Plasma-treated water eliminates Streptococcus mutans in infected dentin model

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Non-mechanical procedures for removing caries-infected dentin are warranted in dentistry. We previously demonstrated the marked sterilization effect for direct irradiation of low-temperature plasma using dentin model infected with Streptococcus mutans. However, it requires 180 s of intraoral plasma irradiation to eliminate bacteria. We alternatively investigated whether plasma-treated water (PTW), i.e., pure water exposed to plasma in an atmosphere, has a same bactericidal activity with the plasma irradiation. In the infected dentin model, the viable S. mutans counts recovered by bur at depth of 0.8–2.4 mm from the cavity floor were 10⁴–10⁶ CFU/round bur. After PTW application for only 10 s, the count was significantly decreased to below the detection limit (2.5 CFU/round bur) or 3.0±5.0 CFU/round bur. Since the bactericidal activity of PTW is rapidly deactivated at body temperature (37°C), PTW is likely to be biocompatible and holds significant potential for non-mechanical procedures for removing caries-infected dentin.

Keywords: Plasma, Water, Sterilization, Streptococcus mutans, Dentin

INTRODUCTION

In modern dentistry, dentin caries is widely recognized as a bacterial infectious disease¹-². Cariogenic bacteria begin to invade the dentinal tubule, which then becomes packed with bacteria, of which the acid demineralizes the peritubular dentin. Through the cracks generated in the peritubular dentin, the acid further penetrates into inter-tubular dentin, dissolving the mineral component of dentin. The pathology underlying the progression of dentin caries together with the histology of dentin was revealed around the beginning of the 20th century³. Since then, the instrumentation for complete removal of bacteria from invaded dentin has become a basic procedure in caries treatment, and is essentially a surgery. Nowadays, however, both dentists and patients seek minimally invasive restorative treatments that are based on internal medicine. Indirect pulp capping (IPC) offers a clinical solution, where the remaining infected dentin can be healed with an application of calcium hydroxide material or polycarboxylate cement combined with a tannin fluoride preparation. IPC has been shown to be effective in killing bacteria, but it requires long-term (3–6 months) application of pulp-capping agents with a stepwise excavation (interval of more than 1 month)⁴-⁶.

To sterilize infected dentin, existing non-invasive sterilization techniques include dental lasers, ozone treatment, and drug therapies; however, these techniques are not yet well established in clinical practice due to some shortcomings and uncertainty about the sterilization effects⁷-¹⁰.

Another newly developed non-invasive method of sterilization that has been attracting attention in dentistry, involves the use of atmospheric pressure plasma. Plasma, the fourth state of matter after solid, liquid, and gas, is formed through electrical discharge and generates numerous types of reactive chemical species. In particular, the gases surrounding the plasma are changed into reactive oxygen and nitrogen species, which are now attracting attention from researchers as a media with sterilization potential.

Many recent studies have investigated the medical use of low-temperature atmospheric pressure plasma, which does not exert a heat load, for sterilization purposes (Fig. 1)¹¹,¹². However, there are few reports about its application for sterilization in the field of dentistry¹³-¹⁶. Yamazaki et al.¹⁷ have previously reported the sterilization effects of plasma exposure in an acidic environment (the reduced-pH method) for a wide variety of oral pathogenic microorganisms, both in suspension and in biofilms. Usui et al.¹⁸ have demonstrated the marked sterilization effects of plasma irradiation, using the reduced-pH method, in an infected dentin model prepared with Streptococcus mutans. However, it had a drawback for clinical application, in that a 180 s (3 min) plasma irradiation period was required to achieve a bactericidal effect below the limit of detection limit, and required extensive equipment use for direct plasma irradiation. Additionally, when intraorally applied, direct irradiation of plasma can yield common chemical compounds, such as hydrogen peroxide, nitrous acid, and ozone, as well as reactive species. These chemicals are inevitably generated during plasma irradiation that...
Plasma, generated under low-temperature atmospheric pressure, does not produce even the slightest damage to a finger.

Consequently, we have focused on the use of plasma-treated water (PTW), i.e., distilled water that had been exposed to low-temperature atmospheric pressure helium plasma, which has a strong bactericidal activity that is enhanced under acidic conditions. Since PTW is deactivated rapidly at human body temperature, it is likely to be highly biocompatible. In addition, PTW can be preserved at a low temperature for a long period after being generated, negating the need for an extensive plasma generator at chair-side.

