Original

Antibacterial effects of three experimental quaternary ammonium salt (QAS) monomers on bacteria associated with oral infections

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Abstract: The aim of this study was to test the antibacterial effects of three experimental quaternary ammonium salt monomers in order to evaluate their potential applications as dental materials. *In vitro* susceptibility testing of the monomers was performed by the broth dilution method on bacteria associated with oral infections: *Streptococcus mutans* ATCC 25175, *Actinomyces viscosus* ATCC 15987, *Staphylococcus aureus* ATCC 29213 and *Lactobacillus casei* ATCC 393. The time-kill kinetics of the monomer with relatively higher antibacterial activity against *S. mutans* were also investigated. It was found that all the tested bacteria strains were susceptible to the three monomers, among which methacryloxylethyl cetyl ammonium chloride (DMAE-CB) exhibited the lowest minimal inhibitory concentrations, ranging from 1.2 to 4.8 µg/ml. The time-kill curve showed that DMAE-CB achieved 99.44% killing at 19.2 µg/ml (4 times the minimal bactericidal concentration) against *S. mutans* after 1 min and 100% killing within 10 min of contact. This result indicates that the quaternary ammonium salt monomer DMAE-CB may be a candidate antibacterial agent for incorporation into dental restorative materials. (J. Oral Sci. 50, 323-327, 2008)

Keywords: quaternary ammonium salt; antibacterial effect; oral pathogenic bacteria; monomer; dental material.

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Introduction

Due to their convenience of application, good esthetics, conservative preparation requirements, and the popularization of adhesive techniques, resinous materials are being used more widely in dental clinics (1). However, cariogenic bacteria such as *Streptococcus mutans* (*S. mutans*) tend to accumulate and propagate more readily on the surfaces of cured composite resin restorations in the oral cavity than on the surfaces of restorations made with other materials. In addition, microleakage caused by the shrinkage of composite resin during curing has always occurred between resin-dentinal interfaces, and allows bacteria to invade deeply, frequently resulting in postoperative complications, including recurrent caries, postoperative sensitivity, marginal discoloration, and pulp inflammation (2). Recently, the concept of minimal intervention dentistry (MI) is being more widely accepted by clinicians (3), so that conservation of the infected tooth structure has become more important. However, this increases the likelihood that some active bacteria will remain in the cavity. Therefore, restorative materials with antibacterial properties may play an important role in controlling the activity of such remnant bacteria.

A number of studies have investigated composite resins impregnated with antibacterial agents such as chlorhexidine, silver ions and fluorides (4-7). The antibacterial effects of all these materials were due to active ingredients released from the composites themselves. Therefore, their effects were time-dependent, and generally short-lasting. The mechanical properties of the carrier material may deteriorate, and the materials released may be toxic if not properly controlled (8,9). To overcome the disadvantages

of agent-releasing composites, some researchers have reported finishing several resin-based restorations with a composite resin, which is incorporated with a type of quaternary ammonium salt monomer, methacryloyl-oxydodecyl pyridinium bromide (MDPB), which exhibits antibacterial activities without leach-out of the incorporated monomer from the material (10-13).

Quaternary ammonium salts (QAS) have been widely used in paint, water treatment, textiles, and the food industry because of their relatively low toxicity and broad antimicrobial spectrum (14). They can also be chemically bound to polymer carriers via active groups, thus integrating QAS monomers with the composite matrix. As compared with conventional antibacterial agents of low molecular weight, the advantages of these polymerizable antibacterial agents include non-volatility, chemical stability, and low permeation through skin (15). However, the application of QAS monomers as dental materials has not been reported, except for MDPB. Therefore, research on the possible application of novel QAS monomers to dentistry may provide more choices for the development of dental antibacterial materials.

For this purpose, our research group synthesized three experimental QAS monomers that are structurally different from MDPB (16,17). The aim of the present study was to assess the *in vitro* antibacterial activities of these three monomers against common pathogenic bacteria in the oral cavity, and to compare their antibacterial abilities. The time-kill kinetics of the most effective monomer against *S. mutans*, the most representative cariogenic bacterium, were also tested in order to evaluate its potential application as a dental material.

Materials and Methods
Structures of experimental QAS monomers

The experimental QAS monomers used in this study were methacryloxylethyl benzyl dimethyl ammonium chloride (DMAE-BC), methacryloxylethyl m-chloro benzyl dimethyl ammonium chloride (DMAE-m-CBC) and methacryloxylethyl cetyl ammonium chloride (DMAE-CB). Their structures are presented in Fig. 1.

