In Vitro Antibacterial and Cytotoxicity Assessments of an Orthodontic Bonding Agent Containing Benzalkonium Chloride

Kayo Saito; Tohru Hayakawa; Rihito Kawabata; Daijiro Meguro; Kazutaka Kasai

ABSTRACT

Objective: To assess the antibacterial activity and cytotoxicity of an orthodontic bonding material containing an antibacterial agent.

Materials and Methods: Superbond C&B (4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane [4-META/MMA-TBB]) resin was mixed with benzalkonium chloride (BAC) to obtain final BAC concentrations of 0.25%, 0.75%, 1.25%, 1.75%, 2.5%, and 5.0% (wt/wt). Antibacterial activity against Streptococcus mutans and Streptococcus sobrinus was evaluated by soaking the BAC-resin in distilled water at 37°C for periods of 30, 90, and 180 days. Antibacterial activity of the BAC-resin was measured by the disk diffusion method, and the inhibition zone around each sample was measured and recorded. For evaluation of cytotoxicity, BAC-resin samples were put into cell culture inserts placed above human gingival cells and were incubated at 37°C for 1, 3, and 6 days. Cytotoxicity was assessed with a tetrazolium bromide reduction assay.

Results: The antibacterial activity of BAC-incorporated resin samples decreased significantly after immersion in water for 180 days, regardless of BAC concentration. The antibacterial activity of nonimmersed resin containing 0.25% or 1.75% BAC was comparable with that of 5.0% BAC-resin immersed for 180 days. In cytotoxicity tests, most cells died when exposed to resins containing 1.75%, 2.5%, and 5% BAC. No difference was observed between resins containing 0.25% and 0.75% BAC at 1, 3, and 6 days of culture.

Conclusions: The addition of BAC to 4-META/MMA-TBB resin confers an antibacterial effect even after immersion in water, and 4-META/MMA-TBB resin containing 0.25% to 0.75% BAC has no significant cytotoxic effect. (Angle Orthod. 2009;79:331–337.)

KEY WORDS: 4-META/MMA-TBB resin; Benzalkonium chloride; Antibacterial activity; Cytotoxicity

INTRODUCTION

Currently, adhesive resin cements are widely used for bonding orthodontic brackets to enamel. Super- bond C&B, a 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate-tri-n-butyl borane (4-META/MMA-TBB) resin, is a unique MMA-based resin that is used for bonding orthodontic brackets; it has earned a reputation for strong bonding.1–6 This resin cement is known as C&B Metabond (Parkell Inc, Farmingdale, NY) in North America. However, little attention has been paid to the antibacterial and biologic properties of adhesive resin cements such as 4-META/MMA-TBB resin.

Matasa7 reported that orthodontic composite adhesive can host and nurture a variety of microorganisms, and that microbial accumulation may lead to weakening of the bond and attack of the tooth. Some investigators have reported attempts to inhibit plaque accumulation on the surfaces of teeth and restorations with the use of antibacterial varnishes or resin materials containing antibacterial agents.8–11 The incorporation of chlorhexidine into glass-ionomer cements produced antibacterial activity.12

An experimental antibacterial bonding system (Ku-
disks of uniform size (8.0 mm in diameter) were made from all BAC-composites and 5.0% (wt/wt), according to previous reports.15,16 Afterward, BAC polymers were mixed with the monomer to the required concentration. Six types of BAC polymers were prepared by adjusting the mixing ratio of 50% BAC-added polymer and 50% original Superbond C&B polymer. Final BAC concentrations in the BAC-added polymer was further diluted with Superbond C&B polymer (wt/wt). The BAC-added 4-META/MM-TBB resin ranged from approximately 10 to 20 MPa.

In the clinical situation, sustained release of the antibacterial agent is essential. The behavior of BAC release will influence the antibacterial activity of the adhesive resin. Moreover, antibacterial compounds sometimes exhibit cytotoxicity and pose a safety problem. However, little research has investigated the cytotoxicity of antibacterial adhesive resins. Their cytotoxic effects should be documented along with their antibacterial activity.

