Abstract: To develop a root canal filling material with high antimicrobial activity, we prepared gutta-percha supplemented with the cationic surfactant cetylpyridinium chloride (CPC). Thermoplastic gutta-percha was supplemented with 0.05%, 0.2%, or 0.8% CPC. The gutta-percha containing CPC was tightly packed at the bottom of a 24-well plate. Its antimicrobial activity against eight representative endodontic pathogens—including gram-positive and gram-negative bacteria and fungi—was evaluated by adding 0.5 mL of liquid samples containing pathogens to the wells. After 24 h of cultivation under appropriate conditions, microbial growth was analyzed by counting colony-forming units (CFU). Gutta-percha alone (without CPC) partially inhibited microbial growth, probably through the antimicrobial effect of some of its components, such as zinc oxide. Addition of CPC dose-dependently increased the antimicrobial efficacy of gutta-percha. Addition of 0.05%, 0.2%, and 0.8% CPC reduced the viable microbial number to below the lower limit of detection (20 CFU/mL) for all tested pathogens except Pseudomonas aeruginosa, which was detected in 0.8% CPC-containing gutta-percha, although the viable number significantly decreased. Gutta-percha with CPC might be useful for preventing microbial infections during root canal therapy. (J Oral Sci 58, 277-282, 2016)

Introduction
Prevention of microbial contamination during root canal therapy requires aseptic treatment of the root canal (1,2). In general, the root canal is shaped, prepared, and then chemically irrigated and disinfected. However, sterilization of the root canal is not always straightforward, because of its fine and complicated structure (3-6). Even with proper treatment, infection caused by coronal or apical microleakage remains a concern (7-10).

To reduce the risk of root canal reinfection, methods of incorporating antimicrobial activity in root canal filling materials have been investigated. Zinc oxide (ZnO) is present in many root canal filling materials and has antimicrobial activity, although it is used mainly as a biocompatible, astringent chemical (11,12). ZnO generates reactive oxygen species that damage the microbial cell membrane, but its antimicrobial activity is limited (13-15). Several studies have reported the development of root canal filling materials supplemented with more effective antimicrobial chemicals. Martin and Martin developed gutta-percha containing iodoform (16); however, the antimicrobial activity of this material is unclear. Some studies found that gutta-percha containing iodoform exhibited only a slight (17,18) or no increase (19,20) in antimicrobial efficacy as compared with gutta-percha alone. A gutta-percha supplemented with tetracycline has also been developed, but it had no antimicrobial effect on fungi such as Candida albicans (21), a common cause of endodontic infections. Furthermore, some reports indicate that addition of chlorhexidine...
increases the antimicrobial activity of gutta-percha (22-24). However, for this material, too, only a limited (25,26) or small increase (27) in antimicrobial activity was reported. Although the possibility of a root canal filling material supplemented with antimicrobial chemicals has great potential, a satisfactory material has not yet been developed.

In this study, we used cetylpyridinium chloride (CPC) to enhance the antibacterial activity of gutta-percha. CPC, a quaternary ammonium compound and a cationic surfactant, is used in a wide variety of antiseptic products and drugs, including mouth rinses, dentifrices, and lozenges (28). Although the antimicrobial mechanisms of CPC are not well understood, it appears to damage microbial membranes, thereby eventually killing microbes (29).

In this study, we prepared gutta-percha supplemented with CPC and examined its antimicrobial activity against representative endodontic pathogens, including gram-positive and gram-negative bacteria and fungi.

### Materials and Methods

#### Microbes and culture conditions

The microbes and specific culture conditions used in this study are summarized in Table 1. The microbes were used in experiments after they had been cultivated until the late logarithmic phase and were prepared at an optical density at 600 nm (OD600) of 0.01 or 0.1 by dilution in appropriate media (Table 1).

