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Microbicidal efficacy of ozonated water against *Candida albicans* adhering to acrylic denture plates

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Background/aims: Ozone is known to act as a strong antimicrobial agent against bacteria, fungi, and viruses. We examined the effect of ozonated water on *Candida albicans* on acrylic denture plate.
Methods: The heat-cured acrylic resins were cultured with *C. albicans*. After treatment of flowing ozonated water, the number of attached *C. albicans* was counted. In some experiments, the test samples were treated with ozonated water in combination with ultrasonication.
Results: After exposure to flowing ozonated water (2 or 4 mg/l) for 1 min, viable *C. albicans* cells were nearly nonexistent. The combination of ozonated water and ultrasonication had a strong effect on the viability of *C. albicans* adhering to the acrylic resin plates. There were no significant differences in antimicrobial activity against *C. albicans* between plates immersed in ozonated water with ultrasonication and those treated with commercially available denture cleaners. In addition, electron microscopic analysis revealed that small amounts of *C. albicans* remained on the plate after exposure to flowing ozonated water or immersion in ozonated water with ultrasonication.
Conclusion: Our results suggest that application of ozonated water may be useful in reducing the number of *C. albicans* on denture plates.

Key words: *Candida albicans*; denture cleaning; denture plaque; disinfection; ozonated water

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Microbial plaque that accumulates on the fitted surfaces of dentures is composed of several oral microorganism species including *Candida albicans*, which is a major causative agent of denture-associated chronic atrophic candidosis. A recent study demonstrated that denture plaque control is essential for the prevention of denture stomatitis associated with *C. albicans* (1). In addition, the fitted surfaces of dentures have been shown to be reservoirs of *C. albicans*, which is associated with stomatitis and disseminated fungal infectious diseases, and it is well known that elderly hospital patients have a high risk of aspiration pneumonia induced by microorganisms on dentures (14, 17).

Several denture-cleaning methods are used clinically for the reduction of denture plaque, debris, and stains, and these are generally divided into mechanical and chemical cleaning methods. However, it has been reported that mechanical cleaning methods are insufficient for a complete reduction of microorganisms on denture plates (5). Mechanical methods have also recently been used in combination with magnetic stirrers, agitators, sonic vibrators, and ultrasonic devices. However, these methods have not been shown to clean dentures efficiently. Thus, it is considered that chemical cleaning methods are more effective and indispensable for dairy denture care, in which hypochlorites, peroxides, enzymes, and acids are generally employed as immersion-type chemical solutions for denture cleaning. However, some chemical agents used for denture cleaning are known to damage acrylic resin and metal alloy materials, and are also relatively expensive compared to ozonated water used in the present study (11). In this study we propose that it is possible to apply a great enough dose of ozonated water for denture cleaning using a continuous flow, and this method effectively saves expense in a home for the aged.

Ozonated water has been shown to be a powerful antimicrobial agent against bacteria, fungi, protozoa, and viruses (6).

During the formation of dental plaque, oral microorganisms first adhere to tooth and denture surfaces, and then grow in the prevailing environment. It is recognized that dental plaque is a kind of bacterial biofilm (8, 13) in which bacterial growth is the primary factor governing the relative abundance of different microorganisms including *C. albicans.*

Recently, we found that ozonated water was useful in reducing the number of infections caused by oral microorganisms, with gram-negative oral microorganisms somewhat more sensitive than oral streptococci and *C. albicans.* This suggested that oral microorganisms might be inactivated by ozonated water at different doses (10). It has been reported that ozonated water can be mutagenic if used for a long period and in high concentrations (12). It is important to determine the effective concentrations for denture cleaning to avoid hazardous side-effects of ozone. In the present study, we examined the antimicrobial efficacy of ozonated water against *C. albicans* on resin denture plates *in vitro*. Further, we sought to establish suitable conditions for the use of ozonated water to remove *C. albicans* from denture plates.

Material and methods

Growth conditions for *C. albicans* and preparation of test plates

C. albicans ATCC 18804 was cultured in yeast-mold broth (Difco, Detroit, MI) supplemented with glucose (100 μg/ml) at 37°C for 24 h, after which the broth containing *C. albicans* (2×10^7 colony-forming units/ml; CFU/ml) was immediately used as a fungus solution. The experimental denture plates were prepared using heat-cured acrylic resin (15 mm × 15 mm × 3 mm; Acron, GC Dental Industrial Corp., Tokyo, Japan) according to the manufacturer's instructions. The resin plates were ground with #180 emery paper, sonicated in water for 60 min, and immersed in water for 1 day to remove the residual monomer, then dried in air and sterilized with formaldehyde gas overnight. They are then stored for 6 months to completely remove resin monomer. The resin plates were incubated with the fungus solution at 37°C for 120 min without agitation, then gently removed and washed three times with sterile saline to remove loosely attached microorganisms. Finally, the plates were stored in sterile Petri dishes at room temperature for 20 min and used in the experiment.

