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Efficacy of ozone on survival and permeability of oral microorganisms

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In the present study, we examined the effect of ozonated water on oral microorganisms and dental plaque. Almost no microorganisms were detected after being treated with ozonated water (4 mg/l) for 10 s. To estimate the ozonated water-treated *Streptococcus mutans,* bacterial cells were stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. Fluorescence microscopic analysis revealed that *S. mutans* cells were killed instantaneously in ozonated water. Some breakage of ozonated water-treated *S. mutans* was found by electron microscopy. When the experimental dental plaque was exposed to ozonated water, the number of viable *S. mutans* remarkably decreased. Ozonated water strongly inhibited the accumulation of experimental dental plaque *in vitro*. After the dental plaque samples from human subjects were exposed to ozonated water *in vitro*, almost no viable bacterial cells were detected. These results suggest that ozonated water should be useful in reducing the infections caused by oral microorganisms in dental plaque.

Key words: antimicrobial activity; dental plaque biofilm; disinfectant; oral microorganisms; ozone

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In the formation of dental plaque, oral microorganisms must adhere to the tooth surface and then grow in the prevailing environment. It has been recognized that dental plaque is a bacterial biofilm (15, 22). In the dental plaque biofilm, bacterial growth is the primary factor that governs the relative abundance of different bacteria. Many investigators have recognized that mutans streptococci are cariogenic bacteria in the supragingival plaque. Furthermore, it is generally accepted that the unique physiological conditions in mature dental plaque biofilm might influence the growth of subgingival plaque. The development of periodontal disease has been thought to be associated with several restricted members of oral anaerobic species such as black-pigmented *Porphyromonas* species and *Actinobacillus actinomycetemcomitans* in the subgingival plaque (16, 23).

There are many advantages of using ozone as a potent oxidizing agent in food and other industries. It is potentially useful for decreasing the microbial load and the level of toxic organic compounds. Its high oxidizing power and spontaneous decomposition make ozone useful for ensuring the microbiological safety and quality of food (13). Therefore, the use of ozone in the food industry has been investigated with regard to food preservation, shelf-life extension, equipment sterilization, and improvement of food plant effluent (12, 21). In addition, ozonation has been used as a method for treating water in Europe. In France, several municipal drinking water facilities have used ozone as the primary disinfectant since 1906 (14).

Although ozonated water is a powerful antimicrobial agent against bacteria, fungi, protozoa, and viruses, as mentioned above, less attention has been paid to the antibacterial activity of ozonated water in bacterial biofilms. In the present study, we examined the effect of ozonated water on the viability of oral microorganisms such as oral streptococci, endodontopathic or periodontopathic bacteria, and oral fungi. Furthermore, we investigated the effect of ozonated water on plaque biofilms and their formation using experimental dental plaque.

Materials and methods

Growth conditions for microorganisms

Streptococcus salivarius IFO 13956 and *Streptococcus sanguis* ATCC 10506 were cultured in brain heart infusion (BHI) broth (Difco, Detroit, MI) at 37°C for 18 h. *S. mutans* Ingbritt and *Streptococcus sobrinus* AHT-k were cultured in BHI

broth at 37°C for 18 h in an atmosphere of 5% CO_2 in air. *A. actinomycetemcomitans* Y4 was cultured in BHI broth supplemented with 1% (w/v) yeast extract at 37°C for 48 h in an atmosphere of 5% CO_2 in air. *Porphyromonas gingivalis* 381 was cultured in general anaerobic medium broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 24 h under anaerobic conditions, using AnaeroPack® (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). *Porphyromonas endodontalis* ATCC 35406 was cultured in trypticase soy broth (BBL, Becton Dickinson, Cockeysville, MD) supplemented with hemin (500 μg/ml), 2-methyl-1, 4-naphthoquinone (100 μg/ml), and 1% (w/v) yeast extract at 37°C for 48 h under anaerobic conditions. *Candida albicans* ATCC 18804 was cultured in yeast-mold broth (Difco) supplemented with glucose (100 μg/ml) at 25°C for 18 h.

