Effect of sodium percarbonate-containing disinfectant on natural biofilm model of dental unit waterline

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Purpose: Biofilm contamination of dental unit waterlines is an important public health problem. This study evaluated the bactericidal effects of a disinfectant containing sodium percarbonate (Mazak P) against naturally formed biofilms under low corrosion-inducing conditions.
Materials and Methods: Fragments of dental polyurethane tube that retained biofilm were immersed for five minutes into each test solution, and bacterial survival was determined by cultivation of the treated tubes in R2A broth.
Results: The results showed that the bactericidal effects of Mazak P at 10˚C were insufficient and that *Stenotrophomonas maltophilia* predominantly regrew from the treated fragments. However, when a mixture of Mazak P and benzalkonium chloride (BAC) was used for treatment at 10˚C, three of seven treated samples showed no bacterial survival. However, the killing efficacy of this mixture tended to decrease at 25˚C, probably because of BAC separation.
Conclusion: All through the experiment, the most resistant strains against disinfection were within the genus *Methylobacterium,* suggesting that special caution is necessary for red colony-forming opportunistic pathogens.

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Key Words: biofilm, corrosion, dental unit waterline, disinfection, heterotrophic bacteria

Introduction

Biofilm contamination of dental unit waterlines (DUWL) is an important public health problem [1-3]. Both in the guidelines [4] and a summary of infection prevention in dental settings opened in the website, the Centers for Disease Control and Prevention (CDC) recommends that sterile solutions be used as coolant/irrigation in the performance of oral surgical procedures, and that the coolant used in non-surgical dental procedures contains less than or equal to 500 colony forming units of heterotrophic bacteria per milliliter of water, the same as in the EPA (United States Environmental Protection Agency) regulatory standards for drinking water. For all dental associated workers and dental patients, intake of contaminated water/aerosols is a risk of infection, particularly for compromised hosts, including elderly and immune-deficient individuals. In fact, a lethal case of Legionnaires' disease in an 82-year-old woman associated with a DUWL was reported in 2012 [5]. In recognition of this case and the CDC summary, the American Dental Association (ADA) issued a statement and described details including methods for cleaning and maintaining waterlines, in addition to a waterline test procedure.

World-wide, infection controls related to DUWL lack consensus at present. In any case, dental units used for a long periods retain some contamination. Although flushing may reduce bacterial counts in dental unit water [6], it cannot remove biofilm formed in the waterline. Ricci et al. reported that flushing reduced the levels of heterotrophic bacteria in dental unit water, but did not reduce the occurrence of *Legionella* spp. and protozoa [7]. Therefore, other than replacing dental units, effective disinfection methods for DUWL without damaging parts such as metal connections are necessary.

In this study, based on these problems, the bactericidal effects of a disinfectant containing sodium percarbonate against naturally formed biofilms under low corrosion-inducing conditions were evaluated.

Materials and Methods

Disinfectants

Mazak P, a disinfectant for dental vacuum lines containing 98% sodium percarbonate (MP; Morita, Kyoto, Japan), and benzalkonium chloride (BAC; Sanisol; Kao, Tokyo, Japan) were diluted using sterilized deionized water, and were used as test disinfectants. When using these disinfectants simultaneously, the same volumes of each were mixed just before the experiment. Liquid temperature for disinfection was previously adjusted to 10 or 25˚C using a dry bath incubator.

Brass corrosion test

A brass disk (2 cm in diameter and 3 mm in thickness with a 1 mm hole; based on Japanese Industrial Standard (JIS) H-3250 and comprising Cu (59.1%), Pb (3.32%), Fe (0.12%), Fe+Sn (0.4%) and Zn (37.0%); Kits Metal Works, Chino, Japan) was used for this experiment. After weighing, the test disk was hung and immersed individually into disinfectant solution at 25˚C. Weight change for each disk was determined after 24 h of incubation. Results (6 disks per experimental group) were statistically analyzed by the Steel-Dwass method.