Bacterial strains and culture conditions

The tested bacterial strains are listed in Table 1. *S. mutans*, *Actinomyces viscosus* (*A. viscosus*) and *Lactobacuillus casei* (*L. casei*) are common bacteria associated with dental caries (18,19). *Staphylococcus aureus* (*S. aureus*) is related to cutaneous, tonsillar and respiratory infection, and has also been isolated from the mouth (20,21). All bacterial strains were maintained by subculture using brain heart infusion (BHI, Difco, USA) agar plates supplement with 0.5% yeast extract. *S. mutans*, *A. viscosus* and *L. casei* were cultured anaerobically at 37°C. *S. aureus* was cultured at 37°C in ambient air.
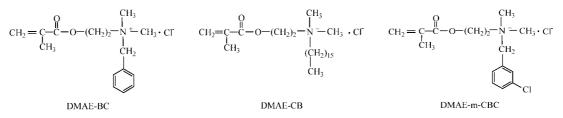

Fig. 1   Structures of the experimental QAS monomers: DMAE-BC, DMAE-CB and DMAE-m-CBC.

Table 1 MIC and MBC values of three experimental QAS monomers

Bacterial strains	MIC*and MBC (µg/mL)			
	Chlorhexidine	DMAE-BC	DMAE-m-CBC	DMAE-CB
S. mutans ATCC 25175	0.6^a (1.2)	1562.5^b (6250.0)	1562.5^b (3125.0)	2.4^a (4.8)
A. viscosus ATCC 15987	1.2^a (2.4)	3125.0^b (3125.0)	1562.5^b (1562.5)	4.8^a (9.6)
S. aureus ATCC 29213	0.3^a (1.2)	1562.5^b (6250.0)	1562.5^b (3125.0)	1.2^a (2.4)
L casei ATCC 393	0.6^a (2.4)	1562.5^b (1562.5)	3125.0^b (3125.0)	2.4^a (9.6)

* Groups identified with the same superscript letter were not significantly different ($P > 0.05$).

   Numbers in parentheses are MBC values

In vitro susceptibility test

Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined by the broth dilution method. The three monomers, DMAE-BC, DMAE-CB, DMAE-m-CBC, were dissolved in BHI broth to prepare a starting concentration, respectively. According to the results of a pilot study, the initial concentrations of the former two monomers were set at 100 mg/ml, and the last one at 5 mg/ml. These solutions were diluted two-fold serially with BHI broth for each experiment. Bacterial suspensions with a turbidity equivalent to that of a 0.5 McFarland standard were prepared by suspending growth from BHI agar plates in 2 ml of sterile saline. Suspensions were further diluted 1:10 twice to obtain final inocula of 1×10^7 colony-forming units (CFU)/ml in BHI broth, then 100 μl of inocula were added to 1 ml of a series of monomer dilution broths. The tubes were read for visible turbidity after 24 h of culture for *S. aureus*, and after 48 h for the other three bacterial strains, with reference to negative and positive growth control tubes. Chlorhexidine (CHX) acetate (Dasheng Chemical, China) was also included as a positive control. MIC was defined as the endpoint where no turbidity could be detected with respect to the controls. A 100-μl aliquot from each test tube without turbidity was inoculated onto BHI agar plates. MBC was determined as the lowest concentration of the tested monomer that produced no colony on the plate. The negative control tube did not contain bacterial inoculum, and the positive control or turbidity tube was free of the monomer. The tests were repeated in triplicate.

Bactericidal kinetics

Based on the results of preliminary experiments, DMAE-CB exhibited the best antibacterial effect. Time-kill determination was therefore performed to clarify its killing
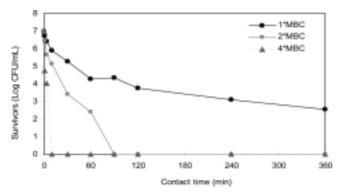

Fig. 2 Time-kill curve for DMAE-CB at concentrations of 1*MBC(●), 2*MBC(■), and 4*MBC(▲) against *S. mutans* ATCC 25175.

kinetics against *S. mutans*. Cultures of *S. mutans* ATCC 25175 with a density of 1×10^7 CFU/ml were exposed to DMAE-CB broth dilutions at three different concentrations. The final concentrations of DMAE-CB were 4.8 μg/ml (equal to MBC value, 1*MBC), 9.6 μg/ml (2 times the MBC value, 2*MBC) and 19.2 μg/ml (4 times the MBC value, 4*MBC). BHI broth without monomer was used as the control. After inoculation, all the solutions were incubated at 37°C under shaking conditions (160 r.p.m). After 1, 3, 10, 30, 60, 90, 120 and 360 min of incubation, aliquots of 100 μl were taken out, serially diluted, and inoculated on BHI agar plates. The plates were then incubated for 48 h anaerobically at 37°C, and the number of survivors (CFU/ml) was determined by counting the colonies. The experiments were conducted in triplicate.

Data analysis

The MIC values obtained from the broth dilution test were assessed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test for post-hoc comparison. Statistical significance was defined as $P < 0.05$.

Results
MIC and MBC determinations

The MIC and MBC values of the tested QAS monomers are presented in Table 1. DMAE-CB showed the best antibacterial activity, with MIC values of 1.2-4.8 μg/ml (2.88~11.50 μM) against the four pathogenic bacterial strains, similar to that of CHX. In comparison, the MICs and MBCs of DMAE-BC and DMAE-m-CBC were significantly higher ($P < 0.05$), and no significant differences were found between these two groups ($P > 0.05$).

