



Antimicrobial effects of ozonated water on the sanitization of dental instruments contaminated with *E. coli*, *S. aureus*, *C. albicans*, or the spores of *B. atropheus*

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KEYWORDS

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Summary

Objectives: Ozone has been used as an alternative method for the decontamination of water, food, equipment and instruments. The objective of this study was to evaluate the antimicrobial effects of ozonated water on the sanitization of dental instruments that were contaminated by *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans* and the spores of *Bacillus atropheus*.

Methods: A total of one hundred and twenty standardized samples of diamond dental burs were experimentally contaminated with *E. coli* (ATCC 25922), *S. aureus* (ATCC 6538) and *C. albicans* (ATCC 18804) and the spores of *B. atropheus* (ATCC 6633) for 30 min. After the contamination, the samples were exposed to ozonated water (10 mg/L O₃) for 10 or 30 min. The control group was composed of samples that were exposed to distilled water for 30 min. After the exposure to the ozonated water, 0.1 mL aliquots were seeded onto BHI agar to count the colony-forming units per milliliter (CFU/mL) of *E. coli*, *S. aureus*, and *B. atropheus*. Sabouraud dextrose agar was used to count the CFU/mL of *C. albicans*. The results were subjected to an analysis of variance and the Tukey test.

Results: For all of the microorganisms studied, the ozonated water reduced the number of CFU/mL after 10 and 30 min of sanitization, and this microbial reduction was dependent on the duration of the exposure to the ozonated water.

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E. coli exhibited the greatest reduction in CFU/mL (2.72–3.78 log) followed by *S. aureus* (2.14–3.19 log), *C. albicans* (1.44–2.14 log) and the spores of *B. atrophaeus* (1.01–1.98 log).

Conclusion: The ozonated water was effective in reducing the CFU of *E. coli*, *S. aureus*, *C. albicans* and *B. atrophaeus* spores, suggesting that ozonated water can be used for the sanitization of dental instruments.

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Introduction

The control of infection is one of the most important facets of dental care. The increased incidence of infectious and/or contagious diseases of various etiologies has forced odontological professionals to adopt procedures for the control of microorganisms for the protection of both the odontological team and the patients. Every patient who seeks buccal treatment should be considered a potential carrier of an infectious and/or contagious disease. Therefore, daily, routine measures for the control of microorganisms should be followed rigorously in odontological clinics [1].

All types of dental instruments and materials should be sterilized and/or disinfected. Sterilization methods that involve physical procedures (such as autoclaving and/or heating) are the most frequently used in odontological clinics. These methods are recommended for the majority of clinical instruments, with the exception being instruments that are sensitive to heat. The use of high-level disinfectants is indicated when an article or instrument cannot be subjected to a sterilization process [2]. Additionally, disinfectants are intended for the decontamination of dental instruments that have been contaminated with saliva and blood before the washing and cleaning of these instruments to protect the odontological professionals [3,4].

Numerous disinfectants and sanitizers are used in odontology, and many commercial products are available. Nevertheless, no ideal cleaning product exists for processing all types of dental instruments because each cleaning product has specific indications and restrictions. Alternative methods for the sterilization and/or disinfection of dental instruments are continually being investigated, and the use of ozone as a novel method to clean dental instruments is of great interest [5–7].

Ozone is the allotropic form of oxygen (O₃), and it is a powerful oxidizing agent. It is believed that ozone can oxidize amino acids and destroy the proteins present in the cellular membrane of microorganisms; therefore, ozone may have excellent antimicrobial properties. Ozone can also

inhibit fungal growth and interrupt the viral replication cycle by altering the contact between a virus and the cell *via* peroxidation [8]. Ozone has been used in the treatment of drinking water, in the food industry and in medicine for wound treatment and microorganism control [9,10].

The use of ozone in odontology, either in an oil form or in ozonated water, has been reported by several authors [8,11–13]. However, reports in the literature on the potential use of ozonated water for the sanitization of dental and surgical instruments are scarce. Thus, the objective of the present study was to evaluate ozonated water as a sanitization method for dental instruments that were contaminated by *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and the spores of *Bacillus atrophaeus*.