Disinfection test using naturally formed biofilm

A polyurethane tube for DUWL (4 mm outside diameter and 2 mm inside diameter; Toughrethane; Nippon Valqua Industries, Tokyo, Japan) was connected to the water supply of the spittoon of the dental unit and left for 1 year under normal clinical usage. The tube was then removed from the water supply and its outer surface was wiped with ethanol-absorbed cotton. One centimeter of the tube on each end was removed in order to avoid environmental contamination and the remaining part was cut into 5-mm fragments. All fragments were washed with sterilized water and dried on sterilized filter paper. Randomly selected pieces (6 to 7) were placed into a plastic tube and tested with disinfectant or sterilized water (as non-disinfection control). Plastic tubes were vortexed in order to ensure the inside of each fragment fully contacted with the liquid. After 5-min incubation at 10 or 25˚C, tube fragments were washed with phosphate-buffered saline (PBS) and dried by paper absorption. To detect surviving bacteria, each fragment was placed into 250 μL of R2A broth (Nihon Seiyaku, Tokyo, Japan) in the wells of a 96-well plate. After a two-week incubation, bacterial survival was determined both by optical density (620 nm) in 100 μL of cultured R2A and colony formation from the same culture (10 μL) spread onto a R2A agar. If colony formation occurred on the agar plate, each bacterial isolate was identified using Partial 16S rDNA sequence polymerase chain reaction (PCR) amplicon with reference to the nucleotide database of NCBI. The PCR-based bacterial identification procedure was according to the description in the Japanese Pharmacopoeia (16th Ed.), and sequence identity between query and reference of over 99% and 90-98% were judged to be the same species and the same genus, respectively. Changes in culture OD values and probability of bacterial killing occurrence among experimental groups were statistically analyzed by the Steel-Dwass method and Fisher's exact test, respectively.

Statistical analysis

All statistical analyses were performed by EZR, a graphical interface for R [8].

Results

Corrosion test for disinfectants

As shown in Fig. 1A, MP at concentrations of 3.3 and 6.7% (5 g and 10 g per 300 mL, respectively) significantly reduced the weight of brass disks after 24-h incubation when compared with water-treated controls ($P < 0.05$;

Steel-Dwass test). Therefore, the concentration of MP for subsequent experiments was fixed at 1.7%. When mixtures of MP and BAC (MP+BAC) were used for the same test, disk weight was not significantly different when compared with control and MP (Fig. 1B).

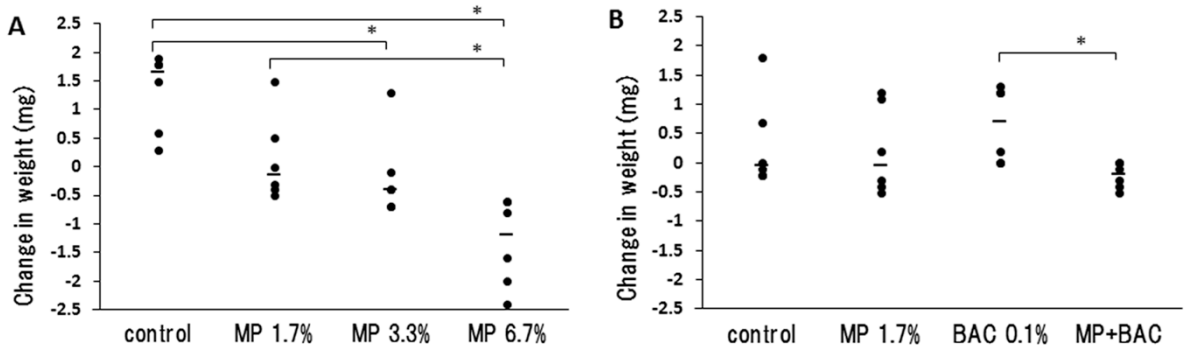

Fig. 1
Weight changes in brass disks after 24-h incubation in disinfectants.
A) Effect of Mazak P (MP) solutions (1.7, 3.3, and 6.7%). B) Effect of MP 1.7%, benzalkonium chloride (BAC) 0.1%, and a mixture (MP+BAC). Bar: median (n = 6), **P* < 0.05 (Steel-Dwass test)