Cleaning procedures

Ozonated water was generated by Neo Ozone Water-S (KORM Electronics, Atsugi, Japan). The concentration of ozonated water in the aqueous phase was determined by a Portable Ozone Monitor (OM-101P; Applics Co., Ltd, Tokyo, Japan) and adjusted to concentrations of 0.5, 2, or 4 mg/l. All test plates were washed three times with sterile saline and immersed in 150 ml of ozonated water (0, 0.5, 2 or 4 mg/l) for 1, 5, 10, 30 or 60 min. In some experiments, all plates were directly washed with several concentrations (0, 0.5, 2 or 4 mg/l) of flowing ozonated water for 1 min at a flow rate of 2 liters/min.

To determine the effect of ozonated water in combination with ultrasonication over a short period of time, the test plates were washed three times with sterile saline and immersed in 150 ml of several concentrations of ozonated water, and sonicated using an ultrasonic device (SW7800, Japan CBM Co., Tokyo, Japan) for 1 min.

Comparison of antimicrobial activity between ozonated water and denture cleansers

A test plate was washed three times with sterile saline. The prewashed plate was sonicated with the ultrasonic device for 1 min in 150 ml of ozonated water (0 or 4 mg/l). The other test plate was immersed in commercially available denture cleansers, such as Polydent® (enzymes; Black Drug Co., Inc., Jersey City, NJ), Pika® (Red) (hypochlorites; Rohto Pharmaceutical Co., Ltd, Osaka, Japan) and Pika® (Blue) (enzymes; Rohto Pharmaceutical Co., Ltd) according to the manufacturer's instructions. Antimicrobial activity was determined as described below.

Determination of antimicrobial activity of ozonated water

After the experiments described above, the plates from each experiment were placed into a centrifugation tube containing 3 ml of sterile saline, and sonicated in an ultrasonic device (SUC-110, Shofu Inc., Kyoto, Japan) for 15 min to release the attached *C. albicans*. In a preliminary test, we confirmed that 95% of the *C. albicans* cells could be recovered by this ultrasonication (15 min). The samples (10 μl) were then cultured on YM agar plates at 37°C for 24 h, after which the number of CFU was determined using the spread plate method.

Electron microscopic observation

After being treated with ozonated water, some test plates from each experiment were fixed with 2.5% (wt/vol) glutaraldehyde in phosphate-buffered saline (PBS; pH 7.2) and dried in air for 72 h. Subsequently, each plate was coated with gold using ion sputter methods (E-1030; Hitachi Co., Ltd, Tokyo, Japan), before being observed with a scanning electron microscope (S-3300 N, Hitachi Co., Ltd).

Results

Microbicidal efficacy of ozonated water against *C. albicans* on resin plates

We examined the effect of immersion in ozonated water on the toxicity of *C. albicans* adhering to resin denture plates. When the plates were washed three times with sterile saline to remove loosely attached *C. albicans and* then immersed in ozonated water (4 mg/l) for 1 min, there was a slight reduction in the number of *C. albicans* (Fig. 1A). Next, we examined the effect of immersion time in ozonated water on the toxicity of *C. albicans*. As shown in Fig. 1B, the viability of *C. albicans* was time-dependently decreased when the *C. albicans*-adhering plates were immersed in ozonated water (4 mg/l). However, it takes more than 30 min for ozonated water to achieve complete microbicidal efficacy. We also examined the cytotoxic activity of flowing ozonated water on *C. albicans* adhering to the resin plates. Such treatment significantly reduced the number of adhering *C. albicans*, even with 0.5 mg/l of flowing ozonated water at a flow rate of 2 liters/min for 1 min (Fig. 2). The combination of ozonated water (2 or 4 mg/l) and ultrasonication had a strong effect on the viability of *C. albicans* adhering to the test plates (Fig. 3).

Comparison of microbicidal efficacy between ozonated water and denture cleansers

We compared the microbicidal efficacy of ozonated water with commercial available denture cleansers [Polydent®, Pika® (Red) and Pika® (Blue)] against *C. albicans* attached to the denture plates. Treatment with ozonated water (4 mg/l) in combination with ultrasonication, Pika® (Red), or Pika® (Blue) eradicated almost all the viable *C. albicans* cells. However, treatment with Polydent® had only a weak effect on *C. albicans* (Fig. 4).

