

Microbicidal effect of ozone gas *in vitro*: interaction with organic compounds and endodontic irrigation agents

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Purpose: The aim of this study was to estimate the utility of an ozone gas generating apparatus (HealOzone) for endodontic treatment by its bactericidal efficiency *in vitro*.

Materials and Methods: Water- or TS broth-suspended microorganisms (*Streptococcus mutans*, *Lactobacillus casei*, *Enterococcus faecalis*, and *Candida albicans*) were exposed to ozone gas for up to 80 s. Serially diluted irrigation agents (H₂O₂, NaOCl) and *E. faecalis* were incubated at room temperature for a minute with or without ozone exposure (20 s). In both experiments, survived microbes were determined by semi-quantitative cultivation.

Results: Eighty second-exposure of ozone gas completely killed the microorganisms suspended in water. However, this treatment had no effect on the same microorganisms suspended in the culture broth, which contained the organic compounds. Combination of ozone gas exposure and H₂O₂ solution showed synergic effect to kill *E. faecalis* in water, but not in the broth. In contrast, ozone inhibited the bactericidal effect of diluted NaOCl solution against *E. faecalis* in water.

Conclusion: Based on the experimental results, probability of sterilization effect of ozone gas either used with or without root canal irrigation agents was doubtful. (Asian Pac J Dent 2012; 12: 21-26.)

Key Words: Healozone, hydrogen peroxide, microbicidal effect, ozone gas, sodium hypochlorite

Introduction

Ozone is one of the strongest oxidizer. It is used in sewage treatment to remove pollutions, and other industrial processes as a disinfectant, a bleaching agent, and a deodorant.¹⁻⁴ While these characteristics of ozone seem to be also useful for dental field, ozone inhalation at high concentration induces acute toxicity for human respiratory tract.^{5,6} Therefore, a system to avoid leakage of ozone gas to oral cavity is necessary for the dental treatment apparatus.

HealOzone (KaVo Dental GmbH, Biberach/Riß, Germany) is well known and the most safety one in this category.⁷ A handpiece of the apparatus releases the gas only when a silicon tip completely attaches to a treated tooth. After treatment, remaining ozone gas is rapidly exhausted from the area by a vacuum pump to prevent both patient and dentist from the gas exposure.

The major target of ozone gas therapy by HealOzone is pit and fissure caries.⁸ Since the traditional invasive process, "Drill and Fill", is stressful for patients, ozone and remineralization therapy for primary caries as minimally invasive dental treatment is very attractive.⁹ The clinical trials in this field have been mostly conducted in UK and the evaluation has been discussed.^{8,9}

For endodontic treatment, on the other hand, the manufacturer of HealOzone also provides a device to get the gas at periapical region. However, root canal has a complex structure and is rich in organic compounds, and the condition for ozone treatment for endodontics should be different from that for enamel caries on tooth surface. Diffused ozone gas in root canal might react with the remaining organic compounds that interfere with disinfectant effect of ozone. Furthermore, penetration efficacy of ozone gas into wet tissues is not established.

Based on the backgrounds, we planned the experiments *in vitro* to expect effectiveness of ozone gas application in clinical endodontic field. The purposes of this study were to investigate: a) microbicidal effect of

ozone gas on the various microorganisms with or without the organic compounds, and b) influence of coexistence of irrigation solutions and ozone gas on their bactericidal effect.

Materials and Methods

Ozone generating apparatus

HealOzone was used as the ozone treatment apparatus.

Microorganisms

Streptococcus mutans (*S. mutans*) ATCC25175, *Lactobacillus casei* (*L. casei*) ATCC393, *Enterococcus faecalis* (*E. faecalis*) ATCC19433, and *Candida albicans* (*C. albicans*) ATCC18804 were used. For all experiments, each test strain was inoculated into Tryptic soy broth (TS broth; Becton, Dickinson and Company, Sparks, MD, USA) and incubated at 37°C overnight under an aerobic condition. The culture was then centrifuged at 10,000 g for 1 minute. The cell pellet was washed twice by sterile water and then suspended in water as approximately 10¹⁰ cfu/mL. The sample was subdivided and stored at -80°C before use.