Materials and methods

Microorganisms

Reference strains of *E. coli* (ATCC 25922), *S. aureus* (ATCC 6538), *C. albicans* (ATCC 18804) and the spores of *B. atrophaeus* (ATCC 6633) were used. The strains of *S. aureus* and *E. coli* were seeded on Brain Heart Infusion agar (BHI, Difco, Detroit, MI, USA), and *C. albicans* was seeded on Sabouraud dextrose agar (Difco, Detroit, MI, USA). The plates were incubated at 37 °C for 24 h. After the growth of the microorganisms, the strains were individually suspended in phosphate-buffered saline (PBS; 0.07 M, pH 7.0) to produce a concentration of approximately 0.5 on the McFarland Scale.

B. atrophaeus was cultured on nutrient agar plates (Difco, Detroit, MI, USA) at room temperature for one week, and several well-growing colonies were suspended in 20 mL of sterilized distilled water. The suspension was then heated at 70 °C for 20 min and used as the spore suspension of *B. atrophaeus* [14].

Contamination of dental instruments

A total of one hundred and twenty sterilized diamond burs (1016, KG Sorensen) were used as

standardized samples. For the contamination, the samples were prepared in sterilized Petri plates containing 20 mL of a suspension of each microorganism for a period of 30 min at room temperature. After this incubation period, the samples were placed in sterilized Petri plates for heated drying at 37 °C for 2 h.

Ozonated water

Ozonated water was prepared using an ozone generator (Ozone, model MVO – UV, Anceros) that was developed by the Physics Department of the Aeronautic Technology Institute (ITA, São José dos Campos, SP, Brazil). The ozone generator was connected to a cylinder of pure oxygen (White Martins, Taubaté, SP, Brazil) that was calibrated to release oxygen at 0.4 mg/L per min. For the production of the ozonated water, 250 mL of autoclaved distilled water was placed in the system with a glass tube coupled to the ozone generator. Next, O₃ was bubbled through the water for 20 min, thereby producing O₃ at a concentration of 10 mg/L/min.

Sanitization of dental instruments with ozonated water

Each standardized sample was submerged for 10 or 30 min in a plastic tube containing 1.5 mL of the ozonated distilled water at an initial concentration of 10 mg/L. After the sanitization period, the ozone was neutralized with 0.1 mL of 0.1 M sodium thio-sulfate, and the tubes were agitated individually for 30 s (Vortex). Serial dilutions were performed in PBS, and 0.1 mL aliquots of the pure material and of each dilution were seeded in duplicate on BHI agar for the *E. coli*, *B. atrophaeus* and *S. aureus* strains and in duplicate on Sabouraud dextrose agar for *C. albicans*. The plates were incubated at 37 °C for 48 h for the analysis of the colony-forming units (CFU/mL).

For the control group, the contaminated samples remained in distilled water for 30 min. A total of thirty standardized samples were used for each microorganism according to the following experimental groups: ozonated water for 10 min ($n = 10$), ozonated water for 30 min ($n = 10$), and the control group ($n = 10$).

Statistical analysis

The CFU/mL counts were converted into a logarithmic form and subjected to an analysis of variance (ANOVA) and the Tukey test. A P value < 0.05 was considered to indicate a statistically significant difference.

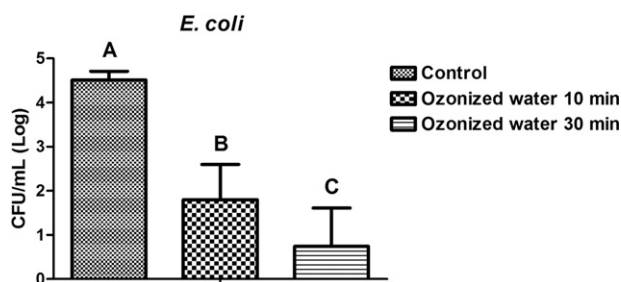


Figure 1 Mean CFU/mL (log) and standard deviations for *S. aureus* exposed to the following experimental conditions: physiological solution (control), ozonated water for 10 min and ozonated water for 30 min. Different letters represent statistically significant differences among the groups (Tukey test, $p \leq 0.05$).

