

Original Paper

Comparison of the antimicrobial activity of three different concentrations of aqueous ozone on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* – in vitro study

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Keywords

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Abstract

*The root canal treatment has been dramatically changed since the emergence of ozone therapy. Due to its potent antimicrobial activity and biocompatibility, ozone can be applied as an agent to improve decontamination procedures and possibly increase success rates. The aim of this study was to compare the antimicrobial activity of 3 different concentrations of aqueous ozone on *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*. This study used a standardized suspension of 3 bacteria. These suspensions were cultivated and spectrophotometrically adjusted to a final concentration of 4.46×10^8 CFU/mL. Then they were submitted to groups: Group I – aqueous ozone 2 μ g/mL; Group II – aqueous ozone 5 μ g/mL; Group III – aqueous ozone 8 μ g/mL, and Group Control – bidistilled cold sterile water with no ozone. Bidistilled cold sterile water was ozonated for 5 minutes. Then 10 mL of each suspension were added to 90 mL of each group in a glass flask. After 1 minute of contact, 1 mL of each flask was added to 9 mL of 0.1% sodium tiosulfate to neutralize the ozone, and then serial dilution was performed. After 24 hours of incubation, the CFU counting was performed. In parallel, 1 mL of each glass flask was added to 9 mL of TSB broth and incubated for 7 days for visual analyses of turbidity broth. The results showed Group I presented *Enterococcus faecalis* growth. Group II and III presented no CFU counting, but aqueous ozone at 5 μ g/mL presented 2 bleary tubes of *Enterococcus faecalis*, indicating bacterium growth. Group III (aqueous ozone 8 μ g/mL) showed no CFU counting or blurry broth. According to the applied methodology, it was possible to conclude that the aqueous ozone in concentration of 8 μ g/mL was the most efficient to eliminate the three evaluated bacteria.*

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Introduction

Ozone has been recognized as an important tool to improve the success rate and as being suitable to be applied in all odontology specialties, due to its strong antimicrobial potential and the ability to stimulate organisms to heal.

Specifically in root canal treatment, ozone plays an important role to improve decontamination phase, improving the decontamination of the root canal system and dentinal tubules.

Nagayoshi et al. (2004) compared the effectiveness of the ozonated water and irrigant agents used in endodontics. The authors used bovine tooth contaminated with *Enterococcus faecalis* and *Streptococcus mutans*. After the irrigation, it was noticed that the tested microorganism's viability presented in dentinal tubules decreased significantly. When sonication was associated with ozonated water, it showed the same result of 2.5% sodium hypochlorite. The authors concluded that ozonated water is perfectly suitable to endodontic therapy.

Cardoso et al. (2008) evaluated ozonated water's effectiveness as an irrigant agent to eliminate *Enterococcus faecalis* and *Candida albicans* and neutralize endotoxins of the root canal system. Twenty-four teeth, single root, were contaminated with the bacterium suspension, and another 24 teeth were inoculated with endotoxins. It was performed microbiological collecting before and after chemical mechanical procedures on the teeth. In the analysis of the data, Cardoso et al. concluded that ozonated water, as an irrigant agent, has dramatically decreased the number of *Enterococcus faecalis* and *Candida albicans*. Supplementing the ozonated water was not able to neutralize the endotoxin of the root canals.

According to Lynch (2008), ozone has been proposed as an antiseptic agent based on the studies of its antimicrobial activity as it relates to gas and diluted water. The ozone is effective when prescribed in a proper concentration and correctly applied to the intracanal after the basic and traditional procedures of cleaning, disinfecting, and shaping. The improvement in ozone action occurs in areas with less organic debris, an indication to apply it after the final rinse and shaping of the root canal.

Nogales et al. (2008) described the potent antimicrobial activity of ozone and how suitable is the application not only in endodontic therapy but periodontic, surgery, cariology, esthetic and restorative dentistry.

