Antimicrobial and Antibiofilm Effects of Ozonated Water for Prevention and Treatment of Bone and Joint Infections

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Abstract
Antimicrobial effects of ozonated water for the planktonic cells and the biofilm cells of staphylococci were examined for clinical applications in the orthopaedic field. As a result, 7 mg/L of ozonated water showed antimicrobial effects for not only the planktonic cells but also for the biofilm cells. Additionally, antimicrobial activity of ozonated water was examined after destroying the biofilm using 1 M NaCl or proteolytic enzymes (proteinase K and trypsin). Interaction between ozonated water and biofilm destruction was statistically significant in 2 strains (SE21, MR23) out of 8 strains. However, the effects of proteinase K or trypsin with ozonated water were statistically significant only in MRSA MR23. MRSA MR23 was previously reported to be a proteinaceous biofilm producer, and its biofilm was destructed by various proteases.

Key words
Ozonated water, Antimicrobial effect, Biofilm, Bone and joint infection

Introduction
In the treatment of suppurative osteomyelitis (osteomyelitis), clinicians must control the dead space that develops after lesion curettage. The method of continuous irrigation involves placing a tube for inflow and a tube for outflow into the dead space after lesion curettage¹. After this, the irrigation fluid is introduced from one side via drip infusion and removed via the outflow tube from the other side to continuously wash the dead space.

According to a research project conducted by the Japanese Orthopaedic Association, out of all pathogenic bacteria, methicillin-resistant Staphylococcus aureus (MRSA), and methicillin-resistant Staphylococcus epidermidis (MRSE) account for >40% of surgical site infections (SSIs) in 46% of artificial joint replacements and 44% of spinal instrumentation, and the rate of SSIs caused by MRSA and MRSE is increasing². Orthopedists recommend irrigating the surgical site with saline to prevent SSI. However, surgeons have reported that despite their efforts to repeatedly irrigate the surgical field, bacteria may be present in the surgical site before wound closure in 6.3% of cases³. If the surgical field contaminant is a multidrug-resistant organism, then it is difficult to prevent SSI simply by the preventive administration of first- and second-generation cephems and the saline cleansing method. Surgeons should thus clean the surgical field using an antimicrobial irrigation solution containing antimicrobial agents or disinfectants. It has been reported that cleaning the surgical field using 0.35% diluted povidone–iodine solution significantly reduces the rate of SSI⁴. The duration of tissue contact with the irrigation fluid when the surgical field is cleaned is shorter than when the treatment involves continuous irrigation. However, 0.35% povidone–iodine is toxic to chon-
drocytes, and it has been reported that exposure for \( \geq 1 \) min reduces cell viability\(^5\). Therefore, an irrigation fluid that exhibits antimicrobial effects and low tissue toxicity must be developed.

Clinicians require an irrigation solution that displays antimicrobial properties against multidrug-resistant bacteria, has low toxicity to patients, and can be used for extended periods. The term “ozonated water” refers to water in which ozone (gas) has been completely dissolved. Ozonated water exhibits strong antimicrobial activity. Although it has been reported that ozone gas damages the respiratory tissue because of oxidation, no reports indicate that ozonated water exerts any of these adverse effects on the human body. In the medical field, ozonated water is extensively employed to cleanse and disinfect medical instruments such as endoscopes, to clean the oral cavity and disinfect during implant placement in dentistry, and to clean the eye for surgery or treatment in ophthalmology. When used for continuous irrigation, ozonated water exhibits antimicrobial activity, and it does not cause tissue toxicity or tube obstruction even after extended use. Furthermore, following lesion curettage in chronic osteomyelitis, remaining bacteria can form a biofilm that is resistant to treatment\(^6\). If an irrigation solution for continuous irrigation displays antibiotic effects, then it would be an effective irrigation solution for the treatment of chronic osteomyelitis and also could be used as a solution to clean the surgical field. Here we examine and report the potential clinical applications of ozonated water as an irrigation solution and examine its effectiveness against biofilms.

