INTRODUCTION
Oral infectious diseases result from an imbalance between bacterial load and the host defense that leads to the loss of teeth, implants and supporting bone. Since bacterial adhesion and colonization are considered the main causative factors for the initiation and progression of periodontal and peri-implant disease, implants and periodontal structures require protection from bacterial invasion and subsequent infection. Early bacterial colonization of periodontal and peri-implant pockets is characterized by an

SYNOPSIS
Ozone nano bubble water (NBW3) seems to be suitable as an adjunct treatment to oral infectious diseases as for antimicrobial effects and compatibility with oral tissues to overcome disadvantage of conventional mouth rinses.

The aim of this study was to determine the effect of NBW3 on sterilization and cytotoxicity. We analyzed the bactericidal effect of NBW3 against oral infectious bacteria. Cytotoxic ability was estimated using human gingival fibroblast cells stained with PI/Calcein-AM and fluorescence microscopy. The reduction in the number of bacteria in the NBW3 group was significantly greater than those in the CHX group and similar to that of Listerine against P. gingivalis. Cytofluorometric assays revealed higher biocompatibility of NBW3 against fibroblasts.

NBW3 had significantly bactericidal activity against oral infectious bacteria and biocompatible with mammalian cells compared with conventional mouth rinses. The oral application of NBW3 might have potential as an adjunctive treatment for oral infectious diseases.

Key words: biocompatibility, ozone, oral disinfection, bactericidal activity

INTRODUCTION
Oral infectious diseases result from an imbalance between bacterial load and the host defense that leads to the loss of teeth, implants and supporting bone. Since bacterial adhesion and colonization are considered the main causative factors for the initiation and progression of periodontal and peri-implant disease, implants and periodontal structures require protection from bacterial invasion and subsequent infection. Early bacterial colonization of periodontal and peri-implant pockets is characterized by an
an increase in colonization with organisms such as *Streptococcus sanguinis* that create the milieu for the adhesion of periodontal pathogens which in turn can induce periodontitis and peri-implantitis. The principal objective of periodontal and peri-implant therapy is to eliminate sub-gingival bacteria and to prevent or minimize recolonization of the sub-gingival area by pathogenic microflora using supra-gingival plaque control measures. Mechanical techniques might be able to eliminate sub-gingival microflora as they are the most popular method for controlling the growth of dental plaque. However, the effectiveness of mechanical control is limited by factors such as individual motivation, the inaccessibility of periodontal pockets, concave tooth surfaces and the margins of restorations.

Therefore, supplementation with antimicrobial agents or other alternatives might enhance mechanical methods of plaque removal. Adjunctive agents in the form of topical or systemic antibiotics or topical antiseptics have been proposed to augment mechanical debridement and improve the non-surgical outcomes of treating oral diseases. Adjunctive antibiotic drugs or antiseptic mouth rinses indeed enhance the outcome of mechanical treatment and might even support therapy for oral infectious diseases.

Numerous antiseptic mouth rinses have already been available for use as part of a daily oral hygiene routine. The formulations contain actives that inhibit microbial growth and enzymatic reactions in the mouth. However, simultaneously, some mouthwashes were reported to induce noxious activities on eukaryotic cells, particularly genetic damage and/or cellular death. They may damage oral tissues and inhibit their regeneration. Ideally, mouth rinses components should be both antiseptic for oral infectious bacteria and compatible with oral related tissues.

In this regard, ozone is currently being discussed as a possible antiseptic agent in dentistry. A study of water purification and food preservation has shown that ozone offers effective bactericidal activity without the development of drug resistance. Ozone is effective against oral pathogenic bacteria, and the role of ozone in the treatment of oral diseases is currently a focus of intensive research. *Streptococcus* eradication requires longer periods of exposure to ozone at conventional high doses. The anaerobic bacteria associated with periodontitis and periimplantitis such as *Porphyromonas gingivalis* might be more sensitive to ozone.