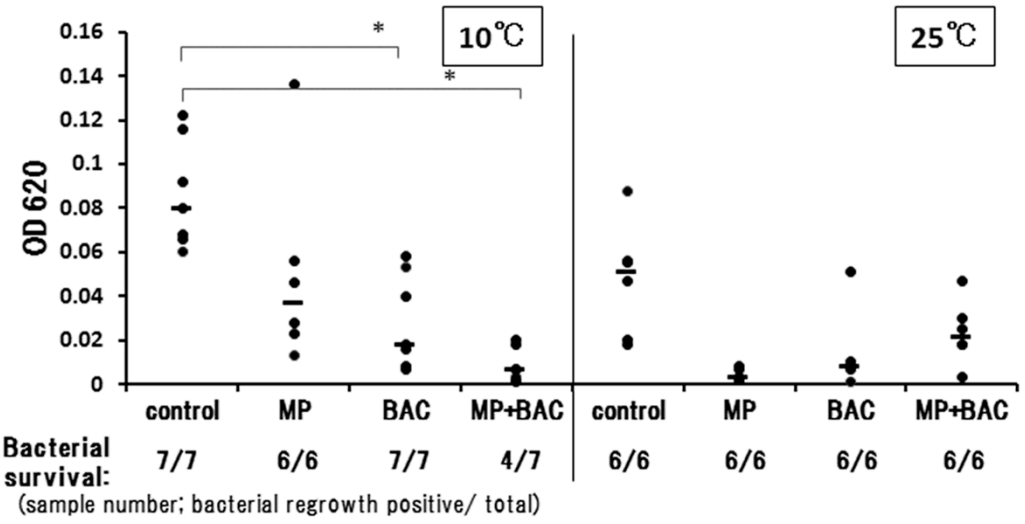

Fig. 2
Optical density increases and bacterial survival in R2A culture for each disinfected tube fragment.
After disinfection treatment, each tube fragment was cultured for 2 weeks in R2A broth. OD620 values for 100 μL culture were then determined. MP: MP treatment, BAC: BAC treatment, MP+BAC: MP and BAC mixture treatment. Bar: median (*n* = 6 or 7), **P* < 0.05 (Steel-Dwass test; compared among 8 groups).
In order to check bacterial survival in each culture, 10-μL aliquots were spread onto R2A agar plates and incubated for 2 weeks. Colony growth observed in the sample was judged as 'bacterial regrowth-positive'. Probability of bacterial survival was not significantly different among the experimental groups (Fisher's exact test; when compared among 8 groups).

Disinfection test using naturally formed biofilm

Optical density was significantly lower in R2A cultures of BAC and MP+BAC treated groups (10˚C) when compared with controls (10˚C) (Fig. 2; $P < 0.05$; Steel-Dwass test). However, as shown in Fig. 2 and Table 1, bacterial colonies were regrown from all R2A cultures plated onto agar plates, except for the 3 samples treated with MP+BAC at 10˚C. Identification results for colonies grown on R2A agar are also summarized in Table 1.

Table 1 Surviving bacteria isolated from disinfected samples

Temp.	Disinfectant	Colony color	Positive samples/total	Bacterial identification results
10˚C	DW (control)	yellowish white	7/7	*Stenotrophomonas multophilia*
	MP	yellowish white pink black	6/6 2/6 1/6	*Stenotrophomonas multophilia* *Methylobacterium popli* (fungi)
	BAC	pink, red	7/7	*Methylobacterium aquaticum, M. popli*
	MP+BAC	pink, red	4/7	*Brevibacilllus agri, M. popli, M. aquaticum, Methylobacterium branchiatum*
25˚C	DW (control)	white yellow red	4/6 5/6 5/6	*Bradyrhizobium japonicum, Bradyrhizobium* sp. *Sphingomonas panni, Sphingomonas paucimobilis* *Brevibacillus agri*
	MP	pink, red black	6/6 1/6	*Brevibacilllus agri, M. popli* (fungi)
	BAC	pink, red	6/6	*M. aquaticum, M. branchiatum, Brevibacilllus agri*
	MP+BAC	pink	6/6	*M. branchiatum, Brevibacilllus agri*

Discussion

According to ADA recommendations, dental units newly installed in clinics will be equipped with a separate water supply system and filter for bacterial removal, which may reduce waterline contamination. However, as the tubes inside the units cannot be dried after the end of daily use, it is impossible to prevent biofilm formation in the waterline. Porteous et al. reported that in a basic experiment using a tube made of *N*-halamine (novel biofilm-controlling material) and ultrapure water, biofilm formation could not be completely prevented [9].

