Antibacterial activity and dentin bonding ability of combined use of Clearfil SE Protect and sodium hypochlorite

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The aim of this study was to evaluate the antibacterial activity and dentin bonding ability of a commercial self-etch adhesive Clearfil SE Protect (Kuraray Noritake Dental, Tokyo, Japan) in combination with sodium hypochlorite (NaOCl). Agar disc diffusion tests and measurement of minimum inhibitory/bactericidal concentrations (MIC/MBC) against *Streptococcus mutans* were performed to evaluate antibacterial effects. The mixture solution of 5.25% NaOCl and the primer of Clearfil SE Protect demonstrated less antibacterial activity than primer only. In microtensile bond strength tests using non-carious human molars, pretreatment with 5.25% NaOCl aqueous solution had no influence on the bond strength of Clearfil SE Protect. These results indicate that pretreatment with NaOCl does not influence the bonding ability of Clearfil SE Protect, while their combined use does not enhance cavity disinfecting effects.

Keywords: Adhesive, Disinfection, Quaternary ammonium compound, NaOCl

INTRODUCTION

Caries is treated by surgical removal of the infected tooth structure, however, excavation frequently results in a complex substrate involving areas of caries-infected, caries-affected, sclerotic, and eroded dentin¹. Incomplete removal of infected dentin is sometimes recommended, especially in clinical situations of deep carious lesions, to preserve pulp tissue vitality². Therefore, cavity disinfection to reduce residual bacteria is essential for the successful treatment of caries, and attempts to create restorative materials possessing antibacterial effects remains an important challenge³.

Clearfil SE Protect (Kuraray Noritake Dental, Tokyo, Japan) is an antibacterial self-etch adhesive system. It contains an antibacterial monomer 12-methacryloyloxy dodecylpyridinium bromide (MDPB) in its primer, which is synthesized by combining a methacryloyl group with a quaternary ammonium compound (QAC)⁴. MDPB as a QAC is proposed to interact with negatively charged bacterial cell surfaces, causing a disturbance to the charge balance and damage to the cell membrane⁵. Because of its rapid bactericidal activity against cariogenic bacteria at the unpolymerized stage, MDPB has been incorporated into the self-etching primer of Clearfil SE Protect to provide cavity disinfecting effects. In addition, the ability of MDPB to polymerize is advantageous because it does not influence the bonding ability of adhesives⁶.

Sodium hypochlorite (NaOCl) is frequently used for disinfection of the tooth surface. Several studies have reported the role of NaOCl used as a disinfectant prior to the bonding procedure^{7,8}. It is considered that

the mechanism for the germicidal activity of NaOCl is the inhibition of enzyme activity essential for bacterial growth, damage to the membrane and DNA, and perhaps injury to the membrane transport capacity⁹. Thus, the mechanism underlying antibacterial actions of NaOCl differs from that of MDPB. Therefore, combined use of NaOCl with Clearfil SE Protect may provide additional effects in terms of antibacterial activity. Conversely, Ozturk and Ozer¹⁰ reported that NaOCl application decreased the bond strength of Clearfil SE Bond (Kuraray Noritake Dental) to dentin. Because Clearfil SE Protect has similar components to Clearfil SE Bond except for the inclusion of MDPB in the primer and encapsulated sodium fluoride particles in the bonding resin, it is important to assess the influence of NaOCl pretreatment on the bonding ability of Clearfil SE Protect.

In this study, to examine the first null hypothesis that the use of NaOCl provides additional antibacterial effects to Clearfil SE Protect primer, antibacterial activity was evaluated by the agar disc diffusion test and measurement of minimum inhibitory/bactericidal concentrations (MIC/MBC) against *Streptococcus mutans*. The second null hypothesis tested was that pretreatment with NaOCl does not reduce the bonding ability of Clearfil SE Protect, which was measured by the microtensile bond strength test.

MATERIALS AND METHODS

Agar disc diffusion tests

The materials used for agar disc diffusion tests included a commercial two-step self-etch adhesive (Clearfil SE Protect primer, Kuraray Noritake Dental) and a

commercial 10% NaOCl solution (Neo Cleaner, Neo Dental Chemical Products, Tokyo, Japan). These solutions were mixed or diluted with water, and four test solutions were prepared as follows:

Group 1: Clearfil SE Protect primer (Primer)

Group 2: 5.25% NaOCl solution (NaOCl)

Group 3: Clearfil SE Protect primer+5.25% NaOCl solution with 1:1 vol ratio (Primer/NaOCl)

Group 4: Clearfil SE Protect primer+distilled water with 1:1 vol ratio (Primer/Water).