The aims of the present study were (1) to assess the antibacterial activity of BAC-incorporated 4-META/MM-TBB resin after immersion in water, and (2) to examine the cytotoxicity of BAC-incorporated 4-META/MM-TBB resin using human gingival fibroblasts.

MATERIALS AND METHODS

4-META/MM-TBB resin cement (Superbond C&B, Sunmedical Co Ltd, Shiga, Japan) was used in this study. It was modified by the addition of the antibacterial agent BAC (ICN Biomedicals Inc, Aurora, Ohio) in powder form.

BAC was initially diluted to 50% by mixing with Superbond C&B polymer (wt/wt). The BAC-added polymer was further diluted with Superbond C&B polymer to the required concentration. Six types of BAC polymers were prepared by adjusting the mixing ratio of 50% BAC-added polymer and 50% original Superbond C&B polymer. Final BAC concentrations in the BAC-composites were 0.25%, 0.75%, 1.25%, 1.75%, 2.5%, and 5.0% (wt/wt), according to previous reports.15,16 Afterward, BAC polymers were mixed with the monomer and catalyst according to the manufacturer’s instructions. Disks of uniform size (8.0 mm in diameter × 2.0 mm in thickness) were made from all BAC-composites with the use of custom-made molds. The mixture of polymer, monomer, and catalyst was put into a mold, and the mold was covered with a glass slide under 300 g weight. After resin was cured, excess resin was carefully removed by polishing with 400 grit abrasive paper under running water. The disk shape specimen was removed from the mold. Four disks were tested for each condition in the antibacterial and cytotoxicity assay; three independent runs were performed for each condition.

Antibacterial Activity of BAC Samples

A disk diffusion assay was employed for the evaluation of antibacterial activity. The BAC-composite disk samples were immersed in distilled water at 37°C for periods of 0, 30, 90, and 180 days before antibacterial activity was assessed.

The cariogenic streptococci S mutans 10449 and PS14 and S sobrinus 6715 and B13 were used as test bacteria. They were grown routinely overnight in a brain-heart infusion medium (Difco, Detroit, Mich) at 37°C. The release of BAC into the surrounding agar medium as shown by growth inhibition of S mutans and/or S sobrinus was evaluated. The growth inhibitory effect was determined by measuring the zone of growth inhibition around the BAC-composite disk. Measurements were taken with the same assessor with the use of electric digital calipers (NSK MAX-CAL, Japan Micrometer Mfg Co, Tokyo, Japan).

An overnight broth culture of S mutans or S sobrinus was diluted, and the cell suspension was adjusted to an optical density of 0.5 (550 nm). Then a brain-heart infusion agar plate was inoculated with 80 μL of a 60-folder dilution of the cell suspension. The inoculum was spread evenly on the plate surface with a glass rod to obtain uniform bacterial growth. BAC-composite disks were placed on the surface of the agar, and the plates were incubated at 37°C. After 48-hour incubation, the inhibition zone around each disk sample was measured and the measurements recorded.

Cell Isolation and Cultures

Human gingival fibroblast culture was established from the cellular outgrowth of healthy gingival tissue explants removed from patients undergoing tooth extraction for orthodontic reasons, according to the method of Somerman et al.17 Informed consent was obtained from all patients before the study was begun. The study was conducted according to a protocol reviewed by the ethics committee at Nihon University School of Dentistry at Matsudo (EC 03-019).

After extraction of teeth, a gingival tissue, which was attached to the interdental papilla, was taken and washed twice in phosphate-buffered saline (PBS). The
tissue was dissected into approximately 1-mm cubes and was transferred to 35-mm tissue culture dishes containing α-minimal essential medium (α-MEM, Gibco, Grand Island, NY) supplemented with 100 μg/mL penicillin G (Sigma Chemical Co, St Louis, Mo), 50 μg/mL gentamicin sulphate (Sigma), 0.3 μg/mL amphotericin B (Flow Laboratories, Mclean, Va), and 10% fetal bovine serum (FBS, Cell Culture Laboratories, Cleveland, Ohio). The cultures were incubated at 37°C in a humidified incubator (Forma CO2 incubator MIP-C, Cleveland, Ohio). The cultures were incubated at 37°C in a humidified incubator (Forma CO2 incubator MIP-C, Sanyo Electric Medica System Co, Tokyo, Japan) in the presence of 95% air and 5% CO2. When cells that grew out from the explants reached confluence, they were detached with 0.05% trypsin (580 BAEE Umg⁻¹, Gibco) in PBS for 10 minutes and were subcultured in flasks. Human gingival fibroblasts were seeded at 2 × 10⁴ per well in 24-well culture plates (Falcon 3046, Becton Dickinson, Franklin Lakes, NJ) and were cultured at 37°C for 24 hours.

