



## Post-antibiotic Effects and Post-sub-minimal Inhibitory Concentration Effects of Cetylpyridinium Chloride on *Streptococcus gordonii* and *Streptococcus mutans*

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### Authors' contributions

This work was carried out in collaboration between both authors. Author So Yeon Lee performed the experiments and wrote the first draft of the manuscript. Author Si Young Lee designed the study, managed the analyses of the study. Both authors read and approved the final manuscript.

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### ABSTRACT

**Background and Objectives:** When an antimicrobial agent is removed after the treatment of bacteria for a short period, it takes a long time for the bacteria to return to normal growth, despite the reduction in the antimicrobial agent concentration. This phenomenon is referred to the post-antibiotic effect (PAE). The PAE of cetylpyridinium chloride, which is a common ingredient in oral mouthwash solutions, has not yet been elucidated. We evaluated the post-antibiotic effect (PAE), post-antibiotic sub-MIC effect (PA SME), and sub-MIC effect (SME) for cetylpyridinium chloride in *Streptococcus gordonii*, which is known to be an early colonizer of the tooth surface, and *Streptococcus mutans*, a causative agent of dental caries.

**Materials and Methods:** After cetylpyridinium chloride was applied to bacteria, PAE, PASME and SME were measured to investigate the time to recovery of bacterial growth.

**Results:** The mean PAE times for *S. gordonii* and *S. mutans* were 1 h and 1.07 h, respectively.

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When the PA SME was compared with the PAE, the PA SME was longer than the PAE in both *S. gordonii* and *S. mutans*.

**Conclusion:** In this study, it was shown that cetylpyridinium chloride can cause a PAE, PA SME and SME; therefore, this pharmacodynamic effect should be expected in the clinical application of cetylpyridinium chloride.

**Keywords:** Biofilm; cetylpyridinium chloride; hydrophobicity; oral bacteria; sub-MIC.

## 1. INTRODUCTION

When an antimicrobial agent is removed after the treatment of bacteria for a short period, it takes a long time for the bacteria to recover to normal growth, even though the concentration of the antimicrobial agent is decreased. This phenomenon is referred to as the post-antibiotic effect (PAE) [1]. The PAE is defined as the continued inhibition of bacterial growth after exposure to antimicrobial agents [2]. Depending on the antibiotic concentration and the susceptibility of the bacteria, PAE can last for a long time [2]. When an antimicrobial agent is applied in the oral cavity, the concentration of the antimicrobial agent gradually decreases and remains at a sub-minimum inhibitory concentration (sub-MIC) [3]. The sub-MIC treatment of an antimicrobial agent to a bacterium exposed at supra-inhibitory antibiotic concentration increases the time required for the proliferation compared with that of a bacterium that is not exposed to the antimicrobial agent at the supra-inhibitory antibiotic concentration. This phenomenon is called the post-antibiotic sub-MIC effect (PA SME) [4,5]. In addition, the sub-MIC effect (SME), which is the effect of the sub-MIC antimicrobial treatment on bacteria not exposed to an antimicrobial agent, has been described [4,5].

Cetylpyridinium chloride has a positive charge and is known to exert antibacterial activity in negatively charged bacteria [6,7]. It causes degradation of the lipid bilayer of bacterial cell membranes, which decreases their ability to control cell permeability and induces the leakage of cell contents [8,9]. Therefore, the use of products containing cetylpyridinium chloride can help control biofilm formation and prevent oral diseases such as gingivitis, periodontitis, and dental caries [6].

It is known that various antimicrobial agents can induce a PAE or PA SME, depending on the bacterial species [10]. However, there are not many studies of the PAE effect on oral streptococci. In one study, amoxicillin exhibited

PAE and PA SME effects on *S. gordonii* and *S. sanguis* [11]. However, the PAE of cetylpyridinium chloride, which is widely used in the field of dentistry, has not yet been elucidated. We evaluated the PAE, PA SME, and SME for cetylpyridinium chloride in *S. gordonii*, which is known to be an early colonizer of the tooth surface, and *Streptococcus mutans*, a causative agent of dental caries.

## 2. MATERIALS AND METHODS

### 2.1 Bacterial Strains and Culture Conditions

*S. gordonii* KN1 and *S. mutans* KN88 were used in this study. Bacteria were cultured for 18 h in an incubator containing 5% CO<sub>2</sub> at 37°C by using brain heart infusion broth (BHI, Becton, Dickinson and Company, Sparks, MD, USA).

### 2.2 Determination of Minimal Inhibitory Concentration (MIC)

Cetylpyridinium chloride (Sigma Chemical Co., St. Louis, Mo., USA) was used as the antimicrobial agent. The MIC of cetylpyridinium chloride was measured by the two-fold serial macro dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). Cetylpyridinium chloride (1000 µg / mL) was diluted serially in BHI medium and the bacteria were added at  $5 \times 10^5$  cells / mL. The cells were then cultured in an incubator containing 5% CO<sub>2</sub> at 37°C for 18 h. After incubation, the turbidity was visually observed to determine the inhibition of bacterial growth.