In this study, we tested the antibacterial activity of PTW against S. mutans. The antibacterial activity in suspension was first assessed using a serial-dilution method, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of PTW were determined and compared with those of a sodium hypochlorite solution (NaClO) as a reference. Then, using an infected dentin model, we tested whether PTW had a similar sterilization capability to that of direct plasma irradiation using the reduced-pH method.

**MATERIALS AND METHODS**

**PTW preparation**
To obtain PTW, atmospheric pressure plasma was irradiated to the flowing pure water in an elongated discharge glass tube (length: 1,000 mm, inner diameter: 8 mm), tilted slightly off the horizontal. The PTW generated in this manner flowed slowly and continuously off the end of the tube. A mixture of helium and a low percentage of nitrogen gas was supplied to the glass tube by mass-flow controllers (Model 8500l, Kofloc, Kyoto, Japan). The glass tube was sandwiched by a pair of metal electrodes connected to a high-voltage power supply (FPG 20-10KN5; FID; Burbach, Germany) and atmospheric pressure glow plasma was generated by a dielectric barrier discharge (20 kV, 10 kHz, 5-ns pulse width). This plasma system was cooled by iced water to avoid thermal deactivation of the PTW. The PTW obtained in this manner was rapidly frozen in liquid nitrogen and stored at −80°C. For a sterilization experiment, frozen PTW was melted at about 0°C to retain the bactericidal effect of PTW.

**Bacterial strain and culture conditions**
Frozen stock of S. mutans (ATCC 25175) was thawed and cultured in brain-heart infusion (BHI) broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), overnight, under anaerobic conditions, at 37°C. After inoculation into 10 mL of fresh BHI, the bacterial suspension was subjected to shaking culture under aerobic conditions, overnight at 37°C. The bacterial suspension was then further inoculated into fresh BHI and subjected to another 6 h of shaking culture, under aerobic conditions, to obtain a concentration of $10^7$–$10^8$ CFU/mL as a pre-culture.

**MIC and MBC of PTW against S. mutans**
Thawed PTW (80 µL) was serially diluted with distilled water in a microplate chilled on ice, and mixed with 10 µL of 200 mM citrate-Na buffer solution adjusted to pH 3.5 or to pH 6.5. Then, 10 µL of the pre-culture suspension of S. mutans was added to the PTW mixture, as described above. To determine its thermal stability at human body temperature (37°C), a portion of PTW was first incubated at 37°C for 5 min before mixing with the pre-culture. After the mixture of PTW, buffer, and bacteria was incubated at 25°C for 10 min, 100 µL of 2× concentrated BHI was added, and the samples cultured at 37°C for 24 h. MIC was evaluated by visual inspection of turbidity, and MBC was evaluated by further plating the culture on agar media. Six volume percent of NaClO solution (Wako Pure Chemical Industries, Osaka, Japan), as a reference, was also serially diluted and the MICs and MBCs determined as for PTW.

**S. mutans-infected model**
The study was approved by the Tsurumi University ethics review committee (approval no. 856). Eighteen extracted human molars were stored in physiological saline solution at 4°C until required. The saline solution was replaced with a fresh saline solution once every 2 days. To simulate occlusal caries (Fig. 2-I), a 3 mm diameter cylindrical cavity was prepared in the center of the occlusal surface of each tooth, extending to a depth of 3 mm from the cusp tip, using a Diamond Point FG #301 (Shofu, Kyoto, Japan). After immersion in distilled water, teeth were autoclaved (121°C, 15 min). To achieve demineralization to a constant depth, a swab soaked in 40% lactic acid (30 µL) was placed in the cylindrical cavity for 2 days. Swabs were replaced daily. Pre-culture of S. mutans was precipitated by centrifugation and re-suspended in the culture medium containing 5% carboxymethyl cellulose sodium salt (CMC; Wako Pure...
Chemicals Industries), followed by thorough mixing. The cavity created in the tooth was wiped with a sterile swab, inoculated with 30 µL of the S. mutans suspension prepared above, and cultured under aerobic conditions at 37°C. This inoculation process was performed daily for 7 days to prepare the infected dentin model. Three infected dentin molars were assigned to the experiment in order to determine an optimal application time of PTW and fifteen molars were assigned to the experiment in order to count the S. mutans in dentin at various depth.