**Materials and Methods**

**Bacterial strains**

We used 8 strains of bacteria that had been isolated at Jikei University Hospital, including *Staphylococcus epidermidis* (S. epidermidis: SE4, SE21), methicillin-sensitive *Staphylococcus aureus* (MSSA: MS3, MS18, SH1000), and MRSA (MR10, MR11, MR23). SE4, MS3, and MR10 were isolated from catheterized urine samples. SE21 was isolated from blood samples. MS18 and MR23 were isolated from samples of open wound purulence. MR11 was isolated from the skin. SH1000 was derived from the NCTC8325 strain of *S. aureus*. The bacterial strains were cryopreserved at \(-80^\circ\text{C}\) in a brain heart infusion (BHI; Difco Laboratories, Tokyo, Japan) supplemented with 20% glycerol.

**Generation of ozonated water**

Ozonated water was produced using the ozonated water generator (OZONE ORAL IRRIGATOR POS-100D, Ebara Jitsugyo Co, Tokyo, Japan). The test was performed using ozonated water produced at concentrations of 7 mg/L.

**Examination of the antimicrobial activity of ozonated water against planktonic cells**

i) Preparation of the bacterial suspension and measurement of viable bacterial count  

Bacteria was grown in 5 mL of BHI broth at 37°C with shaking for 24 h. To measure the viable bacterial count, the serially diluted bacterial suspension was spread on BHI medium containing 1.5% agar and incubated for 24 h at 37°C, following which the viable bacterial count was measured by colony counting.

ii) Examination of the antimicrobial activity of ozonated water  

To collect the bacterial cells, 1 mL of the bacterial culture was centrifuged (10000 × \( g \), 5 min, 25°C); after removal of the supernatant, the cell pellet was washed with 1 mL of saline before being resuspended in 1 mL of saline. The suspension was adjusted to an OD\(_{595}\) of 1 using a spectrophotometer. After mixing 1 mL of the suspension with 39 mL of 7 mg/L ozonated water and allowing it to stand for 1 h at 25°C; thereafter, 20 μL of 1 M sodium thiosulfate was injected to stop the reaction by deactivating the ozone. Then the viable bacterial count was measured. Controls were setup for this group in which sterile purified water was used instead of ozonated water.

**Examination of the antimicrobial activity of ozonated water against biofilm cells, and the effect of biofilm destruction methods on the antimicrobial activity of ozonated water**

i) Biofilm formation  

Staphylococcal biofilms were grown using the method described by Chiba et al.\(^7\) with slight modification. A biofilm was formed on the bottom of a 50-mL conical tube after 100 μL of the overnight culture of bacteria and 20 mL of BHI broth containing 1% glucose were added and incubated for 24 h at 37°C.

ii) Examination of the antimicrobial activity of ozonated water  

After removing the supernatant, the biofilm was suspended in 20 mL of saline and then, centrifuged (5000 × \( g \), 10 min, 4°C); the resulting bacterial cells were collected. The bacterial cells were mixed with
40 mL of 7 mg/L ozonated water and left standing for 1 h at 25°C, deactivating the ozone as in the method described above. Then, the viable bacterial count was measured. Controls were setup for this group in which sterile purified water was used instead of ozonated water.

iii) Examination for the effect of biofilm destruction methods on the antimicrobial activity of ozonated water

Destruction of staphylococcal biofilms was performed using the method described by Sugimoto et al. with slight modification. After destroying the biofilm using 1 M of NaCl or the proteolytic enzymes (proteinase K and trypsin), we measured the antimicrobial activity of ozonated water.

· 1 M NaCl-treated group

Biofilms were prepared using the method described above; subsequently they were resuspended in 20 mL of 1 M NaCl. The suspension was centrifuged (5000 x g, 10 min, 4°C) and the supernatant was removed to leave the pellet, which was then washed using 1 M NaCl solution. After washing, the pellet was mixed with 40 mL of 7 mg/L ozonated water, and the viable bacterial count was measured according to the method described above.