### 2.3 Post-antibiotic Effect (PAE) Assay

The PAE was measured by using previously reported methods [4,5]. Bacteria were grown until the exponential growth phase was reached, diluted in BHI medium to  $10^9$  CFU / mL, and exposed to  $10 \times$  MIC cetylpyridinium chloride for 1 min at 37°C. After treatment with cetylpyridinium chloride, the bacteria were

washed three times with phosphate-buffered saline (PBS, pH 7.2) to remove the antimicrobial agent. The control bacteria were not treated with cetylpyridinium chloride but were also washed three times with PBS. To determine the PAE, the antimicrobial-treated bacteria and unexposed control bacteria were cultured at 37°C in a 5% CO<sub>2</sub> incubator and bacterial growth was monitored hourly through measurement of the OD<sub>660</sub>. The PAE was calculated from the equation  $PAE = T - C$ , where T is the time required for the chlorhexidine-treated cultures to reach 50% of the maximum absorbance and C is the corresponding time for the unexposed control to reach an equivalent value. Each experiment was performed in triplicate.

## 2.4 PA SME and SME Assays

To determine the PA SME, the cells were exposed to 10× MIC cetylpyridinium chloride at 37°C for 1 min and then washed three times in PBS. Then, cetylpyridinium chloride at 0.1, 0.2, and 0.3× MIC was added to the culture medium of the post-antibiotic phase and cultured at 37°C in a 5% CO<sub>2</sub> incubator. Bacterial growth was monitored hourly through the measurement of the OD<sub>660</sub> of the bacterial culture. The PA SME was determined by the following equation;  $PA\ SME = TPA - C$ , where TPA is the time required for the cultures previously exposed to the 10× MIC of chlorhexidine followed by sub-MIC chlorhexidine to reach 50% of the maximum absorbance, and C is the corresponding time for the control.

The SME was measured by using control cultures that were not exposed to 10× MIC chlorhexidine but were exposed to 0.1, 0.2, or 0.3× MIC. The SME was determined from the equation  $SME = TS - C$ , where TS is the time required for the cultures exposed only to the sub-MICs to reach 50% of the maximum absorbance, and C is as previously defined above.

## 3. RESULTS

### 3.1 Minimum Inhibitory Concentration (MIC)

The MIC of cetylpyridinium chloride against *S. gordonii* and *S. mutans* was 0.2441 µg / mL.

### 3.2 PAE, PA SME, and SME Assay

The mean values of the PAE, PA SME, and SME of *S. gordonii* and *S. mutans* for cetylpyridinium

chloride are shown in Table 1. The mean PAE for *S. gordonii* was 1 h and the mean length of the PA SME was 1.13 h (0.1× MIC), 1.37 h (0.2× MIC), and 1.73 h (0.3× MIC). The mean SME times were 0.13 h (0.1× MIC), 0.20 h (0.2× MIC), and 0.23 h (0.3× MIC). The mean PAE for *S. mutans* was 1.07 h and the mean time for the PA SME was 1.43 h (0.1× MIC), 1.80 h (0.2× MIC) and 2.33 h (0.3× MIC). The mean SME times were observed at 0.00 h (0.1× MIC), 0.10 h (0.2× MIC), and 0.03 h (0.3× MIC). The PAE for *S. gordonii* was shorter than that of *S. mutans*. The PA SME was longer than the PAE in both *S. gordonii* and *S. mutans*. In both bacteria, the PA SME value increased as the sub-MIC concentration was increased, but the SME did not change.

**Table 1. The average duration for the PAE, PA SME, and SME of cetylpyridinium chloride on *S. gordonii* and *S. mutans***

	<i>S. gordonii</i> KN1	<i>S. mutans</i> KN88
PAE	1.00 ± 0.61 <sup>a</sup>	1.07 ± 0.31 <sup>a</sup>
<b>PA SME</b>		
0.1x MIC	1.13 ± 0.64 <sup>a</sup>	1.43 ± 0.32 <sup>a</sup>
0.2x MIC	1.37 ± 0.84 <sup>a</sup>	1.80 ± 0.30 <sup>a</sup>
0.3x MIC	1.73 ± 0.99 <sup>a</sup>	2.33 ± 0.35 <sup>a</sup>
<b>SME</b>		
0.1x MIC	0.13 ± 0.12 <sup>a</sup>	0.00 ± 0.00 <sup>a</sup>
0.2x MIC	0.20 ± 0.10 <sup>a</sup>	0.10 ± 0.10 <sup>a</sup>
0.3x MIC	0.23 ± 0.06 <sup>a</sup>	0.03 ± 0.06 <sup>a</sup>

<sup>a</sup> : Mean ± standard deviation (in h)

