

Treatment of Root Canal Biofilms of *Enterococcus faecalis* with Ozone Gas and Passive Ultrasound Activation

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Abstract

Introduction: Biofilms of resistant species such as *Enterococcus faecalis* pose a major challenge in the treatment of root canals with established periapical disease. This study examined the effects of gaseous ozone delivered into saline on biofilms of *E. faecalis* in root canals of extracted teeth with and without the use of passive ultrasonic agitation. **Methods:** Biofilms of *E. faecalis* were established over 14 days in 70 single roots that had undergone biomechanical preparation followed by gamma irradiation. The presence and purity of biofilms were confirmed using scanning electron microscopy and culture. Biofilms were treated with saline (negative control), 1% sodium hypochlorite for 120 seconds (positive control), ozone (140 ppm ozone in air at 2 L/min delivered into saline using a cannula for 120 seconds), saline with passive ultrasonic activation (70 kHz and 200 mW/cm² applied to an ISO 15 file held passively within the canal, for 120 seconds), and ozone followed immediately by ultrasonic agitation. After treatment, samples were taken from the biofilm and serially diluted for plate counting. **Results:** Analysis revealed that 1% sodium hypochlorite was the most effective disinfecting agent followed by ozone combined with ultrasonic agitation, ozone alone, and finally ultrasonic alone. **Conclusions:** 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 adjunct for endodontic disinfection. (*J Endod* 2012;38:523–526)

Key Words

Biofilms, disinfection, enterococci, ozone, ultrasonics

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In order for bacteria to remain viable in a treated root canal system, they must be able to survive chemomechanical preparation and medication phases and persist in a low-nutrient environment. *Enterococcus faecalis* is commonly isolated in root canal systems with persistent disease (1–7), and it resists the antimicrobial actions of sodium hypochlorite and medicaments (1, 8, 9). When present in a biofilm, *E. faecalis* presents a particular challenge because of poor antimicrobial penetration, a low growth rate, and altered gene expression (10).

One possible agent of interest for dealing with persistent organisms is ozone. This thermodynamically unstable short-lived oxygen decomposes to pure oxygen in a short period and generates oxygen-free radicals when in an aqueous solution (11). It is a strong oxidizing agent that causes lipid peroxidation and alters membrane permeability and function (11, 12). The bactericidal and virucidal properties of ozone are well recognized (13–17) and have shown to reduce the level of viable *E. faecalis* in dentine tubules (16). Several endodontic pathogens including *E. faecalis* are rendered nonviable upon exposure to 2 and 4 ppm (mg/L) ozone in water (17, 18). Nagayoshi et al (16) found that combined with sonication, aqueous ozone (4 ppm) gave a similar reduction in colony-forming units as 2.5% sodium hypochlorite; however, the effects on biofilms were not investigated nor have effects of gaseous ozone delivered directly into water or saline in the root canal.

Numerous studies have shown that the activation of irrigants with ultrasonic energy increases their bactericidal effects (13, 19–23). Previous work in industrial settings has shown the enhancement of biofilm removal by ozone followed by ultrasound (22). To date, there have been no studies of endodontic applications of ozonated fluids agitated by ultrasonics. The aim of the present study was to assess the antimicrobial efficiency of a gaseous ozone delivery system with and without ultrasonic agitation of the canal contents against biofilms of *E. faecalis*.

Materials and Methods

Sample Preparation

A total of 70 single roots from extracted caries-free anterior teeth were used. The roots were sectioned and the cut surface polished using a diamond-polishing wheel to give a consistent length of 12 mm. A 3-mm-deep reservoir in the coronal aspect of the root canal was created using a #5 Gates Glidden bur. The coronal portion of the canal was then further instrumented using a number 3 Gates Glidden bur before instrumentation with K3 rotary nickel-titanium (NiTi) files. The canals were prepared in a crown-down fashion using NiTi rotary instruments in a torque-controlled handpiece to ISO 35 with a 6% taper, so that a size 25 K-file would pass to a length of 1 mm beyond the apex of the tooth (24). One percent sodium hypochlorite and 17 % EDTA were used as sequential irrigants between each instrumentation cycle. A total of 10 mL of each irrigant was used per canal. A final 2-minute rinse of EDTA followed by sodium hypochlorite was undertaken, after which an ISO 10 file was placed through the apical foramen to ensure patency.