· Proteolytic enzyme-treated groups

After destroying the biofilm using proteinase K or trypsin, we measured the antimicrobial activity of ozonated water. Biofilms were cultivated in BHI broth containing 1% glucose. After the supernatant was removed and the biofilm washed with 20 mL of saline, the biofilm formed bacterial cells that were collected by centrifugation (5000 x g, 10 min, 4°C) and resuspended in 10 mL of phosphate buffer solution (PBS). The proteinase K (final concentration: 10mg/L)-treated group, the trypsin (final concentration: 10mg/L)-treated group, and the untreated group were prepared and incubated for 1 h at 37°C to allow the enzyme to react. Thereafter, the bacterial cells collected by centrifugation (5000 x g, 10 min, 4°C) were resuspended in 20 mL of sterile purified water and washed. This washing procedure was repeated, after which the collected bacterial cells were mixed with 40 mL of 7 mg/L ozonated water, and the viable bacterial cells were measured according to the method described above.

Statistical analysis

T-test was used to analyze differences in the antimicrobial activity of ozonated water for the planktonic cells. Two way-Anova was used to analyze differences in the effect of ozonated water for biofilm cells and biofilm destruction, and how an antimicrobial response is affected by these two factors. Tukey’s honestly significant difference test was used to analyze factors influencing the interaction. Logarithmic value was used for analysis. All statistical procedures were performed with SPSS software, with significance set at P<0.05.

Results

Examination for the antimicrobial activity of ozonated water against the planktonic cells

In a bacterial suspension of 8 strain adjusted to 10⁸–10⁹ colony-forming units (CFU)/mL, the viable bacterial count exhibit 5 log reduction against SE4, SE21, MR11, MR23 and 6 log reduction against MS3, MS18, SH1000, MR10 after 7 mg/L ozonated water was applied (Fig 1). These differences were statistically significant in all strains.

Examination for the antimicrobial activity of ozonated water against biofilm cells, and the effect of biofilm destruction methods on the antimicrobial activity of ozonated water

The effect of ozonated water was statistically significant in all 8 strains (p<0.001) (Fig 2, 3, 4). Importantly, antimicrobial effects of ozonated water against biofilm cells were attenuated compared to those against planktonic cells in all strains. The interaction between ozonated water and biofilm destruction was statistically significant in 2 strains out of 8 strains (S. epidermidis SE21; p<0.05, MRSA MR23; p<0.005) (Fig 2, 3, 4). Then, factors influencing the interaction were analyzed in these 2 strains. The effects of proteinase K or trypsin with ozonated water were statistically significant in MRSA MR23 (proteinase K; p<0.05, trypsin; p<0.05) (Fig 4). The effects of proteinase K, trypsin, or 1 M NaCl with ozonated water, however, were not statistically significant in S. epidermidis SE21 (Fig 2).

Discussion

The antimicrobial mechanism of ozone is believed to involve damage to reaction-prone sites in proteins and lipids that constitute the bacterial cell wall and membrane. In the present study, ozonated water exhibited strong antimicrobial activity against S.epidermidis, MSSA, and MRSA. We found antimicrobial effects of 7 mg/L ozonated water for the planktonic cells. Regarding clinical applications, we recommend irrigation using fresh batches and large
amounts of ozonated water to contact, as in reach, the bacteria because ozone is inactivated by organic matter, such as blood.

In this study, staphylococcal biofilms were exposed to ozonated water without irrigation. Tachikawa et al. established a biofilm model of exposure to ozonated water in irrigation and reported that when *Pseudomonas fluorescens* was exposed to ozonated water (0.92 mg/L) irrigation for 5 min, the survival rate sank below 1%; after they increased the ozone density (3.2 mg/L), the survival rate decreased further. There is the potential for a further decreased survival rate of staphylococcal biofilm cells using continuous irrigation of ozonated water. Further examinations should be performed using a test model that enables continuous exposure of freshly ozonated water to the biofilm formed cells.