Ozone has been used in clinical dentistry either in gaseous or in aqueous form to eliminate caries pathogens by disinfecting the root canals of avulsed teeth and to improve treatment-induced epithelial wound healing. Gaseous, rather than liquid ozone might be more advantageous for intraoral applications because the physical characteristics and diffusion potentials differ between the two. The downside of gaseous ozone is toxicity if inhaled into the respiratory tract. On the other hand, ozonated water is biocompatible with oral applications as it has optimal cell biological characteristics. New manufacturing techniques have been developed to stabilize ozone gas in nano bubble water; NBW3 (REO Research Facility, Miyagi, Japan).
This novel ozonated water is far more stable than conventionally ozonated water. The procedure for generating nano bubble water has been patented. The ozone concentration of NBW3 is 1.5 mg/L, which is equivalent to the oxidation titer determined by electron spin resonance (ESR). Nano bubbles are gaseous nuclei of < 100 nm in diameter that are produced by collapsing micro bubbles that are ≤ 50 µm in diameter in an electrolyte under ultra-high temperature and pressure. Applying a physical stimulus to the electrolyte causes the micro bubbles to collapse, which results in ions in the solution becoming concentrated around gaseous nuclei; a salting out phenomenon. This in turn prevents the gas from dispersing, which is known as salting-out. Due to this phenomenon, nano bubbles in aqueous solution are stable for long periods. Stabilized NBW3 retains ozone gas in the form of gaseous nuclei that can exert antimicrobial effects for over six months if protected against ultraviolet radiation. Therefore, NBW3 might be useful for treating periodontitis and peri-implantitis or to maintain a healthy oral environment. Here we compared the bactericidal activities of NBW3 and conventional irrigation products against \textit{S. mutans} and \textit{P. gingivalis}. We also examined the cytotoxic effects of NBW3 against human gingival fibroblasts.

**Materials and Methods**

**Ozone nano bubble water (NBW3)**

Nano bubble water (1.5 mg/L of ozone in 0.9 w/v% isotonic sodium chloride) supplied by the REO Research Facility (Miyagi, Japan) is a novel, more stable type of ozonated water than the conventional type because of ionic aggregates around nano bubbles. NBW3 was stored protected from UV radiation.

**Growth conditions for microorganisms**

\textit{Staphylococcus aureus} (RN4220), \textit{Streptococcus sanguinis} (JCM5708), \textit{Klebsiella pneumoniae} (JCM1122) and \textit{Escherichia coli} (JCM20135) and were cultured in Tryptic Soy Broth (TSB; Becton Dickinson) at 37°C for 18 h. \textit{Streptococcus mutans} (NN2025) was cultured in brain heart infusion broth (BHI; Becton Dickinson, Franklin Lakes, NJ USA) at 37°C for 18 h in an atmosphere of 5% CO2 in air. \textit{Porphyromonas gingivalis} (ATCC33277) was cultured in general anaerobic broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) at 37°C for 24 h under anaerobic conditions, using Anaero Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

**Microbacteria culture**

Multiple trials were performed to determined lysis and inactivation of microbacteria and in different aqueous media. From each agar plate, 3 to 5 colonies were inoculated in 10mL of each broth, being incubated for 24 hours at 37°C in broth. \textit{S. mutans} and \textit{P. gingivalis} were incubated at 37°C for 48h.