The roots were then mounted in a system that was modified from that described previously (25, 26). A hole was cut into the lid of a sterile 5-mL specimen container to allow the root to be placed through the lid before being sealed with sticky wax. The external root surface was coated with nail varnish before the samples were sterilized using 20 kGy of gamma radiation (Steritech, Narangba, QLD, Australia).

Biofilm Model

E. faecalis (ATCC 29212) was subcultured onto Trypticase soy agar plates (Oxoid, Basingstoke, UK) containing 5% defibrinated sheep blood at 37°C in 5% CO₂ in air. Gram staining and colony morphology were used to monitor purity, whereas identity was confirmed using API20 Strep (bioMérieux, Marcy l'Etoile, France). A single colony was used to generate a stock inoculum in Trypticase soy broth. Root canals were inoculated using 0.1-mL aliquots each day for 14 days. Complete infection of the root canal was confirmed by the development of turbidity within the broth contacting the apical portion of the root. Biofilm formation was confirmed by scanning electron microscopy of selected roots, which were fixed for 24 hours in 4% buffered paraformaldehyde before being split longitudinally, sputter coated with gold, and examined under low vacuum conditions up to 20,000×. The split roots were examined along the entire length of the canal, with particular attention given to the apical third region.

Experimental Groups and Treatments

Roots were assigned randomly to 1 of 5 groups. Immediately before treatment, the lumen of the canal was filled with 100 μL sterile saline using a 35-G needle. The saline (negative control) group underwent irrigation with 5 mL sterile saline delivered over 2 minutes. In the positive control group, canals were irrigated with 5 mL 1% sodium hypochlorite over 2 minutes and then flushed with sterile saline.

In the ozone-treated group, a sterile cannula attached to a dental ozone system (Prozone; W&H Dental Werk Burmoos GmbH, Burmoos, Austria) was introduced into 100 μL sterile saline in the root canal until 2 mm short of the working length. Ozone-enriched air (140 ppm, 2 L/min) was delivered for 24 seconds as recommended by the manufacturer. The cannula was removed from the canal after each 24-second cycle to prevent room air being delivered during system purging. The canal was then refilled with fresh saline, and the ozonation treatment was repeated 4 times giving a total ozonation time of 2 minutes, after which there was a final saline flush.

In the passive ultrasound group, an ISO 15 K-file placed passively in saline was activated using an ultrasonic scaler (Perio-scan; Sirona, Bensheim, Germany) at 70 kHz and 200 mW/cm² for 30 seconds. The canal was then irrigated with saline for 24 seconds, and the process was repeated until a total irrigation time of 2 minutes was reached.

In the ultrasound-activated ozone group, after the lumen of the canal was filled with sterile saline and the saline was ozonated for 24 seconds, the saline was then subjected to passive ultrasound for 30 seconds. Fresh saline was then placed into the canal, and this process was repeated until a total irrigation time of 2 minutes was reached.

Colony Counting

Immediately after the various treatments, a sterile size 35/02 Hedstrom file was circumferentially filed clockwise in the canal for 20 strokes with light pressure in order to collect as much of the biofilm as possible (27). The intention of the sampling method was to collect well-attached bacteria from deep in the biofilm as well as loosely attached superficial bacteria on the biofilm surface. The file was then placed into 500 μL sterile saline and vortex mixed for 30 seconds to disperse the organisms. The bacterial suspension was serially diluted and 50 μL pipetted onto the surface of triplicate Trypticase soy agar plates, which were then incubated for 3 days. The number of colony-forming units (CFUs) per plate was determined, with serial dilutions of 1:100 and 1:1,000 proving to be the most useful for this purpose.

Analysis of Data

To obtain a conservative measurement of the efficacy of each treatment, the highest bacterial counts for each sample were used. Log-transformed data were analyzed using a Kruskal-Wallis test with post hoc Dunn multiple comparison tests.

Results

Scanning electron microscopic examination of the negative control roots (treated with saline only) confirmed that a dense biofilm was present in the canals before the various treatments (Fig. 1). There was a significant difference between the 5 groups ($P < .0001$, Fig. 2). Overall, 1% sodium hypochlorite was the most effective disinfecting agent followed by ozone combined with ultrasonic agitation, ozone alone, and finally ultrasonic alone.