Antimicrobial activity of ozonated water

Microorganisms were harvested by centrifugation at 10,000 × *g* for 5 min, suspended in saline and adjusted to 1×10^6 cells/ml. After centrifugation to remove saline, the microorganisms were individually exposed to 0.5, 2, and 4 mg/l of ozonated water (Neo Ozone Water-S; KORM Electronics, Atsugi, Japan) for 10, 30, 60, or 120 s. The concentration of ozonated water in the aqueous phase was determined by Portable Ozone Monitor (OM-101P; Applics Co., Ltd., Tokyo, Japan). The suspension of microorganisms (10 μl; 1×10^4 cells) was then cultured on a plate containing an appropriate culture medium. As a preliminary experiment, we confirmed that the antimicrobial activity of ozonated water was reduced when added dropwise on the culture plate. The inoculated plates were incubated at 37°C for 24–72 h depending on the growth rate of the test strains. *P. gingivalis* and *P. endodontalis* were cultured on agar plate media with 10% (v/v) horse whole blood at 37°C for 7 days under anaerobic conditions. Colony-forming units (CFU) are counted by the spread plate method. In some experiments, *Streptococcus mutans* and *C. albicans* were exposed to ozonated water (4 mg/l), povidone iodine (2.3 mg/ml) or benzethonium chloride (40 μg/ml), and the antimicrobial activities of these disinfectants were examined as described above.

Fluorescence microscopic observation

After *S. mutans* was exposed to ozonated water (4 mg/l) for 120 s, the ozone was inactivated by bovine serum albumin (BSA; 5 mg/ml; Sigma Chemical Co., St. Louis, MO). As a preliminary experiment, we confirmed that the antimicrobial activity of ozone was immediately inactivated by addition of BSA. The bacterial cells were harvested by centrifugation at 10,000 × *g* for 5 min and stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit solution (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The bacterial cells were observed with a fluorescence microscope (BX-50; Olympus Optical Co., Ltd., Tokyo, Japan). The excitation/emission wavelengths of the dyes in this kit solution were used at 480/530 nm for SYTO 9 and 520/580 nm for propidium iodide. It has been reported that both viable and nonviable bacterial cells stain fluorescent green, while nonviable bacterial cells stain fluorescent red (3, 9).

Electron microscopic observation

After the exposure to ozonated water (4 mg/l) for 120 s and inactivation by BSA, *S. mutans* was harvested by centrifugation at 10,000 × *g* for 5 min and fixed with 2.5% (w/v) glutaraldehyde in phosphate-buffered saline (PBS; pH 7.2). The fixed cells were washed with PBS and dehydrated in a 70–99.9% (v/v) ethanol-PBS gradient, and lyophilized. The lyophilized cells were coated with gold by ion sputter (E-1030; Hitachi Co., Ltd., Tokyo, Japan), and observed with a scanning electron microscope (S-4300; Hitachi Co.).

Antimicrobial effect of ozonated water on experimental dental plaque

The decalcified human tooth was cultured with *S. mutans* in BHI broth supplemented with 10% (w/v) sucrose at 37°C for 72 h in an atmosphere of 5% CO_2 in air. After the exposure to ozonated water (4 mg/l) for 120 s, the ozone was inactivated by BSA. Experimental dental plaque was also exposed to povidone iodine (2.3 mg/ml). The decalcified tooth with experimental dental plaque was embedded in the tissue freezing medium™ (Leica Instruments, Nussloch, Germany) and frozen immediately. The frozen samples were sectioned in 20-μm-thick sections using a rapid sectioning cryostat CM 1900 (Leica). The thickness of experimental dental plaque was confirmed by modified gram stain (5). The sections were also stained with Via-Gram™ Red$^+$ Bacterial Gram Stain and Viability Kit solution (Molecular Probes) according to the manufacturer's instructions and were observed with a fluorescence microscope. The excitation/emission wavelengths of the dyes were used at 358/461 nm for 4′, 6-diamino-2-phenylindole (DAPI) and 504/523 nm for SYTOX green. It has been reported that viable and nonviable bacterial cells stain fluorescent blue and green, respectively (18, 19).

Effect of ozonated water on the formation of experimental dental plaque

The decalcified human tooth was cultured with *S. mutans* in BHI broth supplemented with 10% sucrose for 24 h. After the tooth with experimental dental plaque was exposed to ozonated water (4 mg/l) or povidone iodine (2.3 mg/ml) for 120 s, the tooth was cultured with *S. mutans* for 24 h. The same procedure was repeated, and then the decalcified tooth was embedded, sectioned, and stained with modified gram stain (5).