Huth et al. (2009) assessed the antimicrobial efficacy of aqueous and gaseous ozone as an alternative antiseptic against endodontic pathogens in suspension and a biofilm model. *Enterococcus faecalis*, *Candida albicans*, *Peptostreptococcus micros*, and

Pseudomonas aeruginosa were grown in planktonic culture or in mono-species biofilms in root canals for 3 weeks. Cultures were exposed to ozone, 5.25% and 2.5% sodium hypochlorite, 2% chlorhexidinedigluconate, 3% hydrogen peroxide, and phosphate-buffered saline (control) for 1 minute, and the remaining colony-forming units were counted. The results showed aqueous and gaseous ozone were dose- and strain-dependently effective against the biofilm microorganisms. Total elimination was achieved by high-concentrated ozone gas and almost total elimination achieved by aqueous ozone. Thus, the authors concluded that ozone was effective in highest concentration in suspensions and in the biofilm model.

Case et al. (2012) examined the effects of gaseous ozone delivered into saline on biofilms of *Enterococcus faecalis* in root canals of extracted teeth with and without the use of passive ultrasonic agitation. Biofilms of *E. faecalis* were established over 14 days in 70 single roots that had undergone biomechanical preparation followed by gamma irradiation. Biofilms were treated with saline, 1% sodium hypochlorite for 120 seconds, ozone (140 ppm in air at 2 L/min delivered into saline using a cannula for 120 seconds), saline with passive ultrasonic activation, and ozone followed immediately by ultrasonic agitation. Although none of the treatment regimes were able to reliably render canals sterile under the conditions used, ozone gas delivered into irrigating fluids in the root canal may be useful as an adjuvant for endodontic disinfection.

Several studies have proven the antimicrobial activity of aqueous ozone but none have compared different concentrations. Such data is fundamental to determine a reliable protocol of use. Thus, the aim of this study is to compare the antimicrobial activity of three different concentrations of aqueous ozone over *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*.

Material and Methods

This study was approved by Ethical Committee of the Dental School of the University of São Paulo.

Standardization of the microbiological essay

For standardization purposes, the technique was performed separately used with suspensions of *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538) and *Enterococcus faecalis* (ATCC 29212) provided by the Microbiology Lab of the Instituto de Pesquisas Tecnológicas do Estado de São Paulo. All procedures were performed under laminar flow.

Initially, an Eppendorf tube of each bacterium was unfrozen and distributed on 3 Petri plates and incubated for 24 hours at 37°C. After this time, a single colony was inoculated in 10 mL of TSB broth and incubated for 24 hours at 37°C.

After 24 hours, the bacteria concentration was adjusted by spectrophotometry using the optical density (OD) by absorbance index (ABS) and calibrated to wavelength of 546 nm according to Table 1.

Bacterium	OD (ABS)
<i>Staphylococcus aureus</i>	0.150 ABS
<i>Enterococcus faecalis</i>	0.800 ABS
<i>Pseudomonas aeruginosa</i>	0.600 ABS

Standardization of aqueous ozone

This experiment used the Philozon Ozone Generator (Philozon, Santa Catarina, Brazil) fed by pure oxygen (Respirox, São Paulo, Brazil) and a flux rate of 1 L/min. Cold and sterile bidistilled water was ozonated in a glass column of 40 cm height and 5 cm diameter.

The oxygen was released and the ozone generator started; it was calibrated at experimental concentrations, and 500 mL of water was submitted to ozone bubbling for 5 minutes.

Experimental Groups

- Group I – Ozonated bidistilled water 2 µg/mL
- Group II – Ozonated bidistilled water 5 µg/mL
- Group III – Ozonated bidistilled water 8 µg/mL
- Control – Bidistilled water with no ozone

90 mL from each experimental group were transferred to a glass flask and, then 10 mL from each bacterium suspension were added to the flask. A contact of 1 minute was established, and then 1 mL of each flask was transferred to 9 mL of 0.1% sodium tiosulfate to neutralize ozone; then serial dilutions and plating were performed. All experiments were performed 3 times. Incubation for 24 hours and then CFU counting were performed.

Enrichment test

After 1 minute of contact of the bacterium suspension with the experimental groups, 1 mL of each experimental flask was transferred to 9 mL of TSB broth and incubated for 7 days at 37°C. This was performed to determine whether there would be bacterium growth.