Cytotoxicity Assay

4-META/MMA-TBB resin disks containing no BAC were used as controls. Human gingival cells were exposed to the resin disks in separate wells of 24-well plates (Falcon 3046). The disks were put into sterile tissue culture inserts (Falcon 3097) with 8-m pore size PET membrane at the bottom and then were placed above the human gingival cells. This experimental design allowed passage of leaching components of resins to reach human gingival cells. This experimental design was a modification of the method of Tang et al.¹⁸ In our study, all samples were incubated with human gingival cells for 1, 3, and 6 days at 37°C.

Cytotoxicity was assessed with a tetrazolium bromide reduction assay kit (Sigma) on the principle that 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) is reduced to purple formazan in the mitochondria of living cells. At 1, 3, or 6 days, the resins were removed, and human gingival cells were washed with PBS after removal of the medium. Then, 100 μL of a 0.5% MTT medium (α-MEM) solution without phenol red was added to each well. After incubation for 3 hours at 37°C, the solution was removed and the resulting intracellular formazan crystals, which were not soluble in MTT medium solution, were dissolved with 100 μL/well of a solubilization solution (10% Triton X-100 plus 0.1 N hydrogen chloride [HCl] in anhydrous isopropanol). The purplish lysate was read with the use of a Microplate Reader (MTP-32, Corona Electric, Hitachinaka City, Japan) with a 560/660 nm filter.

Statistical Analysis

The data are presented as mean and standard deviation (SD). Analysis of variance (ANOVA) was performed to determine whether a significant difference existed among various groups, and Fisher’s test was used for multiple comparisons. Significance for all statistical tests was predetermined at P < .05.

RESULTS

Disk Diffusion Assay Method

The results of the disk diffusion assay method for each bacterial strain are shown in Tables 1 through 4. Multivariate analysis of variance (MANOVA) showed significant differences among various periods of immersion in distilled water (F = 256.983; P < .0001) and among different BAC concentrations (F = 140.915; P < .0001). However, no significant differences existed among the bacterial strains (F = 2.312; P = .0763). MANOVA interactions were not found for the immersion period and for the BAC concentration and bacterial strain (F = 1.082; P = .3428). However, two-way interactions were noted between immersion period and bacterial strain (F = 3.133; P < .005), and between immersion period and BAC concentration (F = 42.719; P < .0001). On the contrary, no significant difference was observed between BAC concentration and bacterial strain (F = 1.326; P = .185).
The antibacterial activity results for *S mutans* 10449 are listed in Table 2. Two-way ANOVA showed significant differences among different immersion periods (F = 126.585; P < .0001) and among different BAC concentrations (F = 64.639; P < .0001). A significant two-way interaction was observed between immersion period and BAC concentration (F = 19.480; P < .0001).

At 0 days, significant differences in antibacterial activity were noted between 5.0% BAC and other concentration levels. At 30 days, no significant differences were observed among 1.25%, 1.75%, and 2.5% BAC groups. At 90 days, no significant differences were seen among 1.75%, 2.5%, and 5.0% BAC groups. At 180 days, no significant differences were detected between 0.25% and 0.75% BAC groups and between 1.25% and 1.75% BAC groups. Higher concentrations of BAC in BAC-composite tended to produce greater decreases in antibacterial activity according to the prolongation of assay time. For 2.5% and 5.0% BAC groups, significant differences in antibacterial activity were evident between 0 and other time groups.