Antimicrobial effect of ozonated water on dental plaque collected from human subjects

The experimental procedure was explained to the subjects and their informed consent was obtained prior to the investigation. The plaque samples were collected from the tooth with no dental caries. One mg of dental plaque sample was collected from each subject and exposed to 30 ml of ozonated water (4 mg/l) for 120 s, and the ozone was inactivated by BSA. After the samples were dispersed by ultrasonication for 30 s, the plaque suspensions (50 μl) were cultured on the plate of Mitis-Salivarius agar medium (Difco) supplemented with 15% (w/v) sucrose at 37°C for 48 h in an atmosphere of 5% CO_2 in air. The cell viability of *S. mutans* was examined by the CFU counting as described above.

Results

Antimicrobial activity of ozonated water

To examine antimicrobial activity, oral microorganisms were exposed to several concentrations of ozonated water. As shown in Fig. 1A, the cell viability of *S. mutans* decreased to 58% after exposure to 0.5 mg/l of ozonated water for 10 s, and *S. mutans* was killed instantaneously in ozonated water (2 and 4 mg/l). The cell viabilities of *S. sobrinus, S. sanguis and S. salivarius* were very similar to that of *S. mutans* when the cells were exposed to ozonated water (Fig. 1B,C,D).

Next, we examined the effect of ozonated water on the cell viabilities of gram-

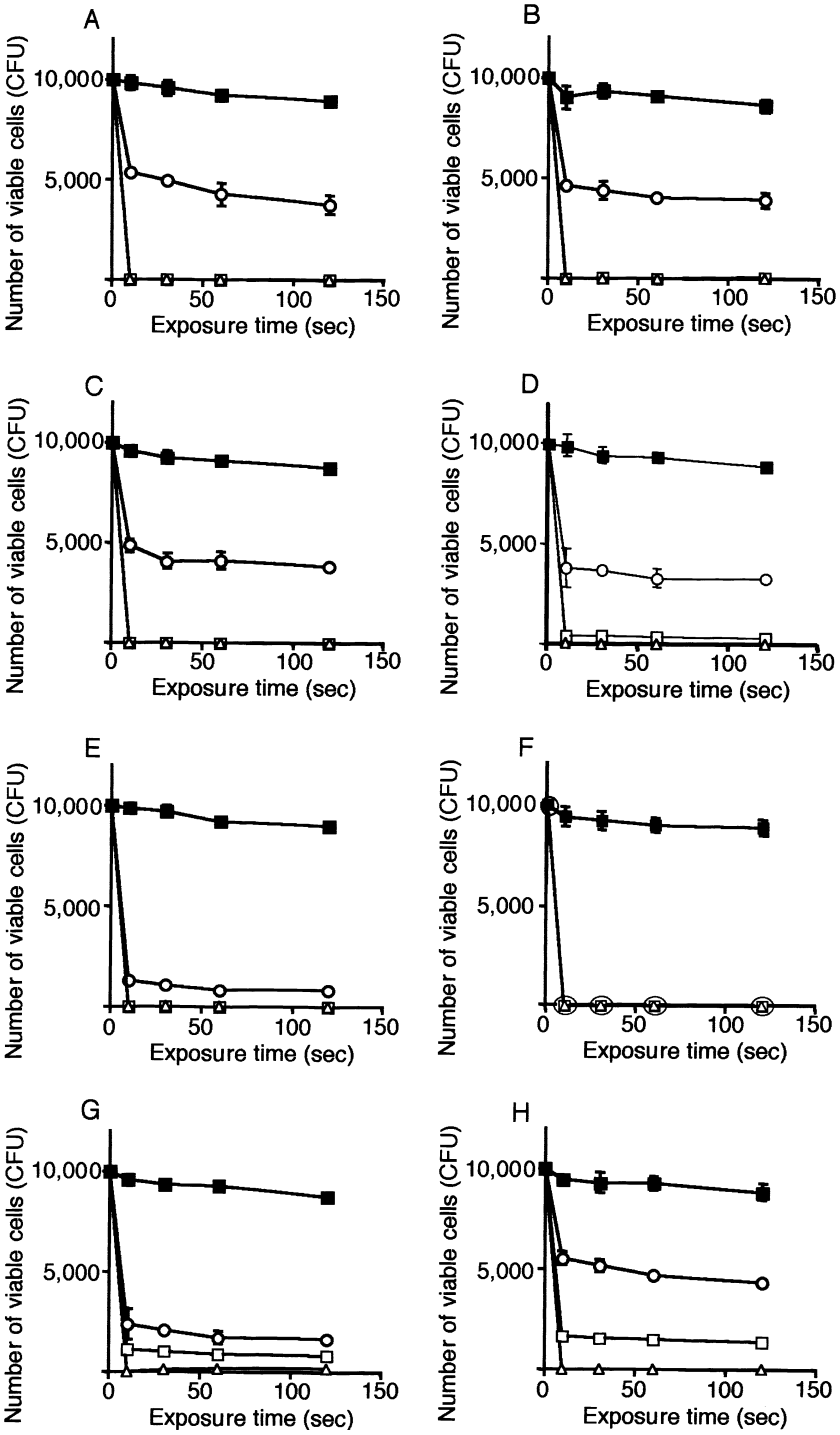

Fig. 1. Antimicrobial efficacy of ozonated water against oral microorganisms. *S. mutans* (A), *S. sobrinus* (B), *S. sanguis* (C), *S. salivarius* (D), *P. gingivalis* (E), *P. endodontalis* (F), *A. actinomycetemcomitans* (G) and *C. albicans* (H) were exposed to ozonated water (open circle, 0.5 mg/l; open square, 2 mg/l; open triangle, 4 mg/l). These strains were also exposed to culture medium (closed square) for 10, 30, 60, or 120 s. The number of viable cells was counted as described in Materials and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. The experiment was performed three times and similar results were obtained in each experiment.

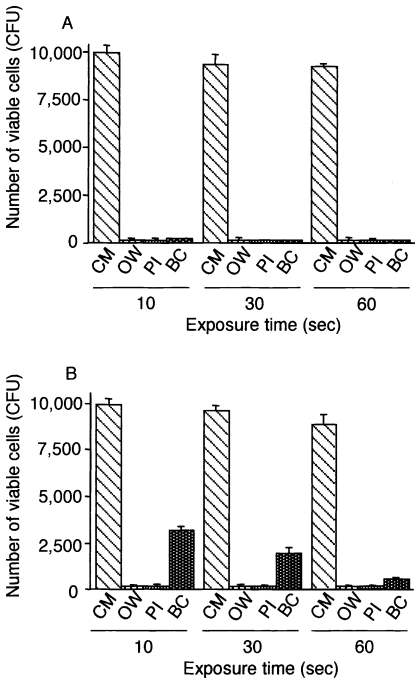

