Sodium Hypochlorite is Effective against Biofilms in Dialysis Equipment

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To test the efficacy of chemical disinfectants against bacterial biofilms in hemodialysis equipment, a Center for Disease Control and Prevention (CDC)-Biofilm Reactor was used to create biofilms. Methylobacterium radiotolerance was isolated from the hemodialysis fluid and used as the test organism. We examined the efficacy of sodium hypochlorite (NaOCl) in elimination of planktonic cells compared to that in the case of biofilms. Planktonic bacteria were completely eliminated at 50 parts per million (ppm) of NaOCl, which is the lowest concentration for clinical use. The viable cell count in the biofilm reached its minimum value around a logarithmic reduction value (LRV) of 6, when the concentration was raised to 1000 ppm and the reaction time was extended by 1 hour or more. Furthermore, at 200 ppm, the LRV was elevated depending on the time. And the LRV while maintaining static conditions for 6 hours at 200 ppm was similar to that of short time at 1000 ppm. These results suggest that NaOCl has sufficient bactericidal activity even for biofilms at a practical concentration and reaction time, and that the CDC-Biofilm Reactor is an effective tool for finding useful disinfection conditions.

Key words: Methylobacterium spp. / Center for Disease Control and Prevention-Biofilm Reactor / highly controlled medical equipment.

INTRODUCTION

Disinfection is essential for the microbial control in hemodialysis systems (ISO 2019, Kawanishi et al. 2009). Sodium hypochlorite (NaOCl) is highly useful for disinfection (Fukusaki, 2006), and is the only approved germicidal disinfectant in Japan listed in the manufacturer's instruction manual for hemodialysis equipment. This is based on the “Test Micro-organism Suspension Method” (TMSM) in appendix G4 of the Japanese Pharmacopoeia 17th edition (HLW 2016), in which it was shown that NaOCl used at clinical concentrations eradicated spore-forming bacteria. However, the effect of NaOCl is controversial, as it is widely known that not all bacteria are eliminated under the conditions recommended in the manual, as proven by evaluation of the bioburden upstream of endotoxin retentive filters (figure 1, right open arrow). One of the underlying reasons is that the use of NaOCl is recommended at relatively low concentrations to prevent the equipment from rusting. Another reason is that the dialysis equipment requires a complicated structure to maintain consistent volume and adequate concentration of dialysis fluid, and flexible silicon tubing is used for hang-over piping, dead-legs, or parallel roots without continuous flow (figure 1). Even if multiple species of bacteria had been isolated from each equipment at initial installation, over the years only...
Methylobacterium spp. has been occasionally isolated as a potential source of endotoxin. This phenomenon has been indicative of compliance with adequate disinfection methods and clean operation to prevent new contamination (Osono et al., 2017a). The spectrum anti-microbial activity of NaOCl may be involved in this selection (Penau et al., 2007).

Here, we examined the practical effects of the use of NaOCl against biofilms in commonly used conditions in hemodialysis facilities. For this, biofilms were created with a clinical strain of *Methylobacterium radiotolerance* isolated from the dialysis fluid, using a Center for Disease Control and Prevention (CDC)-Biofilm Reactor adopted by the United States Environmental Protection Agency (EPA) as a drug evaluation method for biofilms on non-porous hard surfaces (EPA 2017).

**MATERIALS AND METHODS**

**Culture preparation:** *Methylobacterium radiotolerance* clinical strain KOC205 was isolated from hemodialysis fluid. The scarlet-colored colonies were grown on R2A media (Merck, Darmstadt, Germany) and aseptically stored at -80°C. This strain was used in each experiment and cultured in 30 g/L of Tryptic Soy broth (TSB, Becton Dickinson, Heidelberg, Germany) at 24 °C for 3 days on a shaker.

**Preparation of CDC-Biofilm reactor:** Biofilms of *M. radiotolerance* were grown on sterile silicon coupons in a CDC-Biofilm reactor (Model CBR90-1, Bio Surface Technologies Corporation, Bozeman, MT, USA) according to the manuals of the American Society for Testing and Materials (ASTM) international E2562-2017 with partial modifications. The isolated bacteria were grown in 3 g/L of TSB to at least 3 McFarland at 24 °C for 96
h using shear stress provided by baffle rotation at 125 revolutions per minute (rpm). Then, 300 mg/L of TSB were poured at 9.5 to 11.7 mL/min with draining for 24 h in a continuous stirred tank reactor (CSTR) to prevent caramelization.