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Strain</th>
<th>Gram-stainability</th>
<th>Culture condition</th>
<th>Medium</th>
<th>Initial concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>Positive</td>
<td>Aerobic</td>
<td>BHI</td>
<td>0.01 1.0 × 10^7</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>209P</td>
<td>Positive</td>
<td>Aerobic</td>
<td>BHI</td>
<td>0.01 5.0 × 10^6</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>DL-1</td>
<td>Positive</td>
<td>5% CO₂</td>
<td>BHI</td>
<td>0.01 1.0 × 10^6</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>NCTC 10449</td>
<td>Positive</td>
<td>5% CO₂</td>
<td>BHI</td>
<td>0.01 1.0 × 10^6</td>
</tr>
<tr>
<td><em>Actinomyces naeslundii</em></td>
<td>ATCC 12104</td>
<td>Positive</td>
<td>Anaerobic</td>
<td>mGAM</td>
<td>0.1 3.0 × 10^7</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PAO1</td>
<td>Negative</td>
<td>Aerobic</td>
<td>BHI</td>
<td>0.01 8.0 × 10^6</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>ATCC 33277</td>
<td>Negative</td>
<td>Anaerobic</td>
<td>mGAM</td>
<td>0.1 1.0 × 10^6</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>FC18</td>
<td>–</td>
<td>Aerobic</td>
<td>BHI</td>
<td>0.01 2.0 × 10^6</td>
</tr>
</tbody>
</table>

*C. albicans* is a fungus and is not categorized on the basis of gram staining.

The microbes were cultivated at 37°C in air (aerobic); air supplemented with 5% CO₂ (5% CO₂); or 80% N₂, 10% CO₂, and 10% H₂ (anaerobic) conditions. BHI, Brain Heart Infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA); mGAM, modified GAM broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). For measurement of viable microbial numbers, the media were solidified by addition of 1.5% agar.

Microbial cultures (0.5 mL), prepared as described above, were applied to wells containing TG with or without CPC, after which the plates were incubated for 24 h. A well without TG was used as the negative control. Then, a colony-forming unit (CFU) assay was used to measure viable microbial numbers. Briefly, the cultures were diluted in saline solution with 1 mM dithiothreitol, for anaerobes, or without dithiothreitol, for the other microbes (including aerobes and facultative anaerobes), and 50 μL of each dilution was spread on medium solidified with agar. After cultivation under appropriate conditions, the colonies were counted.

#### Antimicrobial activity test of TG with CPC

Microbial cultures (0.5 mL), prepared as described above, were applied to wells containing TG with or without CPC, after which the plates were incubated for 24 h. A well without TG was used as the negative control. Then, a colony-forming unit (CFU) assay was used to measure viable microbial numbers. Briefly, the cultures were diluted in saline solution with 1 mM dithiothreitol, for anaerobes, or without dithiothreitol, for the other microbes (including aerobes and facultative anaerobes), and 50 μL of each dilution was spread on medium solidified with agar. After cultivation under appropriate conditions, the colonies were counted.

#### Continuity of antimicrobial efficacy

Using repeated microbial inoculation, we investigated the continuity of the antimicrobial efficacy of TG supplemented with CPC. Briefly, a *Streptococcus mutans* culture was incubated in wells containing TG with or without CPC for 24 h, as described above. Then, the entire culture was removed from the well, and another fresh bacterial culture was applied to the well and incubated for the same duration. The same procedure was repeated 6 consecutive times, and the respective viable bacterial numbers were measured.
Determination of minimum inhibitory concentration
Serial dilutions of CPC were added to medium solidified with agar. An aliquot of bacterial culture (2 μL), prepared as described above, was placed on the solid media. Microbes were cultivated under appropriate conditions, and the susceptibility breakpoints were used to determine the minimum inhibitory concentration (MIC).

Statistical analysis
Data are presented as mean ± SD. Differences among groups were examined by 1-way analysis of variance followed by the Student-Newman-Keuls (SNK) test for multiple comparisons. A P value of <0.05 was considered to indicate statistical significance.

Results

Determination of experimental conditions
We first examined the experimental conditions to evaluate the antimicrobial activity of TG supplemented with CPC. We applied 0.5 mL of microbial culture per well because the hydrophobic TG repelled aqueous solutions and a volume of 0.5 mL was necessary to cover the entire TG surface. Next, we incubated microbes for 24 h because the viability of microbes incubated for longer periods naturally decreased, which could affect the interpretation of our results. Additionally, we used initial microbial concentrations, as shown in Table 1, because some microbes did not exhibit a reproducible growth pattern over 24 h when lower concentrations were inoculated. We then examined the CPC concentration to be added...
to TG, using \textit{S. mutans}. Addition of 0.05% CPC partly, but reproducibly, reduced bacterial viability. When 1% or more CPC was added to TG, the TG was occasionally discolored. We thus decided to apply CPC at a concentration of less than 1%. Ultimately, we supplemented TG with 0.05%, 0.2%, or 0.8% CPC.