The percent reduction of the CFU/mL for each microorganism studied was calculated by comparing the groups that were sanitized with ozonated water for 10 and 30 min to the control group.

Results

The log-transformed CFU/mL data from the control group and the cultures of *E. coli*, *S. aureus*, *C. albicans* and *B. atrophaeus* that were obtained after sanitization by ozonated water (10 or 30 min) are shown in Table 1. For all of the microorganisms tested, statistically significant differences were observed between the control group, the group treated with ozonated water for 10 min and the group treated with ozonated water for 30 min (Figs. 1–4); therefore, the ozonated water was effective in reducing the microbial levels, and this reduction was dependent on the duration of the exposure to the ozonated water.

The percent reductions in the CFU/mL for the ozonated water groups relative to the control group

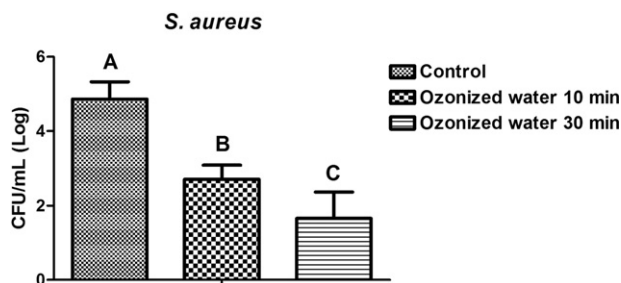


Figure 2 Mean CFU/mL (log) and standard deviations for *E. coli* exposed to the following experimental conditions: physiological solution (control), ozonated water for 10 min and ozonated water for 30 min. Different letters represent statistically significant differences among the groups (Tukey test, $p \leq 0.05$).

Table 1 Descriptive statistics of CFU/mL (log) for *E. coli*, *S. aureus*, *C. albicans*, and *B. atrophaeus* exposed to different experimental conditions: physiological solution (control), ozonized water for 10 min and ozonized water for 30 min.

Microorganisms	Groups	Mean \pm SD	Minimum	Median	Maximum
<i>E. coli</i>	Control	4.51 \pm 0.19	4.03	4.53	4.75
	Oz 10 min	1.79 \pm 0.80	0.00	2.03	2.59
	Oz 30 min	0.73 \pm 0.87	0.00	0.34	2.00
<i>S. aureus</i>	Control	4.85 \pm 0.46	3.79	5.02	5.35
	Oz 10 min	2.71 \pm 0.37	2.07	2.72	3.40
	Oz 30 min	1.66 \pm 0.71	0.00	1.71	2.41
<i>C. albicans</i>	Control	4.66 \pm 0.19	4.37	4.63	5.05
	Oz 10 min	3.22 \pm 0.26	2.73	3.25	3.59
	Oz 30 min	2.52 \pm 0.46	1.65	2.57	3.21
<i>B. atrophaeus</i>	Control	5.31 \pm 0.18	5.05	5.28	5.72
	Oz 10 min	4.30 \pm 0.19	4.04	4.25	4.58
	Oz 30 min	3.33 \pm 0.31	3.00	3.18	3.92

SD: standard deviation.

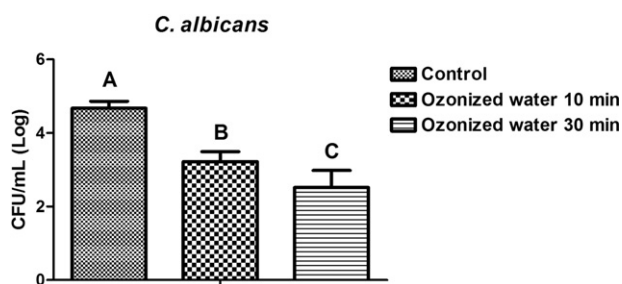


Figure 3 Mean CFU/mL (log) and standard deviations for *C. albicans* exposed to the following experimental conditions: physiological solution (control), ozonated water for 10 min and ozonated water for 30 min. Different letters represent statistically significant differences among the groups (Tukey test, $p \leq 0.05$).

