japan) at a cross-head speed of 2 mm/min.

After debonding, the teeth and brackets were examined at 10× magnification. The debonding condition of each specimen was scored using the adhesive remnant index (ARI) (29). The ARI was scored as follows: score 0 = no adhesive remained on the enamel; 1 = less than half of the adhesive remained on the tooth surface; 2 = more than half of the adhesive remained on the tooth; 3 = all the adhesive remained on the tooth with a distinct impression of the bracket base.

**Statistical Analysis**

The data are presented as the mean and standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine whether a significant difference existed among various groups, and Fisher’s test was used for multiple comparisons. The chi-square ($X^2$) test was used to detect statistical differences in ARI scores and enamel fracture scores among 6 groups. Significance for all statistical tests was predetermined at $p < 0.05$.

**Results**

**Antibacterial activity of catechin–resin disk samples**

The results of antibacterial activity of catechin-resin disk samples are shown in Table 2. One-way ANOVA showed a significant difference among six groups ($F=100.648, p<0.0001$). A significant difference ($p<0.0001$) were detected when each catechin–resin disk was compared with the control resin disk, C.
and EGCg, C and ECg, EGCg and EC, (p<0.01) were detected between C and EGC, EGCg and ECg, EGC and ECg, EGC and EC. No significant differences were detected between C and EC, EGCg and ECg, EGC and ECg, EGC and EC groups.

Next, when the bacterial counts of the resin disk treated with bacterial suspensions were analyzed, one-way ANOVA detected a significant difference (F=453.902, p<0.0001) among six groups (Table 2). A significant difference (p<0.0001) was detected when each catechin–resin-treated suspension was compared with the control resin-treated suspension, and between C and EGCg, C and ECg, EGCg and EGC, EGCg and EC, EGC and ECg, EGCg and EC, (p<0.01) were detected between C and EC. No significant differences were found between C and EC, EGCg and ECg, EGC and EC.

Analysis of catechin release

Figure 2 shows that when the control adjust to 0, the amount of catechin released from the resin disks after immersing in deionized water for 6 weeks. The catechin-composite disks released catechin throughout the 6 weeks. A large quantity of catechin was released from ECg, EC and EGCg resin during one week. The amount of C released was very low.

Comparison of shear bond strengths

The results of shear bond strength measurements (MPa) are listed in Table 3. One-way ANOVA showed no significant difference in bond strength among the six composites, including catechin–containing resins and control resin (F=1.471, p=0.2696). A significant difference was found between control resin and EGCg only (p<0.05).

Comparison of ARI

The ARI scores after debonding were 0 for all six composites including catechin–containing resins and control. The resin was observed only on the bracket after debonding for all six composites. The chi-square test showed no significant difference in ARI scores among the six groups.

Discussion

In orthodontic treatment, the important properties for an antibacterial adhesive are antibacterial durability and oral safety. Otake et al. have shown that tea catechins containing galloyl radicals have inhibitory effects on glucosyltransferase isolated from S. mutans. Moreover, ECg and EGCg inhibit collagenase from Porphyromonas gingivalis, which are periodontopathic bacteria. Hirasawa et al. reported that EGCg is effective in reducing acid production by S. mutans in dental plaque. EGCg inhibits lactate dehydrogenase activity, but does not kill cells cultured in a medium containing sucrose. It was also reported that EGCg inhibits C. albicans growth and that anticandidal activity is pH-dependent. Thus, we hypothesize that the incorporation of catechin as an antibacterial agent is a useful method of preventing dental caries without compromising oral safety. ECg and EGCg are expected to exhibit marked antibacterial activities, because both possess galloyl radicals. The present study showed similar in vitro bactericidal activities with all five catechins tested. The precise mechanism of action of EGCg remains unclear as it appears to have a variety of inhibitory effects on bacteria.