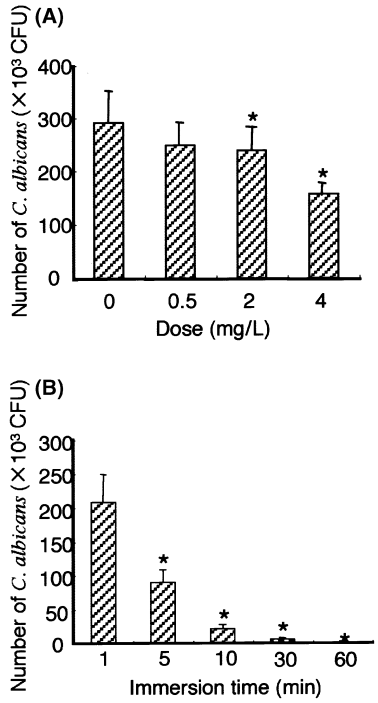

Fig. 1. Microbicidal efficacy of ozonated water against *C. albicans* on the resin plate. A) The test plate was washed three times with sterile saline and immersed in 150 ml of several concentrations of ozonated water for 1 min. Antimicrobial activity against *C. albicans* was determined as described in Material and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. **P* < 0.01(Student's *t*-test) compared with the number of *C. albicans* cells which were immersed in 150 ml of ozonated water (0 mg/l) for 1 min. B) The test plate was washed three times with sterile saline and immersed in 150 ml of ozonated water (4 mg/l) for 1, 5, 10, 30 or 60 min. Antimicrobial activity against *C. albicans* was determined as described in Material and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. **P* < 0.01(Student's *t*-test) compared with the number of *C. albicans* cells immersed in ozonated water (4 mg/l) for 1 min. The experiment was performed three times and similar results were obtained in each experiment.

Electron microscopic observation of *C. albicans* on the resin plates

The morphologic profile of *C. albicans* attached to the resin plates was examined using scanning electron microscopy. Many *C. albicans* cells were detected on the test plates, even when the prewashed plates were immersed in ozonated water (4 mg/l) for 1 min. On the other hand, treatment with flowing ozonated water (4 mg/l) and ultrasonication in ozonated water (4 mg/l) reduced the number of *C. albicans* cells remarkably (Fig. 5).

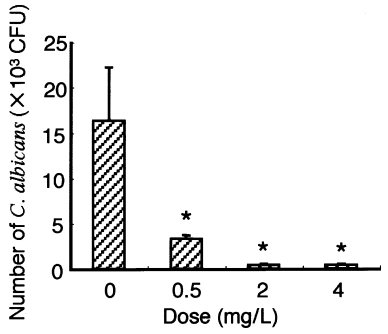

Fig. 2. Effect of flowing ozonated water against *C. albicans* on the resin plate. The test plate was directly washed with several concentrations of flowing ozonated water (0, 0.5, 2 or 4 mg/l) for 1 min at a flow rate of 2 l/min. Antimicrobial activity against *C. albicans* was determined as described in Material and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. **P* < 0.01(Student's *t*-test) compared with the number of *C. albicans* cells which were washed with flowing ozonated water (0 mg/l) for 1 min at a flow rate of 2 liters/min. The experiment was performed three times and similar results were obtained in each experiment.

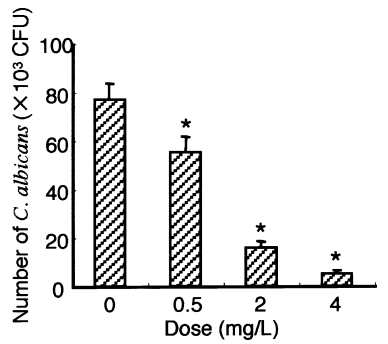

Fig. 3. Microbicidal efficacy of ozonated water in combination with ultrasonication against *C. albicans* on the resin plate. The test plate was washed three times with sterile saline and sonicated with the ultrasonic device for 1 min in 150 ml of several concentrations of ozonated water. Antimicrobial activity against *C. albicans* was determined as described in Material and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. **P* < 0.01 (Student's *t*-test) compared with the number of *C. albicans* cells immersed in ozonated water (0 mg/l) and sonicated for 1 min. The experiment was performed three times and similar results were obtained in each experiment.