Fig. 2. Antimicrobial activity of ozonated water and commercially available disinfectants. *S. mutans* (A) and *C. albicans* (B) were exposed to ozonated water (OW; 4 mg/l), povidone iodine (PI; 2.3 mg/ml) or benzethonium chloride (BC; 40 μg/ml) for 10, 30, or 60 s. The number of viable cells was counted as described in Materials and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. The experiment was performed three times and similar results were obtained in each experiment. CM, culture medium.

negative oral bacteria such as *P. gingivalis, P. endodontalis*, and *A. actinomycetemcomitans*. The number of viable cells was significantly decreased when *P. gingivalis, P. endodontalis*, and *A. actinomycetemcomitans* were treated with ozonated water (0.5, 2, and 4 mg/l) (Fig. 1E,F,G). Although *P. gingivalis* and *P. endodontalis* were cultured on a blood agar plate under anaerobic conditions and *A. actinomycetemcomitans* was cultured on a brain heart infusion agar plate in an atmosphere of 5% CO_2 in air, the cell viabilities of these bacteria slightly decreased even when the cells were treated with distilled water (data not shown). As shown in Fig. 1H, ozonated water was dose-dependently toxic to *C. albicans*. However, *C. albicans* was not completely killed in 2 mg/l of ozonated water for 120 s.

To compare the antimicrobial activity of ozonated water and commercially available disinfectants, povidone iodine and benzethonium chloride were tested in this study. When *S. mutans* was exposed to ozonated water (4 mg/l), povidone iodine (2.3 mg/ml), or benzethonium chloride (40 μg/ml) for 10, 30, or 60 s, no viable cells were detected (Fig. 2A). Although ozonated water and povidone iodine each had a remarkable antimicrobial effect on *C. albicans*, a few thousand viable cells

were still detected when *C. albicans* was treated with benzethonium chloride for 10, 30, or 60 s (Fig. 2B).

Effect of storage temperature on the concentration and bactericidal activity of ozonated water

It is well known that the concentration of ozonated water decreases with time. We examined the effect of storage temperature and time on the concentration and bactericidal activity of ozonated water against *S. mutans*. When ozonated water was maintained at 22°C for 180 min, the concentration and bactericidal activity decreased remarkably with time (Fig. 3A). When ozonated water (4 mg/l; 22°C) was stored on ice, bactericidal activity was maintained for 180 min. In addition, the concentration of ozone was reduced to one-half its initial value when ozonated water was stored on ice for 120 min (Fig. 3B).