Agar disc diffusion tests were performed as previously reported¹¹⁾. Briefly, *S. mutans* strain NCTC10449 was used and cultivated in Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD, USA) broth at 37°C. After anaerobic incubation for 24 h, 300 µL of bacterial suspension was spread onto a BHI agar plate. A 20-µL aliquot of each solution of the four test groups was impregnated into a sterile paper disc (diameter: 6 mm, thickness: 1.5 mm) and placed on an agar plate inoculated with the bacterial suspension. A 20-µL volume was chosen as the optimum volume for impregnation into a paper disc without overflow of the test solution. Plates were incubated anaerobically for 48 h at 37°C, and the diameter of the inhibition halo was measured at three points using a caliper (Mitutoyo, Tokyo, Japan). The sizes of the inhibition zones were calculated by the following equation:

$$\text{Size of inhibition zone} = (I - D) / 2$$

where *I*=mean of three measurements of the diameter of inhibition halo (mm) and *D*=diameter of the paper disc (6 mm). The tests were repeated three times.

MIC and MBC measurements

MIC and MBC of Primer, NaOCl, and Primer/NaOCl as prepared above against *S. mutans* were determined by serial microdilution assay as previously reported¹¹⁾. Serial two-fold dilutions of each disinfectant were made in wells of a 96-well microplate containing BHI broth, and test samples (50 µL; concentrations ranged from 0.000095 to 25% of the original solution) were prepared. *S. mutans* suspension (50 µL) at approximately 2×10^6 colony-forming units/mL was inoculated into each well. The plates were then incubated anaerobically at 37°C for 48 h. The MIC value was determined as the lowest concentration in the well at which turbidity was not observed by visual examination. Suspensions from wells that showed no bacterial growth were inoculated onto BHI agar plates. After subculture for 48 h, the MBC value was determined as the lowest concentration that showed no colony formation on the plates. The tests were repeated five times.

Microtensile bond strength tests

Twenty non-carious human molars were randomly divided into two groups (described below) to test the treatment of dentin surfaces. The occlusal enamel of the crown was removed using a low-speed diamond saw (Isomet 2000, Buehler, Lake Bluff, IL, USA) and the dentin surface was polished with 600-grit silicon carbide paper. To eliminate the influence of differences among

teeth, one tooth specimen was divided into two pieces and one piece from each tooth was allocated to each treatment group.

In the first group, control (SEP), the dentin surface was treated with Clearfil SE Protect (Kuraray Noritake Dental). Briefly, the primer of Clearfil SE Protect was applied to the dentin surface for 20 s using a sponge supplied by the manufacturer. After the surface was dried using a gentle air blower, the bonding resin of Clearfil SE Protect was applied and light-cured for 10 s using a 1,000 mW/cm² LED curing device (PenCure, Morita, Kyoto, Japan). In the second group, the dentin surface was treated with 5.25% NaOCl solution using a cotton pellet for 10 s, washed with water for 10 s, and then dried before application of Clearfil SE Protect.

A resin composite (Clearfil AP-X, Shade A3, Kuraray Noritake Dental) was built up in three layers to a height of 5 mm. Light-curing was performed using the LED curing device (PenCure) for 20 s for each layer, and the specimens were immediately placed in distilled water. After storage at 37°C for 24 h, the bonded specimens were clamped into the adopted holder and sectioned perpendicular to the bonding surface using the low-speed diamond saw to obtain rectangular sticks (1×1 mm, 8–9-mm long). Each specimen was attached to a jig with an adhesive (Model Repair Pink, Dentsply Sankin, Tochigi, Japan) placed in a universal testing machine (EZ Test, Shimadzu, Kyoto, Japan) and the microtensile bond strength test was conducted at a crosshead speed of 1 mm/min. The cross-sectional area of each specimen was measured using a digital caliper (Absolute Digimatic Caliper, Mitutoyo, Kanagawa, Japan) and the bond strength value was calculated by dividing the load by the bonded area. The fracture mode was observed using a stereoscopic microscope (SMZ-U, Nikon, Tokyo, Japan) at 20× magnification to determine failure modes. Failure modes were classified as adhesive failure, cohesive failure within the tooth, or mixed failure (combination of adhesive failure and cohesive failure).

Statistical analysis

The results of agar disc diffusion tests were statistically analyzed using analysis of variance (ANOVA) and Student-Newman-Keuls *post hoc* test with a significance level of $p < 0.05$ (SPSS Statistics 21, IBM-SPSS, Chicago, IL, USA). The results of microtensile bond strength tests were statistically analyzed using Student's *t*-test with a significance level of $p < 0.05$.