Disinfectant efficacy of ozone gas against various microorganisms

The frozen stock of microbial suspension was diluted by either water or TS broth to adjust the concentration to approximately 10⁶ cfu/mL for bacteria and 10⁵ cfu/mL for *C. albicans*. Each suspension was dispensed to the wells of a sterilized micro well plate (Sumilon, Tokyo, Japan) as 100 µL per well, and exposed to ozone gas for 0, 20, 40, and 80 s. After the period, each treated sample was diluted by TS broth and spread onto Tryptic soy agar plates. The amount of bacteria survived was determined by counting colonies grown on the plates after 48-hour incubation at 37°C.

Oxidation- and bacterial killing-extent of ozone gas exposed from agar layer surface

In all of the following experiments, *E. faecalis* was used as a test organism, which is frequently isolated from refractory apical periodontitis.¹⁰ The melting agar (2%) mixed with indigo carmine aqueous solution (0.01%, final) was solidified on a slide glass. Similarly, the *E. faecalis* (10⁶ cfu/mL, final) suspended-agar layer, with or without TS broth, was prepared. Then the surface of each agar layer was exposed to ozone gas for 120 s. The depth and diameter of decolorized area were measured on the indigo carmine gel immediately after exposure, and those of colony-free area in bacteria-suspended gel were determined after 48-hour incubation at 37°C. In the case of the water-prepared gel, melting agar containing two-fold concentrated TS broth was overlaid onto the surface to support bacterial growth within the gel.

Bactericidal effect of simultaneous exposure of ozone gas and irrigation agents

Solutions of sodium hypochlorite (NaOCl; 1%=10,000 ppm) and hydrogen peroxide (H₂O₂; 12%) were serially two-fold diluted either by water or by TS broth. *E. faecalis* suspension (approximately 10⁵ cfu/10mL either in water or in TS broth) was prepared from the stock bacterial suspension. The examinations of bactericidal effect for each dilution series in a micro well plate were performed under three different conditions as follows. 1) Neutralization control: Irrigation solution diluted (50 mL) and bacterial suspension (5 mL) were simultaneously added into 1 mL of 1% sodium thioglycolate solution (STG), which acts as a neutralizer. After a minute-incubation at room temperature, the mixture was serially tenfold diluted and plated onto TS agar plates. 2) Irrigation solution treatment: Bacterial suspension (10 mL) was added to the diluted irrigation solution (100 mL) in a well as the final bacterial inoculation size at 10⁶ cfu/mL, and incubated for a minute. After the treatment period, 50 mL of reaction mixture was added into 1 mL of STG. Following protocol was the same as

in 1). 3) Irrigation solution treatment with ozone: Bacterial suspension added in each irrigation solution was exposed to ozone gas for 20 s. Incubation of bacterium with the solution was continued for a total period of one minute, following the same protocol as that for 1). All agar plates were incubated at 37°C for up to 72 hours, and survived bacterial colonies were counted. For a statistic analysis of interaction of each irrigation agent and ozone, protocols 2) and 3) using the same chemicals in water were independently repeated six times. Survived bacterial colony counts after treatments were analyzed by Wilcoxon rank-sum test.

Results

Effect of ozone gas against various microorganisms with or without the organic compounds

Eighty second-exposure of ozone gas completely killed all microbes examined (*S. mutans*, *L. casei*, and *E. faecalis*; approximately 10^6 cfu/mL, and *C. albicans*; approximately 10^5 cfu/mL) suspended within water, but not within TS broth (Fig. 1).

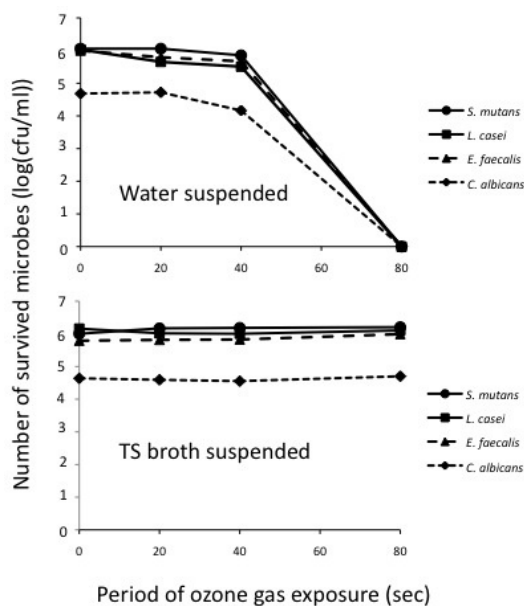


Fig. 1. Microbicidal effect of ozone gas against dental infection-related microbes. Eighty second-exposure of ozone gas killed test organisms suspended in water, but not those in TS broth.