**Reagents for analysis of reaction against biofilm:** The experiment was performed using NaOCl (6% Purelox, Ohyalox, Tokyo, Japan). NaOCl was diluted in sterile water for injection (WFI, Otsuka Pharmaceutical Factory, Naruto, Japan) to 50, 200, and 1000 parts per million (ppm) (1:1200 to 1:60), which was used clinically as per description in the instruction manual of hemodialysis equipment. As outlined in the ASTM E2871-2019, 1:100 phosphate-buffered saline with 1 m mol/L magnesium was used for washing and 11.7 g/L of sodium thiosulfate was used to dilute NaOCl instead of WFI. The pH of the reaction reagent was measured using an Aqua two pH meter S010 (Horiba, Kyoto, Japan). To clarify the influence of pH, washing buffer and 0.1 m mol/L sodium hydroxide solution were used to dilute NaOCl instead of WFI. The pH of the reaction reagent was measured using an Aqua two pH meter S010 (Horiba, Kyoto, Japan). As the number of viable bacteria in the biofilm and the resistance to the drug varied from experiment to experiment, each experimental condition was repeated at least 3 times and represented in the figures.

**Evaluation of chemical disinfectant efficacy:** After the CSTR, biofilm-covered coupons were rinsed in washing buffer to remove planktonic bacteria and placed in 50-mL conical tubes containing 4 mL of the diluted disinfectant or WFI for control. The reaction time was 10 minutes (0.17 hour), and from 0.5 to 6 h. The shortest was specified by EPA 2017, others were the actual time for the equipment disinfection from a single passage to subsequent retention. At the end of the reaction, the diluted disinfectant was decanted out gently and 20 mL of the neutralizing solution was added to equilibrium for 10 min. To remove and break up the biofilm from the coupon, conical tubes were mixed with a vortex mixer for 30 seconds following sonication in an ultrasonic water bath at 45 kHz for 30 seconds. This step was repeated three times. This sample solution obtained was serially diluted in washing buffer, spread onto R2A media, and cultured for 7 days at 24 °C. When the number of viable cells was expected to be low, the sample was filtered from 1 mL to the total volume through a 37-mm, 0.45-μm membrane filter (Pall, Port Washington, NY, USA). The filters were then washed with washing buffer and placed onto R2A media. For the test microorganism suspension method (TMSM) described in the 17th JP, the efficacy of the disinfectants against planktonic bacteria was observed using solutions in the reaction tank. A 9-fold volume of disinfectant solution was added, and after a 0.18 h reaction, a 9-fold volume of neutralization solution was added to stop the reaction.

**Data analysis:** Based on the concentration of the disinfectants and the reaction time, the experimental conditions were obtained from the average of three independent coupons. The logarithmic reduction value (LRV) was determined from the ratio of the number of viable cells determined in the control (using WFI instead of reacting reagent), and the number of viable cells recovered in each test condition. When no colonies were formed, the highest concentration in the dilution series or the largest sample volume of the membrane filter method was assumed to be 0.9 colony-forming units (CFU) for calculation.

**RESULTS**

After 15 experiments, 7.45 ± 0.38 (7.00-8.08) log CFU/mL of *M. radiotolerance* were found in the solution at the end of the CSTR. The number of viable bacteria in the biofilm was 7.93 ± 0.25 (7.52-8.29) log CFU per coupon, and 7.34 ± 0.23 (7.00-7.68) log CFU/cm² under control conditions (figure 2). A weak correlation was observed between the number of planktonic cells and biofilms. As the minimum concentration of NaOCl in each experiment used for 0.17 h eradicated the planktonic
bacteria completely, the LRV variation depended on the difference in the viable cell count at each experiment (figure 3, left). However, the LRV for the biofilm on the coupon remained 4.36 even after using 1000 ppm NaOCl for 0.17 h. When the reaction time was set to 0.5 h, a concentration-dependent enhancement was observed: 1.44 (0.99-1.94) log at 50 ppm, 2.63 (1.92-3.20) log at 200 ppm, and 5.16 (4.77-5.52) log at 1000 ppm (figure 3, right). The LRV was 2 or more in the reaction with 200 ppm NaOCl for 0.17 h, and then increased with the extension of time to 3.61 log in 1 h and further improved to 4.26 log in 6 h (figure 4). On the other hand, at 1000 ppm, the LRV was 4 or more at 0.17 h, and when extended to 0.5 h, the LRV improved by 1 log. However, it was around LRV 6 even after extending to 1, 3, and 6 hours. When the pH was adjusted to enhance cleaning activity, the bactericidal property tended to decrease at high concentrations for a relatively short reaction time, but there was no difference under other conditions (figure 5).