RESULTS

Agar disc diffusion tests

The sizes of inhibition zones produced by agar disc diffusion tests are shown in Fig. 1. All test groups produced inhibition zones against *S. mutans*. A significantly smaller inhibition zone was observed for NaOCl compared with that for Primer ($p < 0.05$, ANOVA, Student-Newman-Keuls *post hoc* test). The size of the inhibition zone for Primer/Water was approximately half of that for Primer. Although the mean size of the

Fig. 1 Size of inhibition zones produced in agar disc diffusion tests.

The bar represents the standard deviation of three replicates. Groups that do not share the same superscript letter are significantly different ($p < 0.05$, ANOVA, Student-Newman-Keuls *post hoc* test).

Table 1 MIC and MBC values of Primer, NaOCl, and Primer/NaOCl against *S. mutans* expressed as the percentage of the original solution (%)

	MIC	MBC
Primer	0.012	0.098
NaOCl	12.5	12.5
Primer/NaOCl	0.024	0.195

inhibition zone for Primer/NaOCl was greater than that for Primer/Water, no significant differences were observed between the two groups ($p > 0.05$, ANOVA, Student-Newman-Keuls *post hoc* test).

MIC and MBC measurements

Table 1 shows the MIC and MBC values against *S. mutans* expressed as the percentage of the original solution. The same results were obtained for five repetitions of the tests. NaOCl demonstrated much greater MIC and MBC values than Primer only. The MIC and MBC values of Primer/NaOCl were two times greater than those of Primer only.

Microtensile bond strength tests

Microtensile bond strengths for SEP and NaOCl+SEP are shown in Fig. 2, and failure modes are presented in Table 2. No significant differences in bond strength were observed between the two groups ($p > 0.05$, Student's *t*-test). For SEP, adhesive failure was observed in all specimens. For NaOCl+SEP, adhesive failure was observed in 50% of the specimens, and cohesive or mixed failure was observed in 20 or 30% of the specimens, respectively.

Fig. 2 Microtensile bond strength of Clearfil SE Protect to dentin with or without NaOCl pretreatment.

The bar represents the standard deviation of ten specimens. No significant differences between the two groups were observed ($p > 0.05$, Student's *t*-test).

Table 2 Results of failure mode analysis

	SEP	NaOCl+SEP
Adhesive	10 (100%)	5 (50%)
Cohesive	0 (0%)	2 (20%)
Mixed	0 (0%)	3 (30%)

DISCUSSION

To improve dental restoration failure rates, much attention has been paid to antibacterial effects of materials applied prior to direct filling materials. Cavity disinfectants are used to eradicate residual bacteria after surgical removal of the infected tooth or tooth preparation for direct/indirect restorative treatments. NaOCl is a well-known, nonspecific proteolytic agent as well as dissolver for magnesium and carbonate ions¹². Therefore, NaOCl is used to remove the smear layer and collagen on dentin surfaces. The role of NaOCl used as a disinfectant on prepared dentin prior to the bonding procedure has been investigated¹³. Due to its potent, Dijken demonstrated that acceptable bonded restorations were achieved after pretreatment of a tooth with NaOCl¹⁴. Since then, the benefit of NaOCl against bacteria on dentin surfaces has also been demonstrated^{15–17}.

QACs, such as cetylpyridinium chloride or benzalkonium chloride, are widely used as antibacterial agents¹⁸. QACs are proposed to interact with negatively charged bacterial cell surfaces, causing a disturbance to the charge balance and damage to the cell membrane⁹. In the 1990s, Imazato *et al.* developed the antibacterial monomer MDPB by combining a QAC with a methacryloyl group¹⁹. As a derivative of QAC, MDPB

has strong antibacterial activity before polymerization. In addition, the antibacterial component is immobilized in a polymer network by polymerization of MDPB, and such immobilized antimicrobial does not leach out from cured resins. A number of studies have reported that MDPB has antibacterial activity against various dental caries-related bacteria^{20–22}. By employing an MDPB-containing antibacterial, self-etching primer, the world's first adhesive system for restoration with antibacterial effects was successfully commercialized in 2004.

S. mutans is one of the major dental pathogens associated with the initiation of dental caries and also well recognized in residual and secondary caries. To evaluate the disinfectant ability of NaOCl in combination with Clearfil SE Protect primer containing MDPB, agar disc diffusion tests against *S. mutans* were conducted. NaOCl demonstrated a much smaller inhibition zone compared with Primer, indicating that the antibacterial activity of NaOCl was fundamentally weak. The size of the inhibition zone for Primer/Water was approximately half of that for Primer. This is because MDPB in the primer was diluted to half of its concentration due to mixture with water. In addition, no significant differences in size of inhibition zones were observed between Primer/NaOCl and Primer/Water. Thus, in agar disc diffusion tests, the additional effects of NaOCl to enhance antibacterial activity of Clearfil SE Protect primer were not observed.