Bactericidal kinetics

Figure 2 shows the time-kill curve for DMAE-CB at concentrations of 1*MBC, 2*MBC, 4*MBC against *S. mutans*. Contact with DMAE-CB at high concentration resulted in immediate killing of *S. mutans*. After contact with DMAE-CB at a concentration of 19.2 μg/ml (45.98 μM 4*MBC) for 1 min, only about 5.6×10^4 CFU/ml cells survived (99.44% reduction), and the cell count was 1.11×10^4 CFU/ml within 5 min of contact (99.889% reduction). Complete lethality occurred after 10 min of contact. However, at a concentration of 9.6 μg/ml (22.99 μM, 2*MBC), DMAE-CB showed slower killing kinetics, bacteria at 260 CFU/ml being still viable after 60 min of culture. The number of survivors after contact with 4.8 μg/ml (11.50 μM, 1*MBC) DMAE-CB for 360 min was 350 CFU/ml.

Discussion

The three antibacterial monomers we investigated were originally developed and selected because of their ability to be immobilized on composite resins through combination of an antibacterial agent (QAS) and a polymerizable group (methacryloyl group). As the antibacterial monomer can copolymerize with other monomers after being incorporated into the resinous material, the polymer network is endowed with antibacterial activity after curing. Therefore, the antibacterial portion of the monomer will not leach from the material, and the composite resin will destroy any bacteria that come into contact with the surface of the composite restoration, acting as a so-called "contact disinfectant". Synthesis of antibacterial monomers and comparison of their antibacterial effects are indispensable for realizing this aim.

Our results showed that the three QAS monomers exhibited various degrees of antibacterial ability. Among them, DMAE-CB was demonstrated to have much lower MIC/MBC values than DMAE-BC or DMAE-m-CBC. The MBC values for DMAE-CB with the four oral pathogenic bacterial strains were within the range 2.4~9.6 µg/ml (5.75~22.99 µM), while those of the other two monomers were within 1562.5~6250.0 µg/ml (5.51~22.04×10^3 µM for DMAE-BC, and 4.91~19.65×10^3 µM for DMAE-m-CBC). Therefore, the MBC value for DMAE-CB was in accordance with those of the representative QAS species, cetylpyridinium chloride (CPC) and benzalkonium chloride (BKC), which have been reported to be 1~8 and 2~8 µg/ml, respectively (22).

The difference in MBC and MIC of these three monomers may be attributed to their overall molecular structure, especially the length of the alkyl chain. It has been shown that increasing the alkyl chain length of the substituents increases the hydrophobic interaction with the lipid bilayer of the cell wall, which further increases the antibacterial activity of the compound (23). DMAE-BC has an alkyl chain with the same length as that of DMAE-m-CBC, while the benzyl in DMAE-BC was substituted by m-chloro benzyl in DMAE-m-CBC. However, both DMAE-BC and DMAE-m-CBC had similar MIC values ($P > 0.05$), and both were significantly higher than that of DMAE-CB ($P < 0.05$), which contains a 16-carbon alkyl chain. Therefore, DMAE-CB was employed for further studies of time-killing against *S. mutans*.

The bactericidal kinetics of DMAE-CB at three different concentrations indicated rapid bactericidal effects against *S. mutans*. At a concentration of 19.2 µg/ml (4*MBC), DMAE-CB killed all the tested bacteria within 10 min of contact. This result was similar to that for MDPB, which killed *S. mutans* cells (99.999% reduction) within 5 min

at 250 µg/ml (4*MBC), and within 1 min at 500 µg/ml (8*MBC) (24). As mentioned previously, the number of carbon atoms in the alkyl chain of DMAE-CB exceeds that of MDPB, which contains a 12-carbon alkyl chain. Therefore, DMAE-CB may have a stronger ability than MDPB to kill oral pathogenic bacteria.

In the present study, chlorhexidine (CHX) was also included as a positive control for comparison of potency of the tested monomers, since CHX has been considered the gold standard for antibacterial application. The results of *in vitro* susceptibility tests indicated that the antibacterial activity of DMAE-CB was similar to that of CHX. However, many studies have reported that CHX can be released in relatively high amounts from various methacrylate polymers and bone cements (25-27), whereas DMAE-CB can be chemically bound within the polymer carrier via a polymerizable group (methacryloyl group) for possible long-term effectiveness.

The bacteria used in this study were mainly Gram-positive and proved susceptible to the tested QAS monomers. Studies of the antibacterial activities of QAS against Gram-negative bacteria and fungi are still in progress. Further investigations will also be needed to clarify whether the antibacterial activities of QAS monomers can be maintained after their incorporation into resin-based restorative materials. In addition, their effects on the mechanical properties of composite resins after incorporation remain to be determined.

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