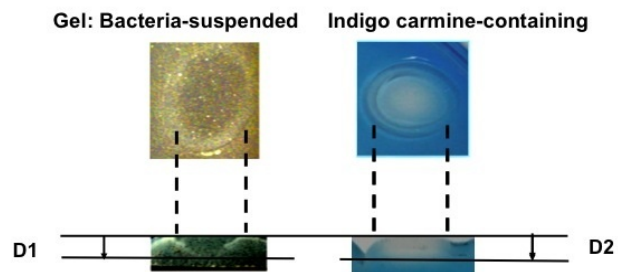


Fig. 2. Extent of bactericidal and oxidation effects of ozone gas on agar gels. Depths of inhibition zone (D1) and of Indigo carmine-decolorized zone immediately after exposure (D2) was matched; approximately 2 mm from the gel surfaces.

Oxidation- and bacterial killing-extent of ozone gas exposed from agar layer surface

Ozone gas exposure for 120 s was effective against *E. faecalis* suspended into the agar gel prepared with water, but not with TS broth. In the former case, the bactericidal area reached to approximately 2 mm depth from the gel surface, which was almost the same as that of the decolorized zone in Indigo carmine-containing gel after ozone exposure (Fig. 2). Diameters of effective zones of ozone were matched to that of the inner space of silicon cap.

Bactericidal effect of simultaneous exposure of ozone gas and irrigation agents against *E. faecalis*

Contamination of the organic compounds interfered the effect of both irrigation agents, especially of NaOCl as shown in Fig. 3. Ozone did not influence on the bactericidal effect of both irrigation agents diluted with TS broth. Under TS-free condition, ozone treatment for 20 s tended to increase the bacterial killing of H₂O₂ at 6%,

and on the other hand, to decrease that of NaOCl at 1.2 ppm. Results of additional examination performed for statistical analysis of these interactions were shown in Fig. 4. Ozone enhanced bactericidal effect of H₂O₂ at 6% (p<0.01) and 12% (p<0.05), and interfered that of NaOCl at 0.6 ppm (p<0.01) and 1.2 ppm (p<0.05).