It is not possible to precisely compare antibacterial activities by using agar disc diffusion tests because diffusivity of NaOCl in the agar might have been interrupted by the components of Clearfil SE Protect primer. Therefore, to compare intrinsic antibacterial activities, the MIC and MBC values of Primer, NaOCl, and Primer/NaOCl against *S. mutans* were determined. The results confirmed that the MIC and MBC values of Primer/NaOCl were two times greater than those of Primer only because MDPB in the primer was diluted to half of its concentration due to mixture with NaOCl. Therefore, based on the results of agar disc diffusion tests and measurement of MIC/MBC values, the first null hypothesis —the use of NaOCl provides additional antibacterial effects to Clearfil SE Protect primer— was rejected. However, we considered that remnant chloride ions after NaOCl application do not obstruct the ability of positively charged MDPB to act on bacteria, even though they do not enhance the antibacterial capacity of the primer.

Several studies reported that NaOCl treatment before application of self-etch adhesives affects bond strengths of the adhesives. Ozturk and Ozer¹⁰ reported that the application of 5% NaOCl for 1 min significantly decreased the bond strength of a self-etching adhesive Clearfil SE Bond because NaOCl removed collagen fibers and consequently prevented the formation of the healthy hybrid layer. In addition, reactive residual free-radicals in NaOCl may compete with propagating vinyl free-radicals during the light-curing procedure for adhesive systems, which might result in immature and incomplete polymerization²³. Conversely, Mohammed

*et al.*⁸) reported that 4% NaOCl application for 20 s increased the bond strength of a self-etching adhesive Adper Easy One (3 M ESPE, Seefeld, Germany) because the penetration of the primer was increased by removing the collagen layer, smear layer, and smear plug by NaOCl. These studies have shown controversial results of bond strength of self-etching adhesives after the pretreatment of NaOCl.

Our results indicated that pretreatment with 5.25% NaOCl for 10 s did not have a negative influence on the bond strength of Clearfil SE Protect. The disagreement between the results of the present study and those of Ozturk and Ozer¹⁰) may be attributed to differences in the processing time of NaOCl. In this study, 5.25% NaOCl aqueous solution was used but applied on dentin surface for 10 s, considering the clinical use of NaOCl as a cavity cleanser. Because the shorter processing time of NaOCl results in decreased removal of the smear layer and reduced generation of residual free-radicals, NaOCl treatment may not affect the bonding ability of Clearfil SE Protect.

In attempts to estimate the lifetime of the bond strength at the tooth-composite interface, it became important to obtain fracture mode data. In the primer only (SEP) group, adhesive failure was observed in all specimens. Conversely, pretreatment with NaOCl before application of the primer (NaOCl+SEP) demonstrated a lower rate of adhesive failure and cohesive or mixed failure was observed, indicating that bond strength between dentin and the adhesive would not be less than the values obtained. These findings support the greater bonding ability of the NaOCl+SEP group to dentin compared with that of the SEP group. Therefore, the second null hypothesis —pretreatment with NaOCl reduces the bond strength of Clearfil SE Protect— was rejected.

Long-term studies have shown that the bond strength of resin-dentin bonds decreases over time due to collagen degradation within the hybrid layer^{24,25}. However, our group has confirmed that Clearfil SE Protect primer or a cavity disinfectant containing MDPB effectively prevents bond strength reduction after 1-year water storage (unpublished data). It is considered that the bonding durability of the adhesive system can be improved by MDPB, which can inhibit matrix metalloproteases and cathepsins^{26,27}. Further studies should evaluate the influence of pretreatment with NaOCl on the bonding durability of Clearfil SE Protect.

CONCLUSION

Employing agar disc diffusion tests and measurement of MIC/MBC values, we found that NaOCl provided no additional effects to the antibacterial activity of Clearfil SE Protect primer containing MDPB. In addition, pretreatment with 5.25% NaOCl aqueous solution for 10 s did not reduce the microtensile bond strength of Clearfil SE Protect to dentin. Within the limitations of this study, it is suggested that enhancement of cavity

disinfecting effects is not expected by the treatment of NaOCl before the application of Clearfil SE Protect, although no influence on dentin bonding ability can be obtained.

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