Once medical devices such as artificial joints develop infection, the bacteria associated with the medical devices form a biofilm that exhibits resistance to...
antimicrobial agents\textsuperscript{6} \textsuperscript{6}, making the condition intractable. Accordingly, if the medical devices are infected, they often must be removed, causing significant functional impairment. For these reasons, the infection of medical devices is a major problem in orthopedics. Therefore, we examined the antimicrobial effect of ozonated water for biofilm cells. The effect of ozonated water was statistically significant in all strains (Fig 2, 3, 4).

These results suggest ozonated water exhibits antimicrobial activity for biofilm cells. Biofilms, however, show a barrier function against ozone. The antimicrobial activity of ozonated water was examined after destroying the biofilm using 1 M of NaCl or the proteolytic enzymes proteinase K and trypsin. The effects of proteinase K or trypsin with ozonated water were statistically significant in only MRSA MR23 (Fig 4). It has been reported that biofilm of MRSA MR23, which was a proteinaceous biofilm producers, and its biofilm was destructed by various

\textbf{Fig 3.} Effect of biofilm destruction treatment of methicillin-sensitive \textit{Staphylococcus aureus} (MSSA) on the antimicrobial activity of ozonated water. Bars indicate standard deviations of triplicate determinations. MS3, MS18, and SH1000 are MSSA.

\textbf{Fig 4.} Effects of biofilm destruction treatment of methicillin-resistant \textit{Staphylococcus aureus} (MRSA) on the antimicrobial activity of ozonated water. Bars indicate standard deviations of triplicate determinations. MR10, MR11, and MR23 are MRSA.
proteases.\textsuperscript{8}) Mizunoe\textsuperscript{11}) and Sugimot\textsuperscript{8}) extracted the extracellular matrix from MRSA MR23 biofilm using 1 M NaCl and identified proteins included in the matrix by quantitative analysis and amino acid sequencing. They reported that 64% were cytoplasmic proteins, 12% were secretory proteins, and 6% were membrane proteins. Although the precise molecular mechanism remains unknown, degradation of biofilm matrix protein which attenuates the effect of ozone, might be reason for the synergistic effect observed. Biofilm destruction methods may have applicability to some resistant cases.

Problems associated with ozonated water include its safety and half-life. Regarding the safety of ozonated water, Hoshi et al.\textsuperscript{12}) observed no change histologically or by transmission or scanning electron microscope in domestic rabbits used in tests of acute toxicity with 20 mg/L ozonated water and subacute toxicity with 4 mg/L ozonated water. The safety of long-term usage of ozonated water, however, is unknown. The half-life of ozonated water is approximately 30 min. Unless it is used immediately after production by an ozonated water generator, its effects will be attenuated. Kawashima et al.\textsuperscript{13}) noted that small ozone bubbles <100 nm in diameter dissolved in ozonated water, but also remain present in the water for a long time and generated ozone nanobubble water dissolved in saline with 0.9% salinity. The strong antimicrobial effect was reported to continue for a long time. Ozone nanobubble water, however, is not currently commercially available. Regarding clinical applications, using electrode-type ozonated water generators, the system should be built to sterilize the circuit that includes the electrodes, and ozonated water should be produced at the bedside or in the operating room and should be used immediately.

In future clinical applications of ozonated water in continuous irrigation and surgical field cleansing, an irrigation-type exposure model should be used to verify environments in which freshly ozonated water can come into contact with bacteria in order to confirm the antimicrobial synergy effect of ozonated water. Furthermore, the efficacy of low ozonated water concentration and the safety of continuous usage should be investigated, and a sterilization circuit should be developed so that ozonated water produced by an ozonated water generator can be used for clinical practices immediately after it is generated.

**Summary**

1. Seven mg/L ozonated water showed antimicrobial effects for not only the planktonic cells but also for the biofilm cells of staphylococci.
2. The effects of proteinase K or trypsin with ozonated water, however, were statistically significant in only MRSA MR23.
3. The efficacy of low ozonated water concentration, the safety of continuous usage, and the clinical application of biofilm destruction methods should be investigated further.

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**References**

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