Discussion

With the onset of the 21st century, infectious disease specialists are being asked to manage a greater number of patients with serious infections who are over 65 years old, and are gaining a new perspective on the emerging problem of geriatric infectious diseases. In 1997, Yoshikawa (18) presented a brief overview of the occurrence of geriatric infectious diseases during 1982–97, and noted their future direction along with aging. Recently, because of growing interest, there have been a great number of clinical studies of a variety of infectious diseases in geriatric populations, and it has come to be generally accepted that the etiology, clinical manifestations, therapy, and prognosis of pneumonia in elderly patients are quite different than in younger adults (9).

In the dental field, it is necessary to find innovative methods and techniques that can prevent, reduce, or eliminate oral microorganism colonization in elderly patients who have denture plates (18). Further, it has been reported that there is a strong association between poor denture hygiene and oral candidal colonization (4). Results of another recent study indicated that compromised elderly patients above 70 years of age had a higher number of *C. albicans* than patients in their 60s (7). These findings suggest that plaque accumulation on the dentures of handicapped elderly patients could create an appropriate environment for the growth of *C. albicans*.

Many researchers have reported that surface roughness is a key factor in the entrapment of microorganisms on denture surfaces. Verran and Maryan (15) found that an increase in surface roughness facilitated *C. albicans* retention on acrylic resin surfaces. However, few microbiological studies have been conducted to develop a cheap and reliable apparatus that is easy to use. In the present study, we prepared the resin plates (15 mm × 15 mm × 3 mm) with a rough surface to create an appropriate environment for the growth of *C. albicans*, and examined the effect of ozonated water on the viability of *C. albicans* in order to understand its effect on dentures.

It is known that ozonated water is very effective against bacteria, fungi, and viruses (6). Since the efficacy of disinfectants is usually evaluated on the basis of the reduction of cultivable microorganisms, we examined the microbicidal activity of an ozonated water produced by Neo Ozone Water-S and found that a range of 0.5–4 mg/l was highly effective for killing oral microorganisms, including mutans streptococci, periodontopathic bacteria and *C. albicans* in pure cultures (10). These findings suggest that ozonated water might be useful to control oral infectious microorganisms.

Ozonated water can be used as a soaking or flowing solution for medical

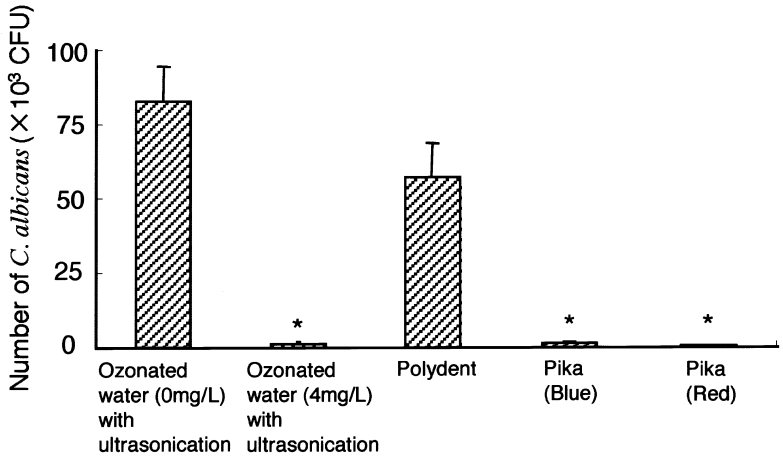

Fig. 4. Comparison of antimicrobial activity between ozonated water and denture cleansers against *C. albicans* on the resin plate. The test plate was washed three times with sterile saline. The prewashed plate was sonicated with the ultrasonic device for 1 min in 150 ml of ozonated water (0 or 4 mg/l). A prewashed plate was also immersed in commercially available denture cleansers, such as Polydent®, Pika® (Red) and Pika® (Blue) according to the manufacturer's instructions. Antimicrobial activity against *C. albicans* was determined as described in Material and methods. Data are expressed as the mean ± standard deviation of triplicate determinations. *****$P < 0.01$(Student's *t*-test) compared with the number of *C. albicans* cells treated with ozonated water (0 mg/l) in the combination with ultrasonication. The experiment was performed three times and similar results were obtained in each experiment.