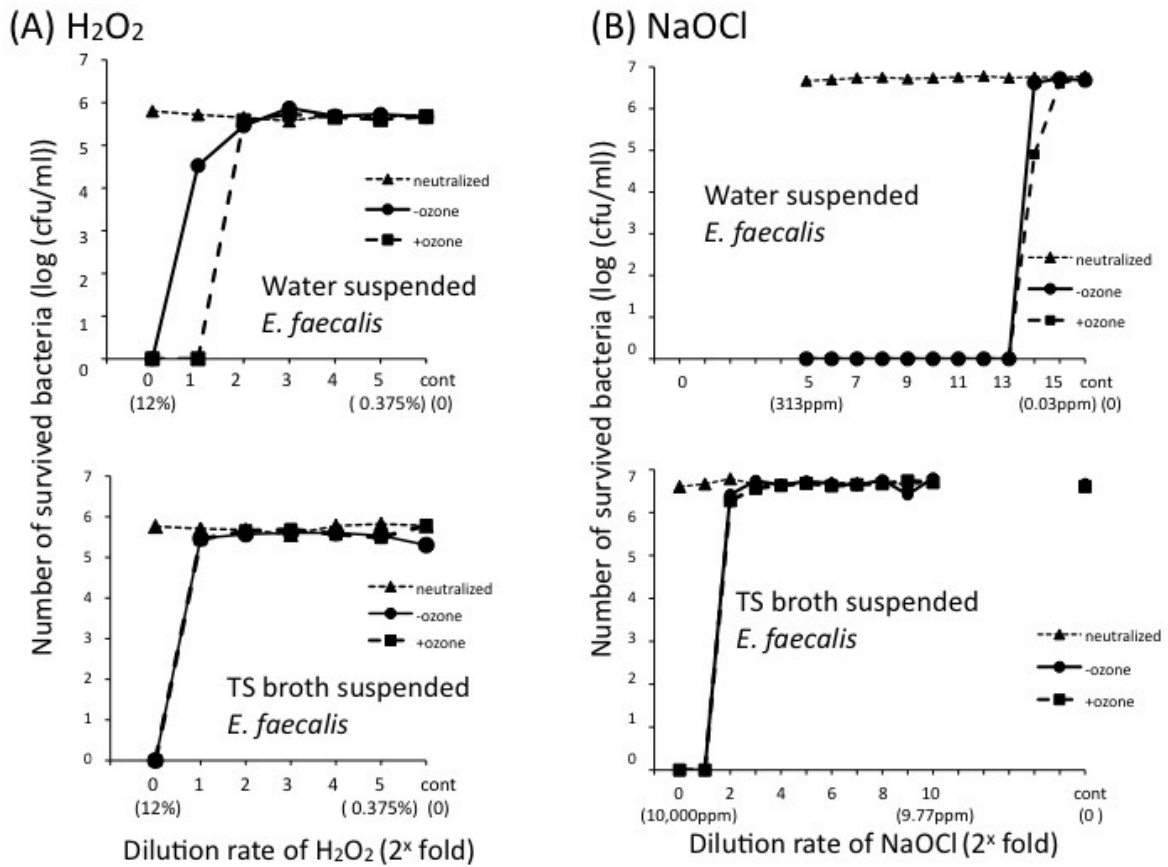


Fig. 3. Influence of ozone gas exposure on bactericidal effect of endodontic irrigation agents: A) H₂O₂, B) NaOCl. Each solution was serially 2-fold diluted using either water or TS broth. Neutralized: irrigants immediately neutralized; -ozone: irrigants alone; +ozone: irrigants with ozone.

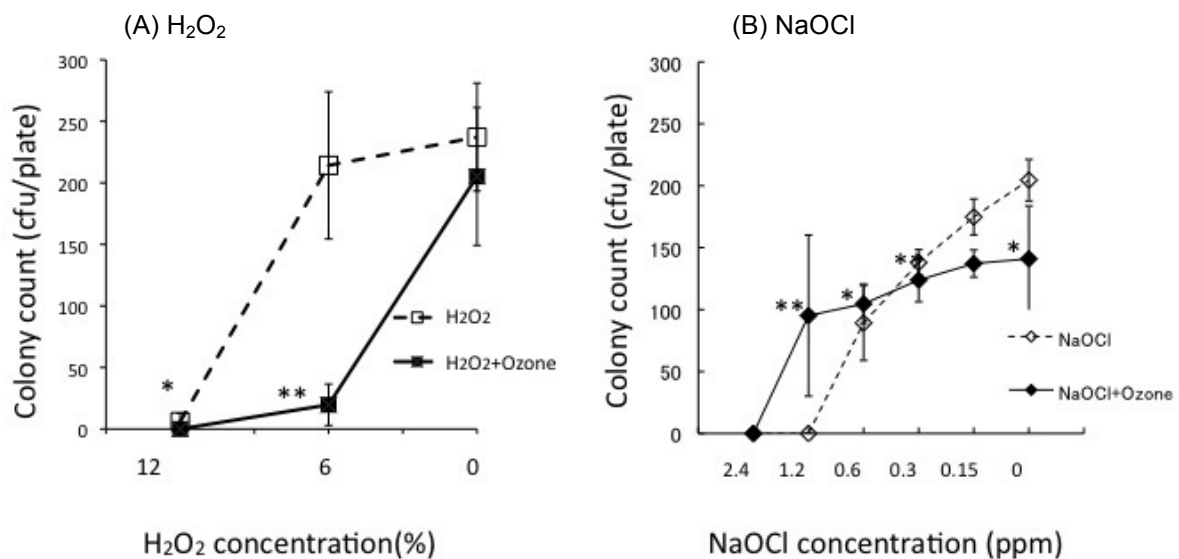


Fig. 4. Survived colony counts of *E. faecalis* after H₂O₂ (A)- or NaOCl (B)-treatment with or without ozone exposure. Maximum concentrations examined 12% for H₂O₂ and 10,000 ppm for NaOCl, respectively.