Microscopic examination of *S. mutans* exposed to ozonated water

To detect total counts of *S. mutans* exposed to ozonated water, we stained bacterial cells with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit. As shown in Fig. 4, fluorescence microscopic analysis revealed that almost all *S. mutans* cells were killed by ozonated water (4 mg/l). On the other hand, only a few dead *S. mutans* cells were detected when treated with culture medium or distilled water. The morphologic change in *S. mutans* exposed to ozonated water was examined by scanning electron microscopy. Treatment of medium or distilled water had no effect on the morphology of *S. mutans*. The disruption of cells were found when *S. mutans* was treated with ozonated water (4 mg/l) (Fig. 5).

Microscopic examination of experimental dental plaque

To detect the *in vitro* antimicrobial effect of ozonated water on experimental dental plaque, we examined the viability of bacterial cells in the plaque by ViaGram™ Red+ Bacterial Gram Stain and Viability Kit. After the exposure to distilled water for 120 s, *S. mutans* cells were mostly alive in experimental dental plaque (Fig. 6A). On the other hand, the treatment with ozonated water (4 mg/l) for 120 s reduced remarkably the number of viable

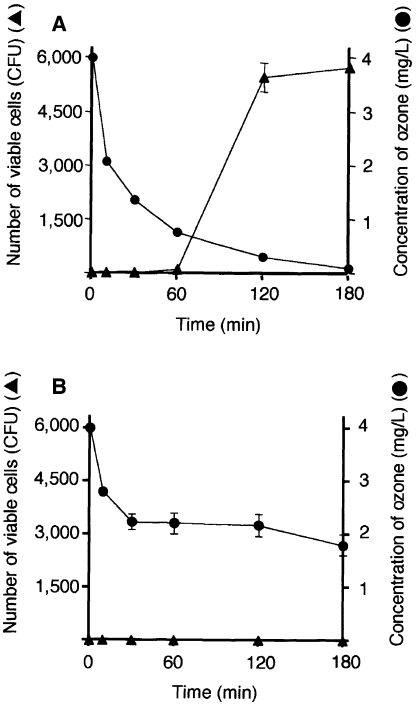

Fig. 3. Effect of storage temperature on the bactericidal activity of ozonated water. The temperature of ozonated water was maintained at 22°C (A) for 10, 30, 60, 120, or 180 min, and its concentration and bactericidal activity were then determined. On the other hand, ozonated water (22°C) was stored on ice for 10, 30, 60, 120, or 180 min (B). After storage at 4°C or 22°C, the concentration of ozone was determined by the dissolved ozone monitor (model OM-101P-20; Silver Reed Co., Ltd., Tokyo. Japan). *S. mutans* was then exposed to ozonated water for 120 s, the number of viable cells counted and the percent toxicity determined as described in Materials and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. The experiment was performed three times and similar results were obtained in each experiment.