**Bactericidal experiment using an infected dentin model**

The cavity of the infected dentin model was washed three times with 30 µL of 200 mM pH 3.5 buffer solution. Before placement of pH 3.5 buffer solution into the cavity, dentin samples were extracted from the cavity using a sterile round bur (#1; diameter: 0.8 mm; DENTSPLY International) (Fig. 2-II), at sampling depth of 0.8, 1.6, and 2.4 mm from the cavity floor. To recover the dentin samples, the round bur containing the dentin sample in the groove was submerged into 150 µL of BHI broth, and subjected to 30 s of ultrasonic treatment (Bransonic 52, Branson, Shanghai, China). Agar plates were seeded with 100 µL of the resulting suspension after serial dilution, and the viable bacteria count (CFU) was calculated. In the buffer application group (Fig. 2-III, A), 30 µL of pH 3.5 buffer solution was placed in the same cavity for 10 s, after which the same procedure as described for Fig. 2-II.

Frozen PTW (1 mL) was thawed and mixed with 100 µL of 200 mM pH 3.5 buffer solution, and then 30 µL of the mixture was placed in the cavity of the model.
teeth for 10 s. After removal of the mixture from the cavity, the dentin sample was collected and cultured, and viable bacteria were counted in the same manner as described for Fig. 2-II. All viable bacterial counts were expressed by unit, CFU/round bur. A dentin sample was corrected from a molar before (Fig. 2-II) and after (Fig. 2-III, A) application of pH 3.5 buffer solution, and after application of PTW with buffer (Fig. 2-III, B). Each sample correction was performed at depth of 0.8, 1.6, and 2.4 mm. Thus, total of 135 dentin samples were corrected from 15 molars.

**Confirmation of the dentin sample site and depth by means of computed tomography (CT) images**

The sample site and depth obtained from the infected dentin model with the round bur were confirmed on the CT image (inspeXio SMX-225CT, 140 kV, 70 μA; SHIMADZU, Kyoto, Japan).

**Statistical analysis**

Data obtained were statistically analyzed using IBM SPSS Statistics Version 19 (IBM, Armonk, NY, USA). All samples were obtained as three groups (viz., before and after application of buffer solution, and after application of PTW) per one molar. Data was analyzed among the same depth, using Wilcoxon’s signed-rank test ($\alpha=0.01$).

**RESULTS**

**Antibacterial activity of PTW against S. mutans**

As shown in Table 1, the antibacterial activity of PTW against S. mutans was determined. The MIC and MBC of PTW at pH 3.5 and 25ºC corresponded to a 1/40 dilution of the PTW stock solution. Alternatively, no antibacterial activity was observed at pH 6.5. Antibacterial activity of NaClO was also confirmed. The MIC and MBC of NaClO was 1/2,560 dilution of the working concentration (23.4 mg/L). To confirm the reduction of the bactericidal capability of PTW at 37ºC, the PTW was pre-incubated at 37ºC for 30 s; this deactivated bactericidal capability of the PTW. In contrast, NaClO demonstrated the same MIC and MBC irrespective of pre-incubation at 37ºC.

**Confirmation of the dentin sample site and depth by means of computed tomography (CT) images**

The sample site and depth obtained from the infected dentin model with the round bur were confirmed visually. As shown in Fig. 3, the sample was obtained

### Table 1  Antibacterial activity of PTW against S. mutans determined by the liquid dilution method. MIC and MBC of PTW and NaClO are presented as reciprocals of the highest dilution

<table>
<thead>
<tr>
<th></th>
<th>PTW</th>
<th>NaClO</th>
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<tr>
<td></td>
<td>pH3.5</td>
<td>pH6.5</td>
</tr>
<tr>
<td>Without pre-warming</td>
<td>1/40*</td>
<td>–</td>
</tr>
<tr>
<td>With pre-warming</td>
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*: MIC and MBC were detected as the same value
—: Inhibitory effect was not observed.