Discussion

As shown in Fig. 1, ozone gas exposure for 80 s completely killed 10^5 - 10^6 cfu/mL of various dental infection-related microorganisms suspended in water. However, coexistence of the organic compounds extremely interfered its ability as a disinfectant. These results simply reflect a basic characteristic of ozone gas: it is a non-specific oxidizer. In clinical endodontic treatment, therefore, ozone gas will equally react to viable microorganisms and the contaminants in root canal, such as host tissue components, dead bacteria, and bacterial extrapolsaccharides. It leads to useless consumption of ozone gas, resulting in a decline of the sterilization efficacy.

Some recent studies reported that ozone treatment had no effect against biofilm of *E. faecalis in vitro*,¹¹⁻¹³ and these results might be also caused by non-specific reaction of ozone and bacterial extracellular products. Ultimately, ozone gas itself might not be useful under the organic compound-rich condition, such as root canal.

When applied onto tooth surface, depth of ozone penetration was reported to be approximately 2 mm.¹⁴ In our basic experiment using 2% agar gel containing indigo carmine as the chemical indicator,^{15,16} oxidizing depth after 120 second-exposure of ozone gas was also approximately 2 mm from the surface. This value corresponded with the extent of bactericidal effect of ozone under the organic compound-free condition. Ozone did not show horizontal diffusion from the directly exposed area, and prolonging the exposure time of the gas (up to 180 s) could not extend the regions of either oxidization or bactericidal effects (data not shown). This result might be partially because of limited penetration of ozone from the exposed surface, and of transfer to aqueous phase.

From the manufacturer's data, HealOzone supplies ozone gas at a concentration of 2,100 ppm (v/v; 5.4 g/Nm³). Under the condition at 25°C, pH 7.0, 10^5 Pa (1 atm) of gas pressure, the concentration of ozone in water is calculated as 0.96 ppm (w/v; 0.96 g/Nm³), which is the maximum value in equilibrium. Nagayoshi et al. reported that although 2-4 mg/L (2-4 ppm, w/v) of ozonated water killed various oral microorganisms within 10 s, 0.5 mg/L (0.5 ppm, w/v) of it was not efficient enough to kill them within 120 s.¹⁷ In addition, when once in aqueous phase, ozone will react with various organic/inorganic compounds, resulting in rapid elimination of the active molecule.¹⁸

To get the best performance of ozone gas as a disinfectant, organic compounds in treatment region should be removed as much as possible before ozone exposure. Therefore, as the next step, simultaneous usage of irrigation solutions and ozone gas was examined to investigate the interaction. H₂O₂ (12 and 6% in water) and ozone (20 s) showed the synergic bactericidal effect. However, ozone gas interfered with the bactericidal effect of NaOCl at low concentration (1.2 ppm and 0.6 ppm in water). Since NaOCl is a popular and strong remover of the organic compounds,¹⁹ this interaction must be considered as a demerit for ozone treatment. Ozone may cause oxidative degradation of active molecules/ions in the NaOCl solution, which is unstable especially at low concentration.

If ozone gas is to be applied for endodontic therapy, therefore, a possible procedure before ozone gas treatment may be NaOCl irrigation followed by H₂O₂ irrigation. However, the synergy of H₂O₂ and ozone seems not to be strong enough to change general treatment protocol of root canal.

Within the limitation of the current experiments, the following conclusions can be drawn. Microbicidal effect of ozone gas was strongly interfered by contamination of organic compounds. Since ozone diminished the bactericidal effect of NaOCl at low concentration, co-treatment of ozone with NaOCl is not recommended.

While H₂O₂ and ozone showed synergy, however, the effect observed in the present study is not strong enough for clinical application.

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