Table 3 shows the antibacterial activity results for *S sobrinus* 6715. Two-way ANOVA detected significant differences among immersion periods (F = 63.284; P < .0001) and among BAC concentrations (F = 28.788; P < .0001). A significant two-way interaction was ob-

### Table 2. Disk Diffusion Assay (*Streptococcus mutans* 10449)*a*

<table>
<thead>
<tr>
<th>BAC wt%</th>
<th>0 day</th>
<th>30 days</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0.25</td>
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<td>0.30</td>
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<td>16.54</td>
<td>2.32</td>
<td>7.10</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*a* Data represent measured distances of bacterial growth inhibition around the BAC-composite disks on agar plates (mm); mean values with different superscripts are significantly different (P < .05); uppercase letters indicate the comparison of bacterial growth inhibition within the same immersion period, and lowercase letters indicate the comparison of bacterial growth inhibition within the same BAC concentration.

*a* BAC indicates benzalkonium chloride; SD, standard deviation.

### Table 3. Disk Diffusion Assay (*Streptococcus sobrinus* 6715)*a*

<table>
<thead>
<tr>
<th>BAC wt%</th>
<th>0 day</th>
<th>30 days</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
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<td>14.58</td>
<td>3.13</td>
<td>7.93</td>
<td>0.29</td>
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</table>

*a* Data represent measured distances of bacterial growth inhibition around the BAC-composite disks on agar plates (mm); mean values with different superscripts are significantly different (P < .05); uppercase letters indicate the comparison of bacterial growth inhibition within the same immersion period, and lowercase letters indicate the comparison of bacterial growth inhibition within the same BAC concentration.

*a* BAC indicates benzalkonium chloride; SD, standard deviation.
served between immersion period and BAC concentration (F = 8.821; P < .0001).

At 0 day, no significant differences in antibacterial activity were observed among 0.25%, 0.75%, and 1.25% BCA groups. At 30 days, no significant differences in antibacterial activity were noted between 0.25% and 0.75% BCA groups. At 90 days, significant differences were present among 0.25% and 0.75% BAC groups. At 180 days, no significant differences were observed between 1.75% and 5.0% BAC groups; 5.0% BAC groups showed significantly higher antibacterial activity at 0, 30, and 90 days. Higher concentrations of BAC in the BAC-composite tended to produce increased antibacterial activity, and the highest level of antibacterial activity against each bacterial strain (P < .05). The antibacterial activity of BAC samples decreased significantly after water immersion for 180 days regardless of the BAC concentration (P < .05). Resin samples with 5.0% BAC still showed greater antibacterial activity than was seen with resin with 0.25% BAC for all treatment periods. The antibacterial activity of 5.0% BAC resin samples immersed in water for 180 days was equivalent to that of preimmersed resin samples containing 0.25% or 1.75% BAC.

Cytotoxicity Assay

The results of cytotoxicity (%) to human gingival fibroblasts are shown in Table 5. Two-way ANOVA detected significant differences in percentage of MTT activity among different BAC concentrations in composites (F = 169.703; P < .0001) and among different incubation periods (F = 19.464; P < .0001). Two-way interaction was observed between BAC concentration and incubation period (F = 3.627; P < .001).

After 1 day of incubation, a significant difference was found between the control resin (0% BAC) and the resins containing 1.75%, 2.5%, or 5% BAC (P < .05). Most did not survive when exposed to resins containing 2.5% and 5% BAC. After 3 and 6 days of incubation, a significant difference was found between the control resin and the resin containing 1.25%, 1.75%, 2.5%, or 5% BAC (P < .05). Most cells did not survive when exposed to resins with 1.75%, 2.5%, and

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Table 4. Disk Diffusion Assay (Streptococcus sobrinus B13)*