**Evaluation of bactericidal activity in vitro**

Microorganisms were harvested by centrifugation at 3,000 rpm for 5 min at 4°C. The pellets were resuspended in saline and the bacterial cell density was adjusted \(1.0 \times 10^6\) - \(1.0 \times 10^9\) CFU/mL, respectively. After centrifugation to remove saline, the microorganisms were individually exposed to saline and NBW3 (0.15 and 1.5 mg/L) for 3min. Suspensions of treated bacteria (100 µL) were diluted with 1% with saline to inactivate any bactericidal activity, which we confirmed in a preliminary experiment. Samples were inoculated on plates containing appropriate culture medium and incubated at 37°C for 24–72 h depending on the growth rate.
of the test strains. Colony-forming units (CFU) are counted by the spread plate method. In some experiments, the suspensions of *S. mutans* and *P. gingivalis* were individually exposed to NBW3 (1.5 mg/L) or conventional irrigation products for 30 sec, 1 and 3 min. The conventional irrigation products comprised Listerine® (Johnson & Johnson K. K. Consumer Company, New Brunswick, NJ, USA) and chlorhexidine mouthwash (20 µg/mL, CHX, ConCool F®; Weltech, Osaka, Japan) and these were applied at the concentrations recommended for medical use by the manufacturers. The antimicrobial activities of these disinfectants were examined as described above. *P. gingivalis* was cultured on agar plate media with 10% (v/v) horse whole blood at 37°C for 7 days under anaerobic conditions.

**Evaluation of cell toxicity**

Cell toxicity was assessed by culturing human gingival fibroblasts (HGFs) with three antibacterial agents. HGFs were obtained from individuals with periodontally healthy gingiva who had provided written informed consent to participate in all procedures associated with the study. Fibroblasts that proliferated from pieces of gingiva were cultured in α-MEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) containing 100 U/mL of penicillin and 100 mg/mL of streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. Cells used in experiments between passages five and fifteen. α-MEM was used as untreated control. After exposure to antibacterial agents for 60 sec, treated HGFs were diluted by 1% with isotonic sodium chloride solution to inactivate any bactericidal activity. Cell morphology was examined by bright field and fluorescence microscopy (IMT-2, Olympus, Tokyo, Japan) and viability was determined optically by Calcein-AM / propidium iodide staining. Viable cells exhibit green fluorescence by a reaction with Calcein-AM while nonviable cells are stained red by propidium iodide. Cells were examined using the IMT-2 microscope immediately after staining. The excitation/emission wavelengths of Calcein-AM and propidium iodide were 480/530 and 520 /580 nm, respectively. Green and red fluorescence was simultaneously recorded using a cooled CCD camera (VB-7000, Keyence, Osaka, Japan). All experiments were repeated in duplicate (n= 5 samples). The Bioethics Board at the Tokyo Medical and Dental University approved the study protocol.

**Statistical analysis**

Data were statistically analyzed using ANOVA and Tukey multiple comparison tests. Differences in effects were taken as significant at *p* < 0.05.

**RESULTS**

**Bactericidal activity in vitro**

To examine antimicrobial activity, oral microorganisms were exposed to several concentrations of NBW3 for 3min. As shown in the results summarized in Table 1, the cell viability of gram-positive bacteria such as *S. aureus* and *S. sanguinis* in degree of 1.0 × 10⁸ CFU/mL were significantly decreased after exposure to 0.15 mg/L of NBW3, and those were killed to >99.99% in 1.5 mg/L of NBW3. The cell viabilities of *S. aureus* and *S. sanguinis* were very similar when the cells were exposed to NBW3.

Then, we examined the effects of NBW3 on the cell viabilities of gram-negative bacteria such as *K. pneumoniae* and *E. coli*. The degree of 1.0×10⁹ CFU/mL cells were insufficiently decreased to 0.15mg/L of NBW3 but those were killed to >99.99% in 1.5 mg/L of NBW3. *K. pneumoniae* showed more resistant to NBW3 than *E. coli*.
### Table 1: Viable bacterial cells after 3min treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>S. aureus</th>
<th></th>
<th>S. sanguinis</th>
<th></th>
<th>K. pneumoniae</th>
<th></th>
<th>E. coli</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
</tr>
<tr>
<td>Untreated control</td>
<td>2.4×10⁸</td>
<td>0</td>
<td>1.5×10⁸</td>
<td>0</td>
<td>7.6×10⁶</td>
<td>0</td>
<td>1.6×10⁸</td>
<td>0</td>
</tr>
<tr>
<td>NBW3 (0.15 mg/L)</td>
<td>b.d.l. *</td>
<td>&gt;99.99</td>
<td>3.3×10⁸</td>
<td>97.8</td>
<td>3.9×10⁵</td>
<td>48.2</td>
<td>6.1×10⁵</td>
<td>61.1</td>
</tr>
</tbody>
</table>