are shown in Fig. 5. The largest percent reduction was observed for the *E. coli* strain after sanitization with the ozonated water for 30 min (99.93%), whereas the smallest percent reduction was observed for the *B. atrophaeus* spores after

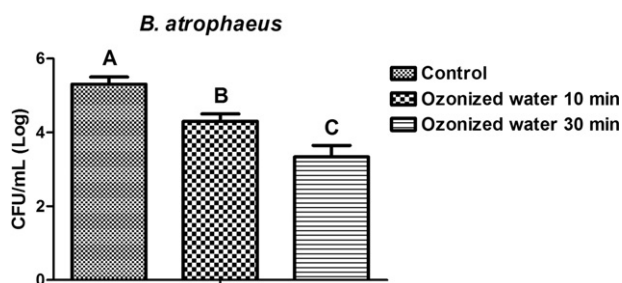


Figure 4 Mean CFU/mL (log) and standard deviations for *B. atrophaeus* exposed to the following experimental conditions: physiological solution (control), ozonated water for 10 min and ozonated water for 30 min. Different letters represent statistically significant differences among the groups (Tukey test, $p \leq 0.05$).

sanitization with the ozonated water for 10 min (90.15%).

Discussion

Ozone is currently being discussed as a possible alternative antiseptic in dentistry because of its reported high antimicrobial power without the development of drug resistance [15]. Recently, both gaseous and aqueous ozone have shown antimicrobial activities against the oral pathogens associated with caries, endodontic infections and periodontitis [16–19]. Moreover, ozonated water has been found to accelerate the healing of the oral mucosa following a tooth extraction process or after surgical interventions because the ozonated water is associated with a hemostatic action [20].

Ozone has also been used as a disinfecting agent in dental unit waterlines and in denture-cleaning bubble solutions [21]. However, reports on the use of ozonated water for the sanitization of dental

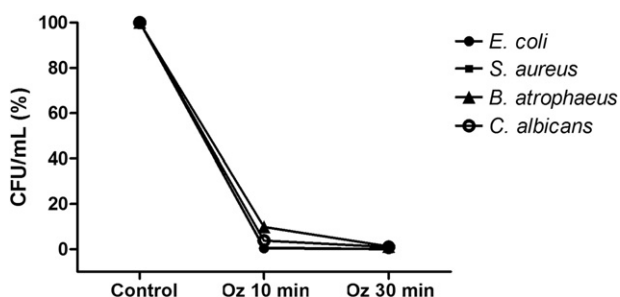


Figure 5 Percent reduction expressed as the mean values (CFU/mL) of *S. aureus*, *E. coli*, *C. albicans*, and *B. atrophaeus* exposed to ozonated water for 10 min (Oz 10 min) or 30 min (Oz 30 min) relative to the control group.

instruments are scarce in the literature. The sanitization of dental instruments, especially sharp instruments, before the washing and cleaning of these instruments is extremely important for the safety of odontological professionals. Therefore, this study evaluated the effectiveness of ozonated water for the sanitization of dental instruments using diamond burs as the standardized samples because these instruments are an appropriate size for experimental tests and provide a rough surface that facilitates the adherence of microorganisms [22].

The use of ozonated water (10 mg/L) for 10 or 30 min was effective for the sanitization of the diamond burs contaminated by *S. aureus*, *E. coli*, *C. albicans* and the spores of *B. atrophaeus*. The percentage of the microbial reduction ranged from 90.15 to 99.33%. Huth et al. [15] evaluated the antimicrobial efficacy of aqueous ozone (1.25–20 mg/L) against both endodontic pathogens in suspension and pathogens that formed a biofilm. These authors verified that the application of aqueous ozone for 1 min was effective and could eliminate *E. faecalis* and *C. albicans* in suspension in a dose-dependent manner when aqueous ozone was used at concentrations of 5, 10 and 20 mg/mL. However, the antimicrobial reduction of *E. faecalis* and *C. albicans* mono-species biofilms only ranged from 86% to 96%, respectively, at ozone concentrations of 10 and 20 mg/mL, suggesting that high concentrations of aqueous ozone are required to eradicate biofilms. Thus, when the microorganisms on dental instruments adhere to an irregular metallic surface, ozonated water at concentrations between 10 and 20 mg/mL is suitable for the sanitization of these clinical instruments.