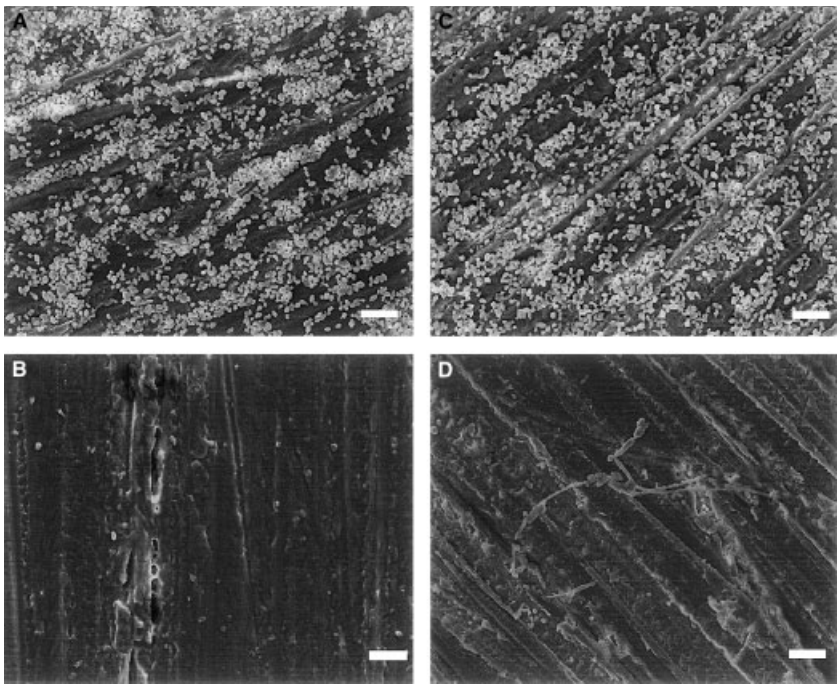

Fig. 5. Electron microscopic observation of *C. albicans* on the resin plates. The test plate was prewashed three times with sterile saline (A), prewashed and immersed in ozonated water (4 mg/l) for 1 min (B), treated with flowing ozonated water (4 mg/l) at a flow rate of 2 liters/min for 1 min (C), and prewashed and sonicated with the ultrasonic device in ozonated water (4 mg/l) for 1 min (D). After the treatment, the test plates were fixed with 2.5% (wt/vol) glutaraldehyde in PBS, coated with gold using ion sputter methods and observed with a scanning electron microscope. Scale bar = 20 μm.

and dental instruments if used properly. However, ozonated water can be mutagenic if used for a long period and in high concentrations (12). It is very important to provide clinical guidelines on the application and safe handling of ozonated water during denture cleaning. In the present study, we found that ozonated water produced by Neo Ozone Water-S had a cytotoxic effect on *C. albicans* when the test plate was prewashed with sterile saline (Fig. 1A). After such a prewash and immersion in ozonated water (4 mg/l) for 60 min, the viability of *C. albicans* was almost completely obliterated (Fig. 1B). Almost no viable *C. albicans* cells were detected after the plates were exposed to flowing ozonated water (2 or 4 mg/l) at a flow rate of 2 liters/min for 1 min (Fig. 2). Treatment with ozonated water (2 or 4 mg/l) in combination with ultrasonication (1 min) had a strong effect on the viability of *C. albicans* (Fig. 3). Further, the present results showed that there were no significant differences in microbicidal activity against *C. albicans* on the resin plates between ozonated water and the commercial denture cleansers Pika® (Red) or Pika® (Blue) (Fig. 4). Since it is possible to apply a high dose of ozonated water while preventing ozone from diminishing by using a continuous flow, a Neo Ozone Water-S apparatus might be useful to provide a continual disinfection environment.

It has been reported that ozone has a strong oxidizing power with a reliable microbicidal effect (2, 3, 12), and it is generally accepted that oxidation mediated by ozone destroys cell wall and cytoplasmic membranes of bacteria and fungi (16). After a membrane is damaged by oxidation, its permeability increases, causing the microorganism to die. As shown in Fig. 5, a scanning electron microscopic analysis revealed that almost no viable *C. albicans* cells remained on resin plates treated with flowing ozonated water or after immersion in ozonated water with ultrasonication. In the present study, electron microscopic observation revealed that many *C. albicans* were detached from the resin plates treated with flowing ozonated water (Fig. 5). These findings suggest that *C. albicans* is detached from the resin plate through functional and structural disorders in the cytoplasmic membranes.

In conclusion, we show that ozonated water was effective for killing *C. albicans* on resin denture plates. Further, a Neo Ozone Water-S apparatus is able to supply a large enough dose of ozonated water in a continual flow and should be useful for cleaning and disinfecting *C. albicans*-adhering denture plates. Although it is necessary to pay attention to the hazardous effects of a high dose of ozone, the ozone sensitivity data obtained in the present

study should provide guidelines on the application of ozone to kill *C. albicans* on denture plates.

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