![Fig. 3](image)  
**Fig. 3**  
CT images of the tooth specimen after the experiment.  
As shown in occlusal view dentin samples under three different conditions were collected per tooth. Sampling depth was shown in the lateral view.  
a: Before application of pH 3.5 buffer solution (Fig. 2-II)  
b: After application of buffer (Fig. 2-III)  
c: After application of PTW with buffer (Fig. 2-III)
Fig. 4 Disinfection effects of PTW.
A. PTW disinfection effects seen after various application times. Viable bacteria count (CFU/round bur) before/after PTW application with pH 3.5 buffer solution after various application times (10, 20, and 30 s).
B. PTW disinfection effects at various depths. There were no statistically significant differences in the viable bacteria count (CFU/round bur) before and after buffer application, at each sampling depth, while the count was markedly reduced after PTW application at all dentin depths. The bur connected with horizontal lines were not statistically different.

A B

Disinfection effects of PTW in the S. mutans-infected dentin model
As shown in Fig. 4-A, before application of buffer or PTW, the viable bacterial count was $10^5$–$10^6$ CFU/round bur. No statistical differences were found in the viable bacterial count between the groups before and after application of pH 3.5 buffer solution. After application of PTW for 10, 20, and 30 s, the viable bacterial counts were less than the detection limit (2.5 CFU/round bur).

Disinfection effects of PTW at various sampling depths in the S. mutans-infected dentin model
As shown in Fig. 4-B, before application of pH 3.5 buffer solution, the viable bacterial counts were $3.1\pm1.8\times10^5$, $5.6\pm3.2\times10^4$ and $2.0\pm1.6\times10^4$ CFU/round bur at depths of 0.8, 1.6 and 2.4 mm, respectively. After application of buffer, the viable bacterial counts were $2.2\pm1.0\times10^5$, $5.4\pm2.6\times10^4$ and $1.6\pm1.0\times10^4$ CFU/round bur at depths of 0.8, 1.6 and 2.4 mm, respectively. There was no statistical difference in the viable bacterial count between the groups above. After the application of PTW for 10 s, the viable bacterial count was less than the detection limit (2.5 CFU/round bur) at 0.8 mm depth, $3.0\pm5.0$ CFU/round bur at 1.6 mm, and $3.0\pm5.0$ CFU/round bur at 2.4 mm depth. Significant differences were found between the groups with and without buffer application vs. the group with PTW application.

DISCUSSION
Plasma irradiation requires 180 s to sterilize an S. mutans infected dentin model\(^{19}\), while the method using PTW in this study resulted in a significant decrease, after only a 10 s application. It is clinically more realistic to apply PTW for 10 s, using an extremely simple method than to apply plasma irradiation for 180 s by means of extensive plasma-generating equipment at the dental chair-side. Moreover, a high concentration of ozone and other gases with unknown active species could be avoided. Bactericidal activity of PTW can be preserved at low temperature\(^{19}\), in other words key bactericidal ingredient in PTW, which is supplied to distilled water by plasma, is stored (not deactivated) if the temperature is low enough. This means longer plasma irradiation to distilled water brings higher concentration of PTW. Although the application of PTW to infected dentin model is only 10 s, the exposure time of plasma to prepare PTW is much longer. So successful disinfection can be achieved by shorter treatment time of the model with PTW than the direct plasma irradiation.

The bactericidal effect demonstrated by plasma irradiation is considered to be due to the superoxide anion radical (O$_2^-$•)\(^{20}\) and the same mechanism may underlie the bactericidal effect of PTW\(^{19}\). O$_2^-$• is a highly reactive compound. Biologically, it can be produced by the catalytic function of enzymes and during hemoglobin oxidation. Plasma irradiation generates active species and their precursors that are absorbed into pure water, and the only water-soluble and relatively long-lasting active species in PTW. In fact, Ikawa et al.\(^{19}\) proved the existence of active species by means of electron spin resonance signals with spin trapping agent and the mechanism by which these are generated. Ikawa et al.\(^{20}\) also provided an explanation for the sterilization mechanism: this
involved further conversion of $O_2^\cdot$ into conjugate acid of HOO$^\cdot$ (O$_2^\cdot$+H$^+$→HOO$^\cdot$, pKa 4.8), particularly under low-pH conditions, delivering a stronger sterilization effect. For this reason, we used PTW with a buffer solution (pH 3.5) in this study. Application of the PTW solution at pH 3.5 had bactericidal effects; however, no bactericidal activity was demonstrated at pH 6.5 (Table 1), indicating that the effect is exerted by PTW under low-pH conditions.