Treatment with Saline served as Untreated control  
*Mean of four experiments  
*b Below detection limit (<10 CFU/mL)  
*p<0.05, significantly superiority was observed in the NBW3 group, compared to the Untreated control group

### Table 2: Viable S. mutans cells after treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30s</th>
<th></th>
<th>1min</th>
<th></th>
<th>3min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
</tr>
<tr>
<td>Untreated control</td>
<td>1.7×10⁶</td>
<td>0</td>
<td>1.7×10⁸</td>
<td>0</td>
<td>1.7×10⁸</td>
<td>0</td>
</tr>
<tr>
<td>NBW3</td>
<td>1.0×10⁵ **</td>
<td>91.92</td>
<td>9.8×10⁷ **</td>
<td>93.31</td>
<td>2.7×10⁷ / **</td>
<td>94.69</td>
</tr>
<tr>
<td>CHX</td>
<td>4.0×10⁵</td>
<td>77.78</td>
<td>3.2×10⁵</td>
<td>79.88</td>
<td>2.5×10⁵</td>
<td>84.08</td>
</tr>
</tbody>
</table>

Treatment with Saline served as Untreated control  
*Mean of four experiments  
*b Below detection limit (<10 CFU/mL)  
*p<0.05, significantly superiority was observed in the NBW3 group, compared to the CHX group  
**p<0.05, significantly inferiority was observed in the NBW3 group, compared to the Listerine® group

### Table 3: Viable P. gingivalis cells after treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>30s</th>
<th></th>
<th>1min</th>
<th></th>
<th>3min</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
<td>Viable bacteria</td>
<td>% killed bacteria</td>
</tr>
<tr>
<td>Untreated control</td>
<td>7.0×10⁷</td>
<td>0</td>
<td>7.0×10⁷</td>
<td>0</td>
<td>7.0×10⁷</td>
<td>0</td>
</tr>
<tr>
<td>NBW3</td>
<td>5.8×10⁷ / **</td>
<td>99.11</td>
<td>8.0×10⁶ / **</td>
<td>99.88</td>
<td>b.d.l. b</td>
<td>&gt;99.99</td>
</tr>
<tr>
<td>CHX</td>
<td>4.0×10⁷</td>
<td>48.33</td>
<td>2.5×10⁷</td>
<td>68.54</td>
<td>3.0×10⁷</td>
<td>61.50</td>
</tr>
</tbody>
</table>

Treatment with Saline served as Untreated control  
*Mean of four experiments  
*b Below detection limit (<10 CFU/mL)  
*p<0.05, significantly superiority was observed in the NBW3 group, compared to the CHX group  
**p<0.05, significantly inferiority was observed in the NBW3 group, compared to the Listerine® group
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Fig 1
Bright-field microscopic observations of human gingival fibroblasts (HGFs). HGFs were incubated with α-minimum essential medium (α-MEM) (A) as untreated controls, NBW3 (B), CHX (C) or Listerine® (D). Scale bar, 100 µm.

Fig 2
Fluorescence microscopic observations of human gingival fibroblasts (HGFs). HGFs were incubated with α-minimum essential medium (α-MEM) (A) as untreated controls, NBW3 (B), CHX (C) or Listerine® (D). Viable cells exhibit green fluorescence, respectively. Scale bar, 100 µm.