For all of the microorganisms tested, the antimicrobial effects of the ozonated water were dependent on the exposure time. The mean reduction (CFU/mL) ranged from 1.01 to 2.72 log and from 1.98 to 3.78 log for the 10 and 30 min exposures, respectively. Bezirtzoglou et al. [20] studied the effectiveness of ozone against the microorganisms colonizing toothbrushes. The bristles of the brushes were soaked in an ozone-saturated PBS solution (3–3.5 mg/L) for 5, 10, 15, 20 or 30 min, and the total microbial population was subsequently reassessed. After 10 min or more of ozonation, statistically significant differences in the microbial concentrations were observed, whereas after 5 min of exposure, only a slight and non-significant decrease of 0.5 log was recorded. Complete sterilization was observed after 30 min of ozonation because no viable CFU were observed for those samples. According to the authors, the application of ozone for short time periods had a

bacteriostatic effect, whereas ozonation for more than 30 min had a bactericidal effect. In contrast, Miguez et al. [23] reported that ozonated water maintained its antimicrobial activity for the first 20 min and that this activity decreased substantially after 30 min because of the instability of gaseous O₃.

Because the ozonated water has a short half-life, and residual ozone can be found in water for a maximum period of 8 h [20], the generation and subsequent storage of ozone cannot be performed. Consequently, ozone must be generated on site when it is required for disinfection [2]. During the production of ozone, care must be taken to prevent the prolonged inhalation of the gas by the operator because ozone inhalation can have harmful side effects. Several ozone delivery systems for use in an odontological clinic are available in the United States and Europe. These systems include a source of oxidizing gas and a dental handpiece for delivering the gas/water to the target tooth [19,24].

One of the main advantages of using ozonated water as a sanitizer is its activity against spores. The spores of *B. atrophaeus* are highly resistant to control methods, such as heating, drying, freezing, radiation, antiseptics, and disinfectants. Indeed, spores are used as a parameter to test the efficacy of physical and chemical microorganism control procedures [14]. The data from this study showed that the antimicrobial activity of ozone against the *B. atrophaeus* spores resulted in growth reductions of 90.15% and 98.74% after 10 and 30 min exposure to ozonated water, respectively. Similarly, Makky et al. [25] found a 99.9% inactivation of *B. atrophaeus* spores after exposure to ozonated water at 10 mg/L for 10 min.

The results of the *in vitro* experiments performed in this study demonstrated that the exposure to ozonated water at a concentration of 10 mg/L for 10 or 30 min was effective at significantly reducing the quantity of microorganisms that adhered to the surfaces of dental instruments. Although ozone appears to exhibit an antibacterial effect on bacteria under *in vitro* conditions, the effect of ozone in different environments *in vivo* is still only speculative [26]. Johansson et al. [26] verified that ozone had a profound capacity to kill cariogenic bacteria, such as *S. mutans*, *L. casei* and *A. naeslundii*, and that the level of killing was reduced in the presence of saliva. Other studies related to the sanitization of dental instruments using ozonated water should be conducted to evaluate the influence of saliva and blood on the antimicrobial activity of ozone. Additionally, new studies are required to verify the activity of ozone

on other microorganisms, such as hepatitis viruses and human immunodeficiency virus.

Conclusion

We conclude that the exposure to ozonated water at a concentration of 10 mg/L for 10 or 30 min was sufficient to reduce the number of CFU/mL of *E. coli*, *S. aureus*, *C. albicans* and the spores of *B. atrophaeus* effectively on the surface of diamond burs, suggesting that ozonated water can be used for the sanitization of dental instruments.

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