**DISCUSSION**

NaOCl showed, indeed, limited efficacy against biofilms formed by *M. radiotolerance* clinical strains isolated from dialysis fluid, but showed complete elimination of planktonic cells even under the weakest conditions (figure 2). In general, disinfectant effectiveness depends on concentration and reaction time. NaOCl biotoxicity was observed to be concentration- and time-dependent under the clinical conditions used for the hemodialysis system. If 200 ppm of NaOCl was retained under static conditions for 6 h, an effect equivalent to 1000 ppm in a short time was obtained, achieving an acceptable level of disinfection against biofilms with LRV 3, which is the standard of sanitizer in European Normal (2005), or 99.9% in EPA 2017. When alkaliized for the purpose of improving the cleaning action (Fukusaki, 2006), it also satisfied the standard as a sanitizer. NaOCl does not penetrate deep into the biofilm at the concentration used for waterworks management (Lee et al., 2011); however, the permeability at relatively high concentrations for clinical use is not clear. At 1000 ppm, the LRV did not increase over time and reached the plateau phase at approximately 6, which did not eradicate as planktonic cells. According to the results of TMSM, the conditions of short-time single pass at 1000 ppm or under static conditions for overnight at 50-300 ppm described in the instruction manual, are not for cleanliness but to avoid rust and deterioration of
**Figure 4.** Time-dependent activity of NaOCl.
The left panel shows NaOCl at 200 ppm and the right panel at 1000 ppm after 1 h or more, assuming static conditions.

**Figure 5.** Effect of pH on the activity of NaOCl.
The left panel shows NaOCl at 200 ppm and the right panel at 1000 ppm. WB is diluted with washing buffer, DW is WFI, and OH is 0.1 mM (0.1 mN) sodium hydroxide solution. The average pH of the solution was determined three times.
the equipment parts. The CDC-Biofilm Reactor forms a biofilm under high shear stress, and has been reported to have higher resistance than other biofilm evaluation methods (Buckingham-Meyer et al., 2007). By simulating the conditions used in the CDC-Biofilm Reactor, it would become possible to show the most clinically useful cleaning and disinfecting method.

There are several special factors to be kept in mind when considering the disinfection of dialysis equipment. First, *Methylobacterium* spp. used in this study survive for several months under conditions of poor nutritional medium, such as tap water (Garrity et al., 2005), and can be found in shower heads (Feazel et al., 2009), bathtubs (Yano et al., 2013), and toilet bowls (Mori et al., 2014), causing pink biofilms. As this species has versatile resistance mechanisms, it can also be isolated from water-treated systems (Hiraishi et al., 1995). In the case of dialysis equipment, 2-6 bacterial strains were confirmed at the time of installation, and *Methylobacterium* spp. was not always the most abundant (Osono et al., 2017a). After that, NaOCl was used for cleaning and disinfection after daily use to prevent secondary contamination. *Methylobacterium* spp. was occasionally detected from the bioburden when evaluated as a performance qualification. Since a single strain with the same genotype was observed in the same equipment for years, it is presumed that the biofilm inhabits the equipment. As the bacteria left behind after disinfection acquired resistance (Penau et al., 2007), NaOCl was considered to be involved in the selection of this clinical strain. Second, this equipment cannot be dried up after washing because the piping structure remains the pivotal. It was considered that the data obtained by the CDC-Biofilm Reactor could be useful for the selection of cleaning and disinfection methods when conducting user validation for each facility and situation. In practice, flush with pure water between replacement of dialysis fluid and disinfectant to ensure disinfection and no disinfectant remains during treatment. It takes 15 minutes to completely replace the solution in the equipment. Assuming the safety margin is doubled before disinfection and tripled before treatment, it is possible to disinfect properly with a single passage with 1000 ppm NaOCl if there is a 3 hour interval before the next treatment. If it can be secured for more than 8 hours and a half, the same effect could be obtained maintaining static conditions at 200 ppm.

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