**Antimicrobial examination of TG with CPC**

After the microbes were incubated in a well containing TG with or without CPC for 24 h, viable microbes were quantified by CFU assay (Fig. 1A). The microbes were also incubated in untreated wells as a control.

The number of viable \textit{Enterococcus faecalis} significantly decreased when incubated with TG alone (without CPC), and decreased even further (to a level below the detection limit) when incubated with TG containing 0.05% or more CPC. Although \textit{Staphylococcus aureus} grew well even on TG containing 0.05% CPC, the addition of 0.2% or more CPC decreased the viable number to levels below the detection limit. Two streptococci, \textit{Streptococcus gordonii} and \textit{S. mutans}, exhibited a similar tendency: their viable numbers significantly decreased when incubated with TG alone, and the addition of 0.2% CPC or more led to a decrease below the detection limit. \textit{Actinomyces naeslundii}, like \textit{S. aureus}, exhibited partial resistance to TG and CPC, but the viable number drastically decreased when incubated with 0.2% CPC-containing TG and dropped below the detection limit for TG containing 0.8% CPC. \textit{Pseudomonas aeruginosa} exhibited the strongest resistance: it was able to grow equally well in the 0.2% CPC-containing TG and the control well without TG. Addition of 0.8% CPC significantly reduced viable numbers, although colonies were still detected. For \textit{Porphyromonas gingivalis}, the viable number decreased when incubated with TG alone, and a significant decrease was observed after addition of 0.05% CPC. Viable numbers dropped below the detection limit for TG containing 0.2% or more CPC. The viable number of \textit{C. albicans} decreased to values below the detection limit for TG containing 0.2% or more CPC, although it grew well when incubated with TG containing only 0.05% CPC.

**Continuity of antimicrobial efficacy**

Using repeated inoculation of \textit{S. mutans}, we investigated the continuity of the antimicrobial efficacy of TG with CPC (Fig. 1B). For all six inoculations, the respective viable bacterial numbers were similar to those shown in Fig. 1A. Thus, bacteria were persistently detected at a concentration of approximately $10^8$ CFU/mL in the control, while TG (without CPC) persistently suppressed viable numbers, to less than $10^4$ CFU/mL. Addition of any concentration of CPC decreased the viable number to below the detection limit for all replicates, with the exception of the 4th inoculation of 0.05% CPC-containing TG, after which bacteria were detected at a concentration of 90 CFU/mL.

**MIC of CPC**

MIC values for CPC are shown in Table 2. The growth of \textit{E. faecalis} and the 2 streptococci was efficiently inhibited by CPC-MIC values were less than 1 μg/mL. \textit{S. aureus}, \textit{A. naeslundii}, \textit{P. gingivalis}, and \textit{C. albicans} were also sensitive to CPC, and their MIC values were less than 10 μg/mL. However, the MIC for \textit{P. aeruginosa} was strikingly high (5,000 μg/mL).

**Discussion**

Our results demonstrate that TG alone (without addition of CPC) arrested the growth of some microbes, especially \textit{E. faecalis}, \textit{S. gordonii}, and \textit{S. mutans} (Fig. 1A). This inhibition is likely due to the presence of antimicrobial agents, such as ZnO, which is present at a concentration of 85% in TG (informal communication from GC Co.). Many of the microbes tested in this study were sensitive to ZnO, as indicated by MICs between 0.01% and 1% (data not shown). The MICs of \textit{E. faecalis}, \textit{P. aeruginosa}, and \textit{C. albicans} are likely to be much higher than 1%. We could not determine exact MIC values because the solubility of ZnO in aqueous media is limited to 1%. While \textit{E. faecalis} was relatively resistant to ZnO, it showed a high sensitivity to TG because it is sensitive to the other antimicrobial components of TG, such as eugenol (30,31).