## 4. DISCUSSION

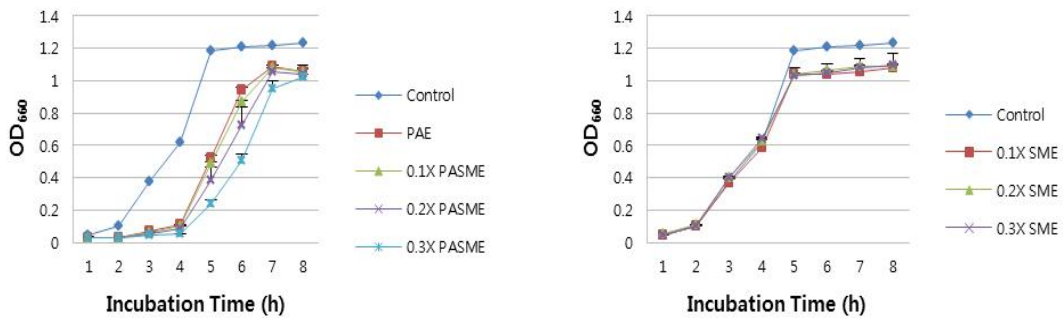
The PAE and PA SME have been extensively studied in the quest for the effective use of antimicrobial agents and to determine the optimal duration of administration [2,12,13]. Although several antimicrobial agents have been reported to induce the PAE, PA SME, and SME in a variety of bacteria [10,13], the use of cetylpyridinium chloride to induce the PAE, PA SME, and SME in oral bacteria has not yet been established. According to Lee [11], amoxicillin induced the PAE, PA SME, and SME in *S. gordonii*; the PAE was 2.0 h. In the present study, the PAE of cetylpyridinium chloride against *S. gordonii* was 1.0 h, which was shorter than that of amoxicillin. In addition, the PAE values of cetylpyridinium chloride differed according to the bacterial species. The PAE, PA SME, and SME for the bacteria may vary according to the types of antimicrobial agents; thus, there may be differences in the duration of

the effect. Other studies have reported that different combinations of bacteria and antimicrobial agents have different PAEs [11,13].

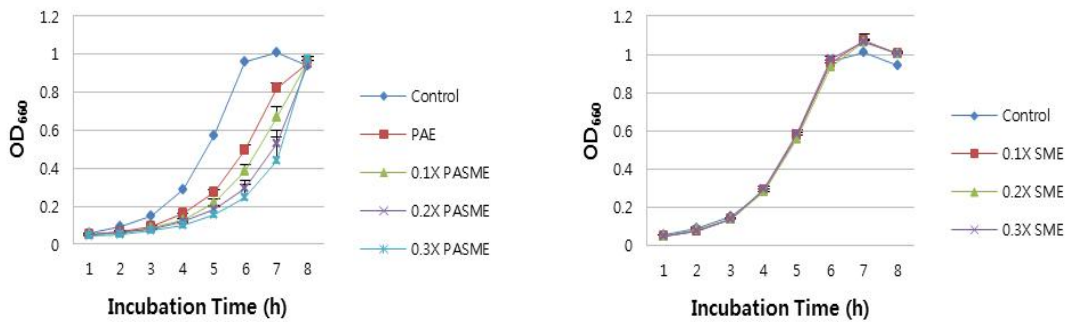
When an antimicrobial agent is applied to bacteria, the concentration of the antimicrobial agent falls to the sub-MIC after a certain time [3]. Therefore, a supra-inhibitory concentration of the antimicrobial agent is applied *in vivo*, followed by a sub-MIC [11]. As it is impossible to remove the antimicrobial agent applied *in vivo* immediately, a period of growth inhibition may occur for a certain period after application of the antimicrobial agent. Thus, the PA SME is more similar to those in situations observed *in vivo*. Previous studies have shown that the PA SME is more apparent than the SME on bacteria that are not exposed to antimicrobial agents [4,5]. The same effect was also observed in this study. In addition, PA SME values were observed to increase with an

increase in the sub-MIC concentrations of antimicrobials. It is thought that the most common mechanism for PAE is the extended presence of the antimicrobial agent at the cellular site after a short period of antimicrobial treatment [14,15]. However, the exact mechanism of the PAE *in vivo* is still not clear.

Cetylpyridinium chloride has been used extensively in the field of dentistry to control and reduce plaque formation and to prevent the development of oral diseases [16,17]. In this *in vitro* study, the PAE, PA SME and SME for cetylpyridinium chloride were determined, but the *in vivo* equivalents of these pharmacodynamics effects have not yet been determined. If the PAE, PA SME, and SME for cetylpyridinium chloride occur *in vivo*, cetylpyridinium chloride therapy may be beneficial because of these pharmacodynamic effects.



(A) *S. gordonii*



(B) *S. mutans*

**Fig. 1.** The PAE, PA SMEs, and SMEs of cetylpyridinium chloride against *S. gordonii* (A) and *S. mutans* (B). The PAE was induced through exposure of the bacteria to 10× MIC cetylpyridinium chloride for 1 min, which was eliminated by washing. The PA SMEs were examined through the addition of 0.1, 0.2, and 0.3× MIC during the post-antibiotic phase, and the SMEs were examined through exposure of the bacteria to cetylpyridinium chloride at sub-MICs only. The data represent the average of three replicate experiments

## 5. CONCLUSIONS

In this study, we demonstrated that cetylpyridinium chloride induced the PAE, PA SME, and SME. When cetylpyridinium chloride is used to prevent oral disease, these pharmacodynamic events may be used to reduce the adverse effects of these drugs *in vivo* through the adjustment of the dosage and administration interval.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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