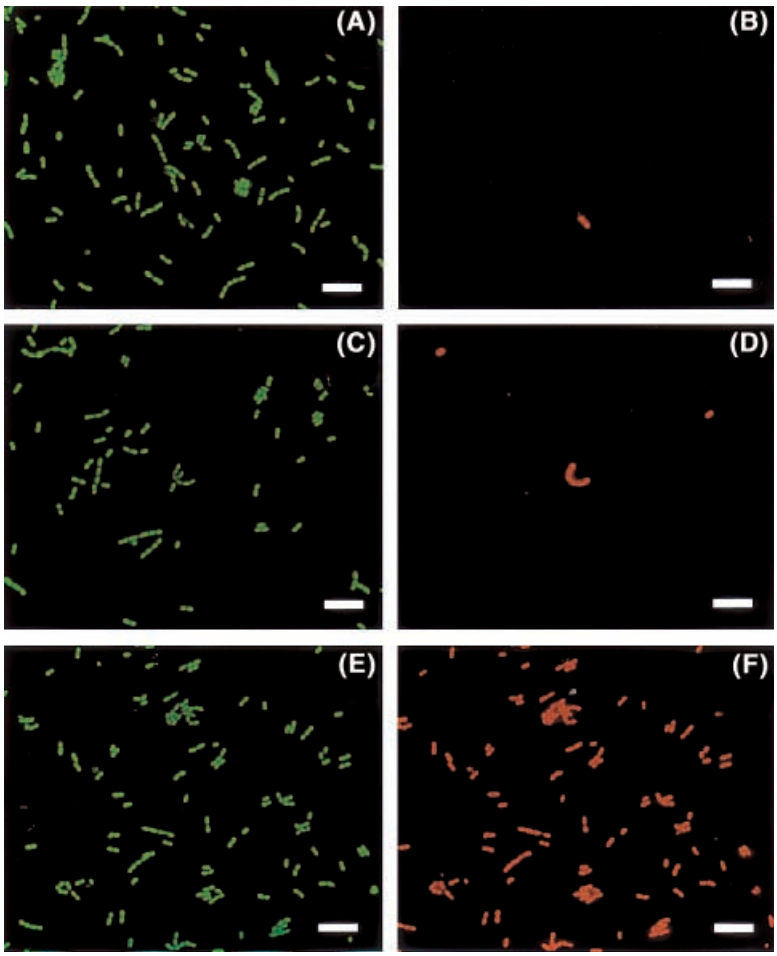

Fig. 4. Fluorescence microscopic observation of *S. mutans* exposed to ozonated water. After exposure to ozonated water (4 mg/l) for 120 s, bacterial cells were stained with LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit solution and observed with a fluorescence microscope (BX-50; Olympus Optical Co., Ltd., Tokyo, Japan). After *S. mutans* was exposed with medium (A, B), distilled water (C, D) or ozonated water (E, F), both viable and nonviable cells exhibited fluorescent green while nonviable cells exhibited fluorescent red. Scale bar = 5 μm.

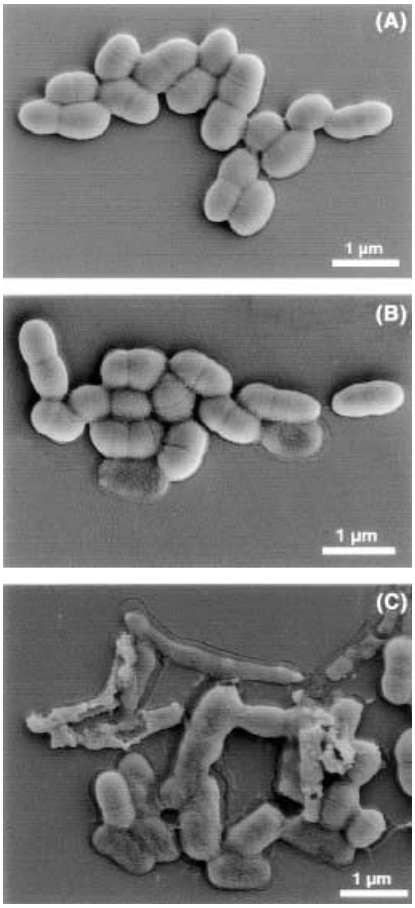

Fig. 5. Scanning electron microscopic observation of *S. mutans* exposed to ozonated water. *S. mutans* was exposed with medium (A), distilled water (B) or ozonated water (4 mg/l) (C) for 120 s. Scale bar = 1 μm.

S. mutans in experimental dental plaque (Fig. 6B). Treatment with povidone iodine (2.3 mg/ml) had almost same effect on experimental dental plaque (Fig. 6C).

Effect of ozonated water on the formation of experimental dental plaque

We examined the effect of ozonated water on the formation of dental plaque on the decalcified human tooth. As shown in Fig. 7A,B,C, treatment of ozonated water (4 mg/l) or povidone iodine (2.3 mg/ml) inhibited strongly the formation of dental plaque biofilm on the decalcified human tooth *in vitro*.

Effect of ozonated water against the bacteria in human dental plaque biofilm

To determine the antimicrobial activity against the bacteria in human plaque biofilm, dental plaque samples from human subjects were treated with ozonated water as well as povidone iodine and