Addition of CPC to TG significantly dose-dependently enhanced the antimicrobial activity of TG against all microbes tested in this study (Fig. 1A). Additionally, antimicrobial efficacy persisted even when the bacterial

<table>
<thead>
<tr>
<th>Microbe</th>
<th>MIC (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{E. faecalis}</td>
<td>0.000098 (0.98)</td>
</tr>
<tr>
<td>\textit{S. aureus}</td>
<td>0.000781 (7.81)</td>
</tr>
<tr>
<td>\textit{S. gordonii}</td>
<td>0.000098 (0.98)</td>
</tr>
<tr>
<td>\textit{S. mutans}</td>
<td>0.000098 (0.98)</td>
</tr>
<tr>
<td>\textit{A. naeslundii}</td>
<td>0.000781 (7.81)</td>
</tr>
<tr>
<td>\textit{P. aeruginosa}</td>
<td>0.5 (5,000)</td>
</tr>
<tr>
<td>\textit{P. gingivalis}</td>
<td>0.000391 (3.91)</td>
</tr>
<tr>
<td>\textit{C. albicans}</td>
<td>0.00195 (1.95)</td>
</tr>
</tbody>
</table>

Three independent experiments showed the same results in all microbes.
culture was repeatedly replaced (6 times) with fresh culture (Fig. 1B). This suggests that CPC is continuously eluted from TG under the present experimental conditions. However, using high-performance liquid chromatography (HPLC), we could not detect CPC in media (or water) incubated for 24 h with TG supplemented with 0.8% CPC (data not shown). The lower limit of detection of CPC was 1 μg/mL in HPLC analysis, which indicates that the concentration of CPC in the medium was lower than 1 μg/mL. MICs of CPC for *S. aureus, A. naeslundii, P. gingivalis*, and *C. albicans* were higher than 1 μg/mL (Table 2), and these microbes exhibited substantial survival when incubated with TG alone. Therefore, it is possible that microbes still grow in TG containing 0.8% CPC, but their viability was significantly reduced in TG containing CPC. This finding indicates that they were efficiently killed by the synergistic effect of CPC and the antimicrobial ingredients of TG. *P. aeruginosa* is intrinsically resistant to many types of antimicrobial agents, including antibiotics and disinfectants (32,33), and was highly resistant to CPC (MIC, 5,000 μg/mL) and ZnO (MIC, >1 μg/mL). Nevertheless, the synergistic effect of CPC and TG was also observed for *P. aeruginosa*, as its viability significantly decreased when incubated with TG containing 0.8% CPC. This suggests that *P. aeruginosa* infection can be controlled by TG supplemented with CPC in root canal therapy, although further improvements will be necessary in order to ensure effective control of bacterial infection.

Because we used a commercially available TG product containing only 1.6 g TG, we mixed a very small amount of CPC with TG. Therefore, we prepared a CPC stock solution dissolved in ethanol. We also attempted to use CPC powder and CPC dissolved in polyethylene glycol, but it was difficult to create a homogeneous mixture of CPC powder and TG. The addition of polyethylene glycol softened TG (data not shown). However, we observed no alteration in TG when CPC dissolved in ethanol was added, until a concentration of 0.8% (data not shown). Clearly, the preparation methods need to be improved before this material can be used clinically.

We examined the antimicrobial efficacy of CPC by adding 0.5 mL of microbial culture to a well containing 1.6 g of TG. This experimental setup differs greatly from conditions during root canal therapy. In the future, we hope to examine CPC-containing TG in experimental conditions more closely resembling a *in vivo* clinical setting, after improving the methods for producing CPC-supplemented TG.

In conclusion, addition of CPC significantly enhanced the antimicrobial efficacy of gutta-percha. CPC-containing gutta-percha might thus be useful for reducing the risk of root canal reinfection in root canal therapy.

**Acknowledgments**

This article is dedicated to the late Yoshifumi Takahashi, who was responsible for the original study design. We thank Fuminobu Yoshimura, in the Department of Microbiology, for providing experimental facilities. We also thank Naoya Higuchi, in the Department of Endodontontology, for many insightful discussions.

**Conflict of interest**

The authors have no conflicts of interest to declare.

**References**


