Potency of ozonated alkali-ion water in inactivating cariogenic bacteria

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Purpose: This study assessed the effects of ozonated alkali-ion water (AW-OZ) on the caries inducing bacteria *Streptococcus sobrinus* (*S. sobrinus*), *Lactobacillus casei* (*L. casei*), and *Actinomyces naeslundii* (*A. naeslundii*) in their planktonic states.
Materials and Methods: Pellets of the above bacteria were prepared from fresh cultures and resuspended. Tokyo Metropolitan City tap water (TW) was used as the source of all experimental solutions and alkali-ion water (AW) was obtained by electrolyzing TW. An ozone delivery device was used to produce ozonated TW (TW-OZ) and AW-OZ. Phosphate buffered saline (PBS) and 0.05% sodium hypochlorite (NaOCl) were included as controls. The bacterial pellets were then inoculated with the solutions and stored at room temperature for 30 minutes. Viability of the bacteria was subsequently measured.
Results: Examining viability following staining with a BacLight viability kit, it was observed that all three species of cariogenic bacteria died in significantly larger numbers with TW-OZ and AW-OZ treatment compared to PBS, TW or AW. For *S. sobrinus* and *L. casei*, the above data were confirmed by viability counting and was also verified by turbidimetric analysis for *A. naeslundii*. However, sparging ozone inevitably reduces the pH levels of the solutions and that this reduction is very significant for TW-OZ. However, in AW-OZ, the pH could be maintained with low alkali levels (8.40).
Conclusion: Both TW-OZ and AW-OZ had an antibacterial effect on the three cariogenic biofilm forming bacteria and AW-OZ might be considered as the safer for human usage.
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Key Words: cariogenic bacteria, ozone, ozonated alkali-ion water.

Introduction

Ozone, in either the gaseous or aqueous phases, has strong oxidizing power with a reliable microbicidal effect.[1,2] 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.[3] The use of ozonated water for treatment of endodontic infections, sterilizing of cavities, root canals, and periodontal pockets has been suggested.[4-6] Nagayoshi et al.[7] observed that ozonated water produced significant reduction in *Enterococcus faecalis* ATCC 29212 and *Streptococcus mutans* Ingbritt viability compared with control samples and have shown nearly the same antimicrobial activity as 2.5% NaOCl during irrigation, especially when combined with ultrasonication. Moreover, one of the major environmental advantages of ozone is its low cytotoxicity, which, in clinical situations, can be caused by a rapid degradation of ozone just after contact with organic compounds.

The oral cavity, one of the most anatomically complex parts of the human body consists of teeth, periodontal tissue, tongue, mucosa as well as secretary organelles and harbors a heterogeneous microbial community. To maintain a healthy oral environment, it is important to keep the normal oral flora predominant relative to pathogenic microorganisms. There are several well established oral care methods now being employed in addition to routine tooth brushing in maintaining good oral health. These approaches primarily target pathogenic microbes in the oral cavity. The most prevalent caries inducing pathogens have now being identified after nearly a century of continuous research.[8] Namely, the mutans streptococci group of oral spreptococci as well as

lactobacillus species have been primarily associated with human dental caries. More recently, actinomyces species have been associated with root caries and root surface biofilm formation.[9,10] However, a simple practical procedure has not yet been developed to specifically control these pathogens.