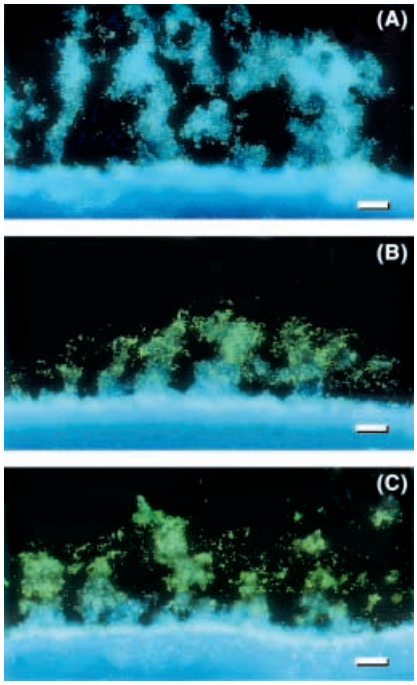

Fig. 6. Microscopic observation of experimental dental plaque exposed to ozonated water and commercially available disinfectants. The decalcified human tooth was cultured with *S. mutans* in BHI broth supplemented with 10% (w/v) sucrose at 37°C for 72 h. After the exposure to distilled water (A), ozonated water (4 mg/l) (B), or povidone iodine (2.3 mg/ml) (C) for 120 s, and the experimental dental plaque was stained by ViaGram^{TM} Red^{+} Bacterial Gram Stain and Viability Kit solution (Molecular Probes). Fluorescence microscopic analysis reveals that viable and nonviable bacterial cells in dental plaque stain fluorescent blue and fluorescent green, respectively. Scale bar = 50 μm.

benzethonium chloride. Not only commercially available disinfectant, povidone iodine, but also ozonated water showed completely antibacterial activity. On the other hand, treatment with benzethonium chloride had less antibacterial activity against *S. mutans* in dental plaque biofilm (Fig. 8).

Discussion

It is well known that ozone, in the gaseous or aqueous phase, can kill bacteria, fungi, and viruses (13). The efficacy of disinfectants is usually evaluated on the basis of a decrease in cultivable microorganisms, as tested in this study (7). The present results showed that ozonated water (0.5–4 mg/l) was highly effective in killing both gram-positive and gram-negative oral microorganisms. Among them, the gram-negative bacteria, such as the endodontopathic bacterium *P. endodontalis* and the periodontopathic bacterium *P. gingivalis*, were

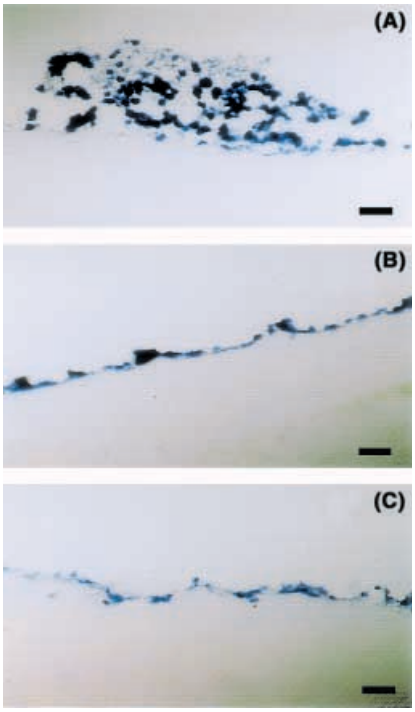

Fig. 7. Effect of ozonated water on the formation of experimental dental plaque. The decalcified human tooth was cultured with *S. mutans* for 24 h. After exposure to distilled water (A), ozonated water (4 mg/l) (B), or povidone iodine (2.3 mg/ml) (C) for 120 s, the sample was cultured with *S. mutans* for 24 h. The same procedure was repeated, and the experimental plaque was stained with modified gram stain. Scale bar = 50 μm.

substantially more sensitive to ozonated water than the gram-positive oral streptococci and *C. albicans* in pure culture (Fig. 1). The ozone sensitivity data suggest

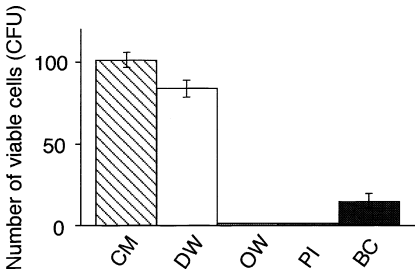

Fig. 8. Antimicrobial activity of ozonated water against dental plaque collected from human subjects. Dental plaque 1 mg was exposed to 30 ml of distilled water (DW), ozonated water (OW; 4 mg/l), povidone iodine (PI; 2.3 mg/ml), or benzethonium chloride (BC; 40 μg/ml) for 120 s. The number of viable *S. mutans* cells in the 10 μl of plaque suspension was counted as described in Materials and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. The experiment was performed three times and similar results were obtained in each experiment. CM, culture medium.

that ozonated water might be especially useful for killing oral infectious microorganisms.