Based on the results of heat deactivation of PTW at 37°C and the bactericidal effect of PTW at low pH, the main active species is likely to be a precursor of $O_2^\cdot$. The results shown in this study agreed well with those of Ikawa et al.\textsuperscript{19}. Furthermore, acidity levels similar to those (pH 3.5) that were shown to be effective in the present study are present in various acidic monomers used for resin adhesives that are widely employed in daily practice. Therefore, negative effects are likely to be negligible within the 10 s application required for bactericidal effects.

Various bacterial strains have been isolated from actual dentin carious lesions\textsuperscript{22}–\textsuperscript{25}, however, the present study focused only on S. mutans, which has high caries-pathogenicity\textsuperscript{22}. Yamazaki et al.\textsuperscript{17} demonstrated that plasma irradiation of bacterial suspensions of Candida albicans and Enterococcus faecalis as for S. mutans resulted in equivalent sterilization; therefore, the plasma sterilization effects obtained in the present study will likely be replicable for other cariogenic pathogens found in carious lesions.

Plasma sterilization was targeted at the “outer layer of carious dentin” encountered in the clinic. In a clinical setting, infected dentin needs to be completely removed during standard caries treatment. We confirmed the softness of the targeted dentin using a spoon excavator and heavy staining using a caries-detector solution (Caries Detector, Kuraray Noritake Dental, Tokyo, Japan).

In order to confirm whether the sterilization effect of PTW worked across various depths, infected dentin was sampled using a round bur of 0.8 mm diameter. This size was chosen after considering that a smaller size may generate less friction heat in the dentin and that a larger size would be more clearly visible and easy to handle during the experiment, particularly in sampling of the dentin at different depths (0.8, 1.6 and 2.4 mm). The round bur cut the dentin from the cavity floor to its greatest depth, and the dentin material that adhered to the grooves on the bur was recovered. Therefore, the sterilization effects of PTW must have extended not only to the surface of the cavity floor, but also to a depth of 1.6 and 2.4 mm below the floor, despite the fact that the infected dentin made contact with the PTW on the surface for only 10 s. The MIC test for PTW showed that the sterilizing effect disappeared rapidly with pretreatment of PTW at 37°C and remaining toxicity was not considered compared to NaClO. Therefore, we decided to use PTW without dilution. Thus, the reactive oxygen species clearly permeated the dentin; however, as their half-life is short, their residual activity within the dentin is limited and biological damage residual is unlikely.

In this study, we achieved complete sterilization by PTW application, with less than the detection limit of bacteria (2.5 CFU/round bur) found at a depth of 0.8 mm after a 10 s treatment. However, at a depth of 1.6 and 2.4 mm, sterilization was incomplete (3.0 CFU/round bur), although a remarkable reduction in bacterial concentration (5 log reduction) was observed. This may be improved by applying a higher concentration of reactive species, or applying the solution for longer. The present study is largely based on patented or patent-pending technology.

Sterilization of infected dentin using practical concentrations of drug solutions, such as NaClO, is not safe in a clinical situation because of the high concentration required\textsuperscript{23–25}. For example the Centers for Disease Control and Prevention has stated that NaClO at the concentration used in household bleach (5.25–6.15%) can produce ocular irritation, or oropharyngeal, esophageal, and gastric burns\textsuperscript{26}. Therefore, application of PTW for effective sterilization of caries-infected dentin represents a revolutionary treatment as a zero-intervention caries treatment, and holds great potential for future clinical implementation.

CONCLUSION

A 10 s application of PTW virtually completely deactivated S. mutans in an infected dentin model up to a 2.4 mm depth, demonstrating the same or better disinfection capability as shown by the reduced-pH method (low-temperature plasma jet under acidic conditions) that requires a 180 s plasma irradiation. For PTW application, no special equipment is needed at the chair-side. Moreover, PTW is regarded as biocompatible since its active oxygen species are rapidly inactivated at body temperature. We therefore consider that PTW holds great potential as an alternative to conventional instrumentation for a-traumatic treatment of infected dentin.

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