As ozone has been shown to be an effective disinfectant in both gaseous and diluted forms, it is of interest to develop safe ozonation procedures for use in the treatment of medical conditions. Among the potential risk factors involved in using ozonated water for oral irrigation or mouth rinses is the alteration of pH. Although several reports have discussed toxicity of ozone for soft tissue,[11] only a few reports have evaluated the effects of ozonated water on pH and on hard tooth structures. The pH at which tooth demineralization beings is known as the clinical pH and is in the vicinity of pH 5.0 to 5.5.[12,13] It was also reported that low pH mouth rinses and soft drinks have variable potential in inducing erosion of enamel.[14-16] Therefore, it is important to control the pH in the oral environment.[14] From our experience, it was observed that the pH falls instantly when ozone is dissolved in water. In many instances the pH falls bellow 4.0 when large amounts of ozone are diluted in aqueous solutions, i.e., tap water (TW) or Milli-Q water. An alkali-ion-water solution with a high alkaline pH range (7.5 to 10.5) is being used as drinking water to minimize acidity in the stomach, to maintain normal blood oxygen levels as well as for other health maintenance purposes. An alkali-ion water electrolyzer is a commercial device which is designed to prevent the generation of health-hazardous substances when tap water is used as an electrolytic solution during water electrolysis. For example, trichloromethane, acetoaldehyde and tributylmethane produce so-called alkali-ion-water (potable alkaline electrolyzed water), a catholyte that contains supersaturated hydrogen. Recently, medical and biochemical studies have clearly indicated that the drinking of alkali-ion–water is good for one's health.[17,18] It was expected that ozonated alkali-ion water (AW-OZ) would have nearly the same effects on planktocnic bacteria as ozonated water (TW-OZ). Therefore, the present study was designed to evaluate the effects of AW-OZ on caries inducing bacteria including representatives of the mutans streptococci, lactobacilli and actiomyces in their planktonic states.

Materials and Methods

Preparation of bacterial suspensions

Laboratory strains of three cariogenic bacteria *Streptococcus sobrinus* 6715 (*S. sobrinus*), *Actinomyces naeslundii* ATCC12104 (*A. naeslundii*), and *Lactobacillus casei* IAM12473 (*L. casei*) were used in this study. The baseline suspensions of *S. sobrinus* in phosphate buffered saline (PBS) at OD_{500}=2 were prepared from 16 hours fresh cultures in Brain Heart Infusion (BHI, Becton Dickinson, Sparks, MD, USA) broth after washing three times with PBS, and stored at 4˚C with gentle stirring. The suspensions of *A. naeslundii* in PBS at OD_{500}=2 were prepared after 48 hours anaerobic culture in BHI broth and washed three times with PBS for storage at 4˚C with gentle stirring. Suspensions of *L. casei* in PBS at OD_{500}=2 were prepared from 48 hours fresh cultures in Lactobacilli MRS Broth (MRS, Becton Dickinson, Sparks, MD, USA) after washing three times with PBS and stored at 4˚C with gentle stirring.

Solution preparation

Solutions used in this study are listed in Table 1. TW was collected from Tokyo Medical and Dental University COE laboratory tap supplied by the Bureau of Waterworks, Tokyo Metropolitan Government. AW was produced by using a water electrolysis device (TK7705, National, Osaka, Japan) using the same TW. The solutions (5 mL each) were sparged with ozone gas from an ozone-generating device (HealOzone, Kavo,

Biberach, Germany) with a range of 300-360 s (approximately 1000 mg/L) and ozonated TW (TW-OZ) and AW-OZ were obtained. PBS, which maintains bacteria at a static condition for several hours and a solution of sodium hypochlorite (NaOCl), a strong antibacterial agent, were also used as controls in this study. The pH and oxidation-reduction potential (ORP) data of the experimental solutions (Table 1) were measured following filtration with a 0.22 μm Millipore filter.

Table 1. Materials investigated.

Solution	Abbreviation	pH	SD	ORP	SD
Phosphate buffered saline	PBS	7.30	0.00	N.D.	
Tap water	TW	7.26	0.25	12.67	11.72
Alkali-ion water	AW	10.54	0.08	-171.67	4.70
Ozonated tap water	TW-OZ	3.50	0.32	204.67	15.63
Ozonated alkali-ion water	AW-OZ	8.40	0.26	-62.33	10.97
0.05% Sodium hypochlorite aq.	NaOCl	10.31	0.05	207.00	0.00

Inoculation of bacteria with the solutions

From the above suspensions of *S. sobrinus*, *A. naeslundii*, and *L. casei,* 1.0 mL aliquots were prepared just before use. After centrifugation, the supernatants were discarded and the pellets were inoculated with 1.0 mL of the each of the test solutions. After 30 minutes incubation, the microtubes were centrifuged, the supernatants discarded, and the pellets resuspended in 1.0 mL of fresh and filtered PBS to analyze the inoculation effects of the different solutions.

Bacterial viability testing after incubation using BacLight straining.

A LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Invitrogen Detection Technologies, Carlsbad, CA, USA)[19] was used to verify potential inoculation effects of the solutions on bacterial cells. One half (500 μL) of each bacterial sample was resuspended in PBS and transferred into dark microtubes followed by staining with 0.5 μL BacLight stain (a mixture of SYTO 9 and propidium iodide). In this staining system, viable bacterial cells exhibit green fluorescence, whereas only nonviable bacterial cells exhibit red fluorescence,[20,21] which allows bacterial cells to be distinguished according to the permeability of the cytoplasmic membrane.[22] The excitation/emission wavelengths of the dyes were approximately 480/530 nm for SYTO 9 (green signals) and 520/580 nm for propidium iodide (red signals). The bacterial cells were observed under a fluorescence microscope (CKX41, Olympus, Tokyo, Japan) using an objective lens (x60).

Colony forming units of *S. sobrinus* and *L casei*

Each bacterial cell suspension (500 μL) was serially diluted (x1,000) and homogenized (UP50H, Dr. Hielscher GmbH, Baden-Wuerttemberg, Germany) before plating with a spiral plating instrument (Eddy Jet, IUL, Barcelona, Spain). *S. sobrinus* samples were plated on Mitis Salivarius (MS) agar medium (Becton Dickinson, Sparks, MD, USA). *L. casei* samples were plated on Rogosa agar medium (Becton Dickinson). All plates were incubated for 48 hours under anaerobic conditions at 37˚C. After 48 hours, the number of colony-forming units (CFUs) was counted with the aid of a microscope.

Turbidimetric analysis of viable *A. naeslundii*

The remaining 500 μL of fluid inoculated with *A. naeslundii* samples were initially homogenized, 100 μL from each sample suspension was then inoculated into 4 mL of BHI broth medium and cultured under anaerobic conditions for 48 hours at 37˚C. They were then vortexed vigorously and 100 μL of each bacterial cell

suspension was transferred into separate wells of 96-well flat-bottom microplate to quantify bacteria by turbidimetric analysis (OD_{500nm}) with a Biotrak II plate reader (Biochrom, Cambridge, UK). A baseline enumeration and controls were carried out as described above.

Statistical analysis

All numerical data were analyzed using the Statistical Package for the Medical Science (SPSS Ver.11 for Windows, Chicago, IL, USA) for statistical procedures. Bacterial viability tests were analyzed using two-way ANOVA and Bonffroni test at 95% level of confidence. Colony forming units and turbidimetric analysis of viable cells were analyzed using Mann-Whitney's U test. All experiments were repeated three times under the same conditions to insure reproducibility.

Results

Fluorescence microscopic observations

BacLight viability data for each sample are summarized in Table 2 and representative photomicrographs are shown in Fig. 1. Clearly, more live cells are visible than dead cells in all groups except for the bacterial cells treated with NaOCl. Essentially all of the cells were killed following NaOCl treatment. Only 1.97% *S. sobrinus*, 2.85% *L. casei,* and 6.98% *A. naeslundii* died after 30 minutes incubation with PBS. Almost the same percentages with similar ratios of dead cells were detected in the case of ozone-free TW. Compared to that, there was a significant increase in the percentage (an average of 10%, $p<0.05$) of dead cells when TW-OZ was used. A similar pattern was also observed in the case of AW-OZ treated bacteria and significantly more bacteria were killed compared to TW and AW ($p<0.05$). Interestingly, more live cells were counted when the bacterial cells treated with AW compared to TW (except for *A. naeslundii*) as well as for bacterial cells treated with AW-OZ compared to TW-OZ ($p<0.05$).

Live bacterial cells are visualized as green and dead cells are red in the same microscopic image following excitation with blue light (Fig. 1). Microscopic images clearly show that large numbers of *S. sobrinus* cells remain alive after 30 minutes inoculation with AW and TW compared to AW-OZ and TW-OZ, indicating more bacteria died following ozone treatment.

Table 2. Mean averages (%) of dead (red) cells, counted from four micro-photographic images taken by a fluorescent microscope stained with LIVE/DEAD BacLight viability kit.