<table>
<thead>
<tr>
<th>BAC conc.</th>
<th>0 day</th>
<th>30 days</th>
<th>90 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
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<td>5.34^a</td>
<td>1.15</td>
<td>5.42^ab</td>
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</tr>
<tr>
<td>0.75</td>
<td>6.41^ab</td>
<td>1.14</td>
<td>6.18^ab</td>
<td>0.24</td>
</tr>
<tr>
<td>1.25</td>
<td>7.18^abc</td>
<td>1.22</td>
<td>7.58^bcd</td>
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<tr>
<td>1.75</td>
<td>9.12^cd</td>
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<td>6.95^e</td>
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</tr>
<tr>
<td>2.50</td>
<td>10.89^f</td>
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<td>7.30^f</td>
<td>0.11</td>
</tr>
<tr>
<td>5.00</td>
<td>14.01^g</td>
<td>2.30</td>
<td>8.21^h</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* Data represent measured semidiameters of bacterial growth inhibition around the BAC-composite disks on agar plates (mm); mean values with different superscripts are significantly different (P < .05); uppercase letters indicate the comparison of bacterial growth inhibition within the same BAC concentration.

b BAC indicates benzalkonium chloride; SD, standard deviation.
5% BAC. No significant difference was observed between resins containing 0.25% and 5% BAC at 1, 3, and 6 days of incubation. No severe cell death was observed for resins containing 0.25% or 0.75% BAC.

**DISCUSSION**

*S. mutans* and *S. sobrinus* are the principal bacterial species associated with caries in humans. Recent studies have indicated that *S. sobrinus* is more frequently isolated from highly caries-susceptible patients than *S. mutans*, and that the cariogenic potential of *S. sobrinus* is greater than that of *S. mutans*. Two different strains of *S. mutans* and of *S. sobrinus* were used in the present study.

BAC hand sanitizer is the most popular rinse-free hand sanitizer formula for normal hand washing. Intranasal products containing the preservative BAC appear to be safe and well tolerated for both long-term and short-term clinical use. Othman et al and Sehgal et al reported that composite resin containing BAC showed antibacterial properties. They reported a continuous and constant release of BAC from the composite over time. Thus, we used BAC as the antibacterial agent to be incorporated into the orthodontic bonding resin.

Our previous study revealed that BAC-added 4-META/MMA-TBB resin produced high levels of antibacterial activity. In the clinical situation, long-term release of the antibacterial agent is essential. Resins with higher concentrations of BAC tended to release BAC rapidly and tended to show a rapid decrease in antibacterial activity. The present study showed that resin containing 1.25% or 1.75% BAC provided relatively constant antibacterial activity during immersion in water for 180 days because of the continuous release of BAC.

Generally, a higher level of antibacterial activity is accompanied by a higher degree of cytotoxicity. In the present study, resins with higher BAC concentration showed stronger cytotoxicity. The cytotoxicity of resin with 0.25% or 0.75% BAC is similar to that of controls during all test periods.

The present results were obtained after immersion of BAC samples in water. It is presumed that the oral environment will influence the release of BAC, as well as the antibacterial activity and cytotoxicity of the BAC-composite. Further research into saliva immersion is needed.

Thus, the present study revealed that resins containing 0.25% or 0.75% BAC exhibited antibacterial activity and little cytotoxicity. Our previous study reported that the shear bond strength of the orthodontic bracket bonded to enamel by resins containing 0.25% or 0.75% BAC was comparable with the shear bond strength of the original 4-META/MMA-TBB resin. A bonding procedure performed in one patient requires approximately 180 mg of bonding composite, and 0.50 mg of BAC is needed to achieve 0.25% BAC concentration. BAC has been used as an antiseptic in contact lenses, but the quantity used in the present study was less than that used in contact lenses.

The present study confirms that the addition of BAC to 4-META/MMA-TBB resin confers antibacterial properties, and that the resulting composite exhibits antibacterial activity produced by release of the antibacterial agent against *S. mutans* and *S. sobrinus*. Further in vivo study of the safety of BAC-incorporated 4-META/MMA-TBB resin is required before it can be tested clinically.

**CONCLUSIONS**

- The addition of BAC to 4-META/MMA-TBB resin confers an antibacterial effect even after immersion in water.
- 4-META/MMA-TBB resin containing 0.25% to 0.75% BAC has no significant cytotoxic effect.

**ACKNOWLEDGMENT**

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