Ikigai et al. reported that the investigated bactericidal functions of EGCg are mainly dependent on charges in the
bacterial membrane. Negatively charged EGCg is bound to the positively charged phospholipids of the membrane and causes damage to the lipid bilayer. Tsuchiya\textsuperscript{a} showed that between geometrical isomers, (+)-EC and (-)-EC were more effective than (+)-C and (-)-C for reducing the fluidity of all liposomal membranes. Catechins reduce membrane fluidity by acting on outer and inner layers of membranes. The membrane effects of catechins are considered to occur by the hydrogen bonding of their polydroxyl groups with the phosphocholine moiety and the interaction of their hydrophobic portions with acyl chains of phospholipids as reported for those of phenolic compounds\textsuperscript{30}. The interaction of their hydrophobic portions with the phodphocholine moiety and the geometrical isomers, (+)-EC and (-)-EC were more effective than (+)-C and (-)-C for reducing membrane fluidity than (-)-EC. 3-Gallate esterification to increase hydrophobicity is the determinant for enhancing the membrane effects and 5'-hydroxylation to decrease hydrophobicity reduces the ability to interact with membrane lipids\textsuperscript{20}.

No clear relationship was observed between the antibacterial activities of catechin disks and the amount of catechin released. Detailed studies are required to assess the antibacterial activities of each catechin.

Concern has been raised as to the safe intake of high doses of green tea polyphenols and led to the recent publication of a systematic review of the safety of green tea extracts by the US Pharmacopeia\textsuperscript{31}. In mice, EGCg was by far the most cytotoxic of the tea phenolics investigated and also most readily collapsed the mitochondrial membrane potential, and a lower concentration of EGCg (150 mg/kg) caused 100 % mortality of male CD-1 mice in 24 h\textsuperscript{21}. In humans, impaired liver function was observed in participants regularly consuming as little as 6 cups per day of green tea or 720 mg/d green tea extracts\textsuperscript{21}. On the other hand, Frank et al.\textsuperscript{33} reported that supplementation by healthy men with a high daily dose of 714 mg green tea polyphenols for 3 wk did not cause adverse effects or impair liver and kidney function and did not improve cardiovascular disease risk biomarkers other than the ratio of total HDL cholesterol. Daily consumption of a high dose of green tea polyphenols, equivalent to 6-8 cups of green tea\textsuperscript{41}, for 3 wk did not affect markers of liver and kidney function in healthy men\textsuperscript{35}, consistent with a comparable trial in healthy Japanese men who consumed 690 mg/d catechins for 12 weeks\textsuperscript{35}. A bonding procedure in one patient requires approximately 180 mg bonding composite, and only 18 mg of each catechin is needed for catechin-resin.

Self-etching primer, and not phosphoric acid, was used for etching in this study. Kawasaki et al.\textsuperscript{36} observed more dissolution of the enamel surface resulting from phosphoric acid etching than from self-etching primer treatment. Sirirungrojying et al.\textsuperscript{37} evaluated the effectiveness of a self-etching primer for bonding orthodontic brackets to enamel with 4-META/MMA-TBB resin, and found that a commercially available self-etching primer, Clearfil SE bond primer, produced no significantly different bond strength compared with phosphoric acid etching. Our previous study suggested that when used with Superbond C&B in bonding orthodontic brackets, Clearfil SE bond primer is superior to phosphoric acid as an enamel preparation agent in providing durable bond strength\textsuperscript{38}. Thus, Clearfil SE bond primer was used in the present study.

The clinically acceptable shear bond strength remains unknown. It has been suggested that a minimum shear bond strength of 6.0 to 8.0 MPa is adequate for bonding orthodontic brackets to teeth\textsuperscript{39,40}. Moreover, Ogaard et al.\textsuperscript{40} reported that debonding forces above 13 MPa may produce enamel fractures or tears, especially if the forces occur at an angle to the prisms. In this study, the shear bond strength of the catechin–resins ranged from 7.78 to 21.94 MPa. These results suggest that catechin-incorporated 4-META/MMA-TBB resins give clinically acceptable bond strength. The results of ARI indicated safe debonding with all catechin-containing composites.

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