		S. sobrinus			*L. casei*			*A. naeslundii*		
Group	Solution	Mean	SD	Category	Mean	SD	Category	Mean	SD	Category
	PBS	1.97	0.66		2.85	1.60		6.98	1.34	
No-OZ group	TW	3.31	1.85	b, c	2.74	1.11	g	7.86	3.53	i
	AW	2.51	0.90	a, c	1.39	1.08	e	11.50	2.01	h
Oz group	TW-OZ	12.81	1.96	b, d	12.90	1.95	f, g	20.78	3.50	i
	AW-OZ	7.60	3.87	a, d	6.05	2.31	e, f	16.61	2.82	h
	NaOCl	101.97	0.00		102.85	0.00		101.78	3.74	

The same letters indicate statistically significant differences ($n=4$, $p<0.05$).

***S. sobrinus* and *L. casei* viable cell counts (CFU)**

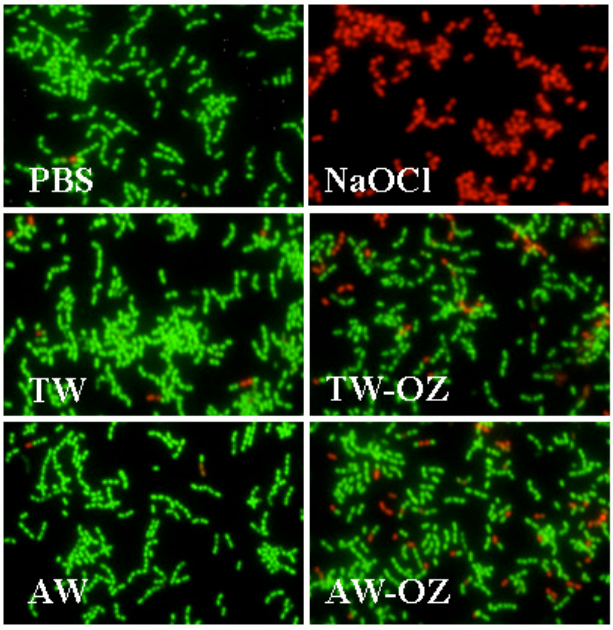
The viability of bacterial cells recovered from each sample is summarized in Table 3. In general, no

remarkable differences can be observed in the results between the bacterial species *S. sobrinus* and *L. casei.* The number of viable bacteria cells after incubation with all solutions used in this study compared to the baseline viable cell counts suspended in PBS or 30 minutes inoculation in PBS were similar. However, significantly more bacteria were viable when the cells were treated with AW and TW compared to AW-OZ and TW-OZ ($p<0.05$). The number of viable bacterial cells treated with NaOCl was lowest among all of the solutions, and almost no bacteria survived (positive control).

Table 3. Number of *S. sobrinus* and *L. casei* cells surviving after treatment.

	Solution	*S. sobrinus*		*L. casei*	
	PBS	3.99		5.44	
No-OZ group	TW	3.88	a	5.30	c
	AW	3.99	b	5.42	d
Oz group	TW-OZ	2.63	a	3.50	c
	AW-OZ	3.69	b	4.08	d
	NaOCl	0.00		0.00	

Data are expressed as the median log10 of colony-forming units per mL (CFU/mL).
The same letters indicate statistically significant differences as analyzed by Mann-Whitney's U test ($p<0.05$), n=3.
Data of TW-OZ and AW-OZ were compared with AW and TW, respectively.



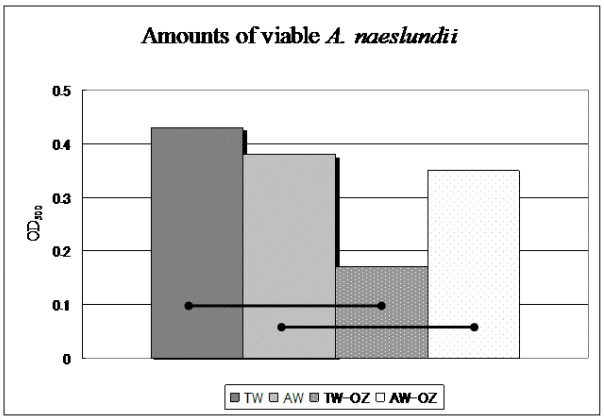


Fig. 2.
The amounts of viable *A. naeslundii* cells are shown in the graph. Data are expressed as the median. Horizontal bars indicates significant differences ($p<0.05$), n=4. Data of TW-OZ and AW-OZ were compared with TW and AW, respectively. Mann-Whitney's U tests revealed that both TW-OZ and AW-OZ had significantly less viable bacteria compared to TW and AW, respectively.