The advantages of ozone in the aqueous phase are its potency, ease of handling, lack of mutagenicity, rapid microbicidal effects, and suitability for use as a soaking solution for medical and dental instruments (17). In the present study, we found that ozonated water had a rapid antimicrobial effect on oral microorganisms in pure culture, and that an ozone concentration of 2–4 mg/l was needed to kill the cells. The results of this study showed that there were no significant differences in microbicidal activity between ozonated water and povidone iodine in pure cultures of *S. mutans* and *C. albicans* (Fig. 1D).

Addy & Wright (1) compared the antimicrobial activities of two antiseptic mouthwashes, 1% povidone iodine and 0.2% chlorhexidine gluconate, and suggested that the lack of prolonged action of povidone iodine in the oral cavity may be related to its reported lack of antiplaque activity. In the present study, we found that ozonated water reduced the live bacteria (Fig. 6B) and the accumulation of experimental dental plaque on the decalcified tooth (Fig. 7B). In addition, there was no significant difference in antimicrobial activity against the bacteria in dental plaque between ozonated water and povidone iodine. These findings suggest that ozonated water with antiplaque activity might be effective as a disinfectant solution for dental instruments and removable dentures.

Membrane permeability is a key element to cell viability, and the changes in permeability involve the loss of several vital processes linked to the cytoplasmic membrane (2, 20). It is generally accepted that oxidation due to ozone induces the destruction of cell walls and cytoplasmic membranes of microorganisms, and that differences in the sensitivity to ozonated water are probably due to differences in the structure of the cell walls of microorganisms (24). After the membrane is damaged by oxidation, the permeability of the membrane increases, and ozone molecules can readily enter the cells (6). It has been thought that the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit allows bacterial cells to be distinguished according to the permeability of the cytoplasmic membrane (11). A scanning electron microscopic analysis revealed some holes in the membrane when *S. mutans* cells were treated with ozonated water. In addition, SYTOX green dye in the Via-Gram™ Red^{+} Bacterial Gram Stain and Viability Kit is used to detect the changes on membrane permeability (18). In the present study, we found antimicrobial activity of ozonated water against the bacteria in experimental dental plaque using SYTOX green dye (Fig. 6B). These findings suggest that the bactericidal activity of ozonated water is through functional and structural disorder in the cytoplasmic membrane.

Although rapid degradation is one of the major environmental advantages of ozonated water, this also produces a rapid decrease in microbicidal activity. It has been reported that the important factors in microbicidal activity are the quantity of ozone transferred to the water, contamination by dissolved organic compounds, temperature, and pH (10). Among these factors, the efficiency of ozone sterilization is particularly dependent on the temperature of the aqueous phase. We therefore examined the effect of the storage temperature of ozonated water on its microbicidal activity. As shown in Fig. 3, both the activity and the concentration of ozone in the aqueous phase decreased faster at 22°C than at 4°C, indicating that the effectiveness of ozone immediately decreases when ozonated water is stored at room temperature. In the present study, we used ozonated water made using Neo Ozone Water-S. It is possible to supply a high dose of ozonated water using this apparatus, suggesting that this apparatus might be useful to maintain continual disinfection.

Bacterial biofilm is defined as a structured community of bacterial cells enclosed in a self-produced polymeric matrix. The microorganisms in biofilms show unique characteristics and a low growth rate as compared with planktonic cells in a pure culture system. Furthermore, microorganisms in biofilms often display resistance to antimicrobial agents when compared with their planktonic counterparts (8). Polymetric substances, which make up the matrix of a biofilm, have been shown to retard the diffusion of antimicrobial agents (4). As shown in Fig. 7, treatment of ozonated water by Neo Ozone Water-S inhibited strongly the formation of biofilm *in vitro*. In addition, ozonated water showed complete antibacterial activity against the bacteria in human dental plaque biofilm (Fig. 8). We examined the cytotoxicity of ozonated water against mouse fibroblast cell line, L929, and found that there were no significant differences in the metabolic activity of L929 cells among PBS, distilled water, and 4 mg/l of ozonated water (data not shown). These findings suggest that ozonated water might be useful against bacterial growth in biofilms.

In conclusion, the present results demonstrated that ozonated water was effective for killing gram-positive and gram-negative oral microorganisms and oral *C. albicans* in pure culture. Furthermore, we found that ozonated water had strong bactericidal activity against the bacteria in plaque biofilm. In addition, ozonated water inhibited the accumulation of experimental dental plaque *in vitro*. The ozone sensitivity data obtained in this study should provide guidelines for further experiments on the application of ozone to dental plaque on the tooth surface or dentures.

Acknowledgments

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