Fig 3
Fluorescence microscopic observations of human gingival fibroblasts (HGFs). HGFs were incubated with α-minimum essential medium (α-MEM) (A) as untreated controls, NBW3 (B), CHX (C) or Listerine® (D). Non-viable cells exhibit red fluorescence, respectively. Scale bar, 100 µm.
To compare the antimicrobial activity of NBW3 and commercially available mouth rinses, *S. mutans* and *P. gingivalis* were incubated with NBW3 (1.5 mg/L), CHX and Listerine® for 30 sec, 1 or 3 min. We initially investigated the effects of NBW3 on the viability of *S. mutans*, which is a cariogenic bacterium. Table 2 shows that NBW3 reduced the viable cell count of *S. mutans* (1.0 × 10^6 CFU/mL) by a factor of 10^-1 within 30 sec, and by > 10^-2 within 3 min. However, exposure to NBW3 for 3 min did not completely kill *S. mutans*. The viable cell count of *S. mutans* significantly decreased after exposure to Listerine® for 30 sec, but not to CHX for 3 min. The bactericidal activity of NBW3 against periodontopathic *P. gingivalis* was different from that against *S. mutans*. Table 3 shows that NBW3 reduced the bacterial activity of *P. gingivalis* (1.0 × 10^8 CFU/mL) by a factor of 10^-2 after 30 sec. NBW3 decreased the cell counts of *P. gingivalis* more effectively than that of *S. mutans* within 30 sec and killed all *P. gingivalis* within 3 min. In contrast, CHX did not obviously reduce the cell count of *P. gingivalis* (1.0 × 10^8 CFU/mL) within 3 min. Listerine® was more powerful and exerted more rapid bactericidal effects against *P. gingivalis* than CHX. NBW3 was more effective against *P. gingivalis* than *S. mutans* and the bactericidal activity was similar to that of Listerine®, especially against *P. gingivalis*.

**Cell toxicity**

The cytotoxicity of NBW3 against HGFs was evaluated. The effects of NBW3, CHX and Listerine® on HGFs were examined by bright field and fluorescence microscopy and morphological changes were also examined by bright field microscopy. Morphologically, all treated cells with NBW3, CHX and Listerine® were not definitely different from untreated cells. They seemed to be no damaged by all treatments, respectively (Fig. 1). Simultaneously, viable and non-viable cells were visualized by fluorescence microscopy. Viable cells emitted green fluorescence and non-viable cells emitted red fluorescence, respectively. Similar numbers of viable HGFs which emitted green fluorescence were detected after treatment with NBW3 and CHX (Fig. 2B and 2C), and there were observed few non-viable cells which emitted red fluorescence (Fig. 3B and 3C). On the other hand, there were observed few green emitted cells after treated with Listerine® (Fig. 2D) and these cells strongly emitted red fluorescence (Fig. 3D). These results indicate that Listerine® was the most toxic among the three antibacterial agents for HGFs.

**DISCUSSION**

Supra-gingival plaque control is a key to the prevention of gingivitis and thus the occurrence or recurrence of chronic periodontal diseases. Using chemical adjunctive agents would appear to offer a means of overcoming deficiencies in mechanical dental hygiene habits. Although many chemical agents have already been considered as methods of periodontal plaque control, some antiseptic mouth rinses could induce genetic damage for oral tissues. To overcome these disadvantages, ozone might be expected as a candidate antiseptic mouth rinse. Recently, ozone has attracted attention because it is antimicrobial and does not induce microbial resistance, features that were originally identified from the aspects of water purification and food preservation. Gaseous and aqueous ozone is active against specific periodontal pathogens.

However, conventional ozonated water has a half-life of about only 20 minutes and ozone will revert to oxygen very rapidly, so it should be applied within 5 – 10 minutes of generation to
assure potency. Thus, ozone manufacturing techniques have targeted the stabilization of nano bubble water to overcome this disadvantage and a new, more stable type of ozonated water called NBW3 has been generated 29-32. This half-life of nano bubbles in NBW3 is several months when protected from ultraviolet radiation and thus NBW3 remains stable over the long-term. Therefore, NBW3 might be useful to maintain a healthy oral environment.

Although the bactericidal mechanisms of NBW3 remain unknown, they might be essentially similar to those of extant ozonated water. Ozone is a potent oxidizing agent 37 and ozone in water reacts with and decomposes organic molecules via free radical-mediated oxidation 38. Ozone changes to oxygen during reactions with organic substances. This process generates the most reactive of all oxidative species, namely hydroxyl radicals (-OH). These free radicals might then help to destroy bacterial pathogens.