Therefore, disinfection/washing methods for DUWL should be further investigated and the old-type dental units with biofilm already formed require particular attention. A serious problem for disinfection of old dental units is the possible damaging of parts of during treatment. Various metals with different chemical resistances may be present in such dental units.

In this study, to obtain information on the corrosion effects of disinfectants, a simple immersion test using brass disks was performed. An immersion period of 24 h can be converted into a total of 288 5-min units. If disinfectant treatment is repeated once a week, this would be equivalent to a maintenance period of 5.3 years. Based on this calculation, the low corrosion-inducing concentration of the test disinfectant, MP, was determined to be 1.7% (5 g/300 mL). MP is a vacuum disinfectant containing sodium percarbonate and the original working solution is prepared by dissolving 30 g of powder in 300 mL of water (10%). However, the disk immersion test suggested that MP at a concentration of 3.3% (10 g/300 mL) or more is corrosive for brass, an alloy frequently used in the connections of tubing systems. As another disinfectant, BAC, was also nominated for waterline disinfection because of its relatively strong growth suppressive effects against *Methylobacterium*, a disinfection-resistant heterotrophic bacteria [10]. BAC (0.1%) did not reduce, but tended to increase, the weight of brass disks, suggesting a possible separation of the chemical.

For the disinfection experiment, naturally formed biofilm within polyurethane tubes was used and the results showed that natural biofilm, even within the same DUWL, may not be uniform (Table 1). Optical density of the first culture from treated samples in R2A did not reflect bacterial survival, but only the growth rate of survivors. For this reason, re-cultivation from R2A culture onto R2A agar plates was necessary to evaluate the bactericidal effect of disinfectants. Interestingly, even in the water-treated control samples, remaining bacterial species differed between 10˚C and 25˚C. The reason for these results cannot be explained; however, washing-out efficiency may influence the remained bacterial species.

The effects of MP were strongly influenced by reaction temperature. At a low temperature (10˚C), a rapid growing bacterium, *S. maltophilia* was isolated from all MP-treated fragments, as well as from the water-treated controls, while predominant surviving bacteria from BAC and MP+BAC were *Methylobacterium* and *Bravibacillus agri*. At 25˚C, the main surviving bacteria were within the genus of *Methylobacterium*, followed by *B. agri* in all disinfection groups, while the various bacteria including *B. agri*, *Sphingomonas* and *Bradyrhizobium* grew from the control fragments. According to the results, when MP is solely used, reaction temperatures should be considered carefully, in addition to reagents containing similar compounds.

In contrast, MP+BAC treatment at 10˚C showed the strongest bactericidal effects among all experimental conditions. Three of seven samples treated with the mixture were free of bacterial regrowth. At 25˚C, however, bacterial survival was cultivated from all 6 samples treated by the same disinfectant. This suggests the poor stability of the mixed disinfectant.

Unfortunately, when used solely, both of MP and BAC incompletely kill *Methylobacterium* within 5 min, thus suggesting that cycling disinfection using these agents is a poor countermeasure against waterline biofilm. Species in *Methylobacterium*, the opportunistic pathogens frequently isolated from waterline and producing the antioxidant red pigments, are known to be resistant to many disinfectants [11]. Although disinfection may induce substitution of bacterial components in biofilm to that rich in *Methylobacterium*, stronger pathogens such as *Legionella* and rapidly growing opportunistic pathogens such as *S. maltophilia* should be reduced by chemical treatment. Therefore, for maximal benefit in clinical practice, further investigation to optimize usage conditions of MP+BAC or other disinfectants is essential.

Also, a simple test for brass corrosion and a natural biofilm disinfection model shown here will contribute to search for optimal disinfection conditions for disinfecting DUWL contaminated with previously formed biofilms.

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Conflict of interest
Morita Corporation, a provider of some materials used in this study shown above, did not participate the experiments and their interpretation, and writing of this study.

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