Results

Arithmetical average of the action of the experimental groups over *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* is shown in Table 1. Only *Enterococcus faecalis* in the lowest concentration of aqueous ozone was able to detect CFU.

The evaluation of the enrichment test has confirmed the antimicrobial activity of aqueous ozone. After 7 days of incubation, the TSB broth containing the bacterium suspension was analyzed looking for broth turbidity. The quantity of tubes containing blurry broth is presented in Table 2. A total of 90 tubes, 30 tubes for each bacterium, provided bacterium growth in Group I and Group II, and no turbidity was noticed in Group III.

Table 1 – Colony Forming Unit counting of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* after been submitted to experimental groups

Microorganism	Initial Bacterium Concentration	Control	Group I – Aqueous Ozone 2 µg/mL	Group II – Aqueous Ozone 5 µg/mL	Group III – Aqueous Ozone 8 µg/mL
<i>Pseudomonas aeruginosa</i>	4.46X10 ⁸	3.08X10 ³	ND	ND	ND
<i>Staphylococcus aureus</i>		1.22X10 ⁵	ND	ND	ND
<i>Enterococcus faecalis</i>		3.59X10 ⁵	1.52X10 ⁴	ND	ND

ND = not detectable

Table 2 – Quantity of blurry broth after 7 days of incubation according to experimental groups

Microorganism	Total of tubes with broth incubated	Group I – Aqueous Ozone 2 µg/mL	Group II – Aqueous Ozone 5 µg/mL	Group III – Aqueous Ozone 8 µg/mL
<i>Pseudomonas aeruginosa</i>	30	0	0	0
<i>Staphylococcus aureus</i>	30	0	0	0
<i>Enterococcus faecalis</i>	30	14	2	0

Discussion

The selection of any chemical substance as part of endodontic protocol is based on several criteria, most importantly criteria related to the antimicrobial activity.

The use of ozone in odontology has earned importance in the past few years. This is proven by the number of studies have been carried out. Most of them are in vitro studies that certify the potent ozone antimicrobial activity.

The lack of a protocol of use specifically in endodontic therapy has kept ozone from being used. The establishment of an optimal concentration is the first step to determine the correct protocol. Thus, it was the aim of this study, through comparing 3 different concentrations in vitro, to allow future studies to determine the optimal procedure to find the correct process for applying ozone and to improve the results of root canal treatment.

The results of this assay showed that the highest concentration of aqueous ozone (8 µg/mL) was the most effective against the microorganism tested. The concentration of 5 µg/mL provided no CFU counting, but it was possible to notice that 2 TSB broth tubes of *Enterococcus faecalis* blurred, indicating bacterium growth.

Differently from Nagayoshi et al.'s (2004) study, which showed that 4 µg/mL of aqueous ozone was effective against *Enterococcus faecalis* and *Streptococcus mutans*, this study showed 8 µg/mL of aqueous ozone as the optimal concentration. Cardoso et al. (2008) proved the efficacy of aqueous ozone in concentration of 24 mg/L against *Enterococcus faecalis* and *Candida albicans*, and Huth et al. (2009) showed the highest concentration was effective against the microorganism tested.

Using a different protocol of ozone production, Case et al. (2012) assessed the activity of ozone-enriched air bubbled into saline solution over biofilm of *Enterococcus faecalis*. Even though a completely different methodological application and production of ozone, the results were positive.

Lynch (2008) has preconized ozone as part of endodontic protocol. The best results will be reached after the basic procedures have been taken. Linking to the results of this assay, ozone has the potential to become an important part of the endodontic treatment and can probably improve the success rate. Further studies, especially clinical trials, are required to provide a safe and reliable protocol of application and thus to enable dental professionals to enjoy the benefits of ozone. This way endodontic therapy will be faced as a painless and safe therapy.

Conclusion

Besides the methodological restriction, the comparison of 3 different concentrations of aqueous ozone showed the 8 µg/mL to be the most effective concentration against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis*.

Reference

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