To evaluate the effectiveness of novel ozonated water of NBW3, we examined the bactericidal activities based on a decrease in the number of cultivable microorganisms instead of using PCR 39. Because the disadvantage of PCR is that it detects both viable and non-viable bacteria, the effectiveness of antimicrobial agents is difficult to assess and the prognostic value of PCR is compromised in this study. Only viable microorganisms are detected in culture, this method is suitable for assessing cell viability in this study.

The present results showed that ozonated water (1.5 mg/L) was highly effective in killing both representative gram-positive and gram-negative microorganisms. We found the degree of the gram-positive bacteria and the gram-negative bacteria which were sensitive to 1.5 mL/L of NBW3 in pure culture (Table 1). Among them, the gram-negative bacteria, such as K. pneumonia and E. coli, were rather more sensitive to NBW3 than the gram-positive bacteria, such as S. aureus and S. sanguinis. The sensitivity data might be especially useful guidelines for treating oral infectious microorganisms with NBW3.

We also compared the bactericidal activities among NBW3, CHX and Listerine®. The present study found that the antimicrobial activities of NBW3 and Listerine® against P. gingivalis did not significantly differ during a period of 3 minutes (Table 3), whereas those of NBW3, CHX and Listerine® against S. mutans (Table 2) and those of NBW3 and CHX against P. gingivalis significantly differed within this time frame. For a method to be regarded as antibacterial, at least 10^-3 CFU (99.9%) must be killed according to the American Society of Microbiology. Based on this criterion, NBW3 was antibacterial against periodontopathic P. gingivalis. The differences in microbial sensitivity to NBW3 were probably due to structural differences in their cell walls. The three-dimensional cell architecture profoundly differs between gram-positive and -negative bacteria. The membrane barriers of Gram-positive bacteria consist of a relatively thick but porous cell wall comprising inter-connected peptidoglycan layers surrounding a cytoplasmic membrane. Conversely, the cell envelope of Gram-negative bacteria comprises an outer membrane, a thinner peptidoglycan layer, and a cytoplasmic membrane 40.

To apply for clinical treatment with NBW3, we must concern about its cytotoxicity. As previously noted, the cytotoxicity of conventional antiseptic agents is clinically relevant, because they contact resident oral cells such as epithelial or mucosal cells. Some conventional mouthwashes such as Listerine® and CHX that have powerful antimicrobial
activity are also associated with side-effects such as oral pain, mucosal desquamation, impaired wound healing, fibroblasts attachment to tooth surfaces, tooth staining and altered taste sensation. Before using NBW3 as an oral treatment, its cytotoxic potential needs to be compared with that of established agents. Therefore, we directly compared the cytotoxicity of NBW3 with that of established conventional antiseptic mouthwashes.

Bright field microscopic images showed that cells incubated with all three chemical agents seemed morphologically vital, whereas almost all cells incubated with Listerine® were killed. Listerine® contains ethanol, which is present in concentrations of 21.6%, that might have fixed the HGFs. Regardless, Listerine® significantly damaged HGFs compared with NBW3, and these findings were consistent with those of other reports.

Chlorhexidine also did not induce toxic effects on HGFs in the present study. However, we applied 20 µg/mL of CHX which the Minister of Health, Labour and Welfare in Japan has approved for local application to the oral cavity and throat. Chlorhexidine at such a low concentration might not be toxic when applied as oral irrigation, but it negatively affects fibroblast and keratinocyte proliferation in a concentration- and time-dependent manner.

In conclusion, the present study showed that NBW3 killed both gram-positive and -negative bacteria and was not cytotoxic, unlike the conventional mouthwash, Listerine®. Therefore, NBW3 fulfills the optimal biological characteristics for oral application. However, further study is required before NBW3 could be used clinically to treat oral infections. Although NBW3 exerted bactericidal effects in vitro, whether or not the effect would be similar in vivo remains to be determined because saliva and surface biofilm in the dental milieu are highly resistant to various antiseptics.

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