Fig. 1. Fluorescence photomicrography of *S. sobrinus* cells stained with a BacLight bacterial viability kit. Live cells are visible as fluorescing green and dead cells visible as red after 30 minutes inoculation in different solutions (PBS, NaOCl, TW, TW-OZ, AW, and AW-OZ).

Turbidimetric analysis of viable *A. naeslundii*

The amounts of viable *A. naeslundii* cells are after 30 minutes incubation are shown in the graph (Fig. 2). Mann-Whitney's U tests revealed that both TW-OZ and AW-OZ had significantly less viable bacteria compared to TW and AW, respectively. However, the difference between AW and AW-OZ was very small, while the difference between TW and TW-OZ was large. At the same time the median of NaOCl was 0.022.

Discussion

Ozone has been applied for water disinfection purposes for almost a century. There is frequent debate on the

role of OH radicals and ozone for disinfection processes in drinking water within the research community. Some authors state that ozone is the main disinfectant[20,21] while others suggest that OH radicals may play an important role for disinfection.[22,23] However, Von Gunten critically reviewed the role of OH radicals in the inactivation of *C. parvum oocysts* and *B. subtilis* spores and concluded that the effects of OH radicals in disinfection processes can generally be neglected.[24] Data in the present study indicated some induction of bacterial inactivation to some extent by AW water, which contains significant amounts of free OH radicals compared to the TW. On the other hand, ozone's inactivation potency has been well established after nearly a century of investigations. Although ozone is quite unstable when it is sparged into any solution, as was done in TW and AW in the present study, both ozonated solutions showed significant disinfection activity on three cariogenic bacteria. These results definitely indicate that incorporation of ozone into these solutions made a significant difference. To our knowledge, inactivation of *A. naeslundii* by ozonated water was observed for the first time in this study.

However, the antibacterial effect of ozonated water was still far less than that of NaOCl. According to previous reports, direct application of gaseous ozone appeared to be more effective than dissolved ozone in solutions, mainly because ozone decay begins to occur instantly when mixing is done. On the other hand, although in the oral cavity gaseous ozone could be used, obtaining sufficient access to specific target sites becomes difficult in many cases. Bocci has suggested that ozone can be toxic but, when properly used, can be medically useful.[25] The toxic effects of ozone are a function of the gas concentration and exposure duration. Risks always exist in delivering ozone gas directly to inner portions of the oral cavity because gaseous ozone can cause damage to the lung lobes if inhaled in large amounts. Instead, AW-OZ might be much safer for delivery to most parts of the oral cavity.

Clearly, TW-OZ had more antibacterial ability than AW-OZ, especially in case of *A. naeslundii*. Here, one important issue should be discussed; i.e., the pH and ORP levels of the ozonated water. In the present study, the pH and ORP of TW-OZ were below 4 (3.5 on average) and above 200 mV (204.67), respectively. These conditions are definitely problematic for use in the oral cavity. In contrast, the pH and ORP for AW-OZ were above 8 (average of 8.40) and below 1 (average of -62.33), respectively. These pH and ORP levels are considered to be much safer for periodic use in the oral cavity. Moreover, an unexpected characteristic of AW was explored recently (Gyo et al., 84th IADR, Brisbane, 2006; Abst. #1267), i.e., it can effectively dissolve glucan from the cariogenic biofilms. Therefore, a double benefit may be achieved from AW-OZ if it can dissolve glucan as well as mildly inactivate oral pathogens. This may be also important for maintaining the normal oral flora by not drastically reducing the levels of beneficial oral bacteria.

The results of the present study do not confidently indicate any possibilities of therapeutic use in the clinics, neither for TW-OZ nor AW-OZ. If considered for prophylactic use for gargling at home it can be suggested that AW-OZ should be preferred instead of using TW-OZ. Further evaluation will be necessary to draw firm conclusions in this regard.

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