In the positive control group, exposure to 1% sodium hypochlorite for 2 minutes produced a 93.5% reduction in viable CFUs compared with the saline negative control, whereas passive ultrasonication produced a 50.2% reduction. Exposure to ozone-enriched air for a total period of 2 minutes resulted in a 71.6% reduction in viable CFUs compared with the control group, whereas treatment with ozone gas combined with ultrasonic agitation resulted in an 83.8% reduction in viable CFUs.

There was a consistent trend for ultrasonic activation to increase the efficacy of ozone, but this did not reach statistical significance (Table 1). Of note, none of the treatment regimes were able to reliably reduce counts to zero under the exposure conditions used (Fig. 2).

Discussion

The present study shows that ozone-enriched air bubbled into saline exerts an antimicrobial action against an established biofilm, which is enhanced by ultrasonic agitation. This positive result contrasts with past work, which compared the efficacy of ozone with other antimicrobial agents using short-term broth cultures rather than biofilms (28, 29). Past work has used very low doses of ozone (18), whereas the present study assessed the capability of ozone in terms of its antibacterial actions against an intact biofilm of *E. faecalis* under exposure conditions that more closely resemble those used in clinical practice.

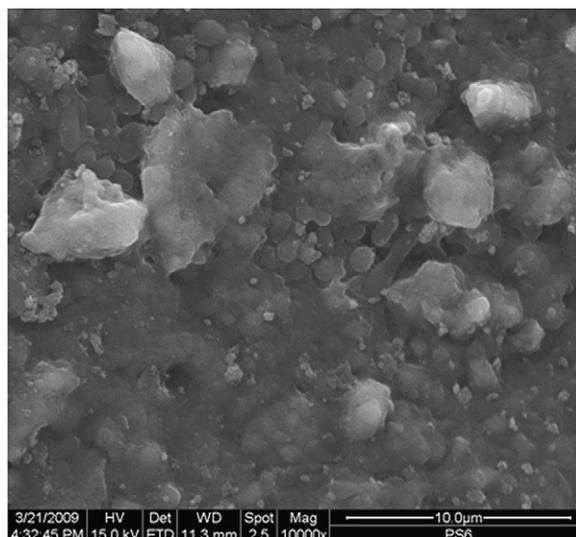


Figure 1. A scanning electron microscopic view of *E. faecalis* biofilm in a control root (group 1) (10,000×). Individual cocci can be seen embedded in a dense matrix. Several stacks can be seen rising from the biofilm surface.

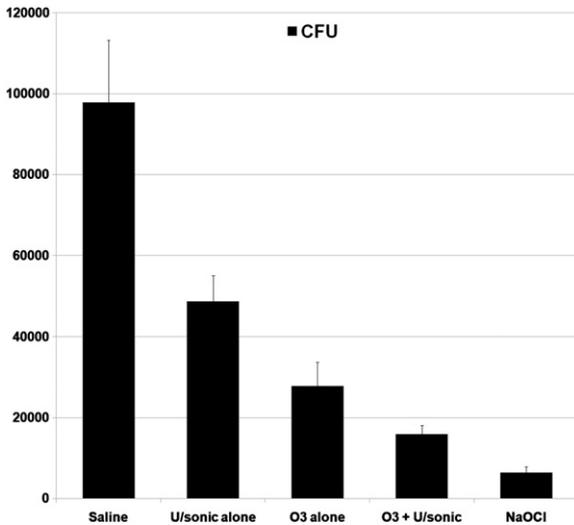


Figure 2. The reduction in viable CFUs (mean and standard error).

The present results indicate that saline-filled canals treated with ozone or with ozone followed by ultrasonic agitation contained fewer viable bacteria compared with those treated with ultrasonically agitated saline. There was a trend toward a greater reduction in viable bacteria when ultrasonic agitation was combined with ozone compared with ozone used alone, but this failed to reach statistical significance at the $P < .05$ level. These results indicate some potential for locally delivered ozone to be used as an adjunct to existing methods for root canal disinfection.

Not surprisingly, the positive control treatment involving 1% sodium hypochlorite was found to be the most effective, giving a statistically greater reduction in surviving bacteria than ozone. The better performance of 2.5% sodium hypochlorite over gaseous ozone has been reported in previous studies using planktonic *E. faecalis* bacteria (30). Unlike this previous work, in the present study, an exposure time of 2 minutes to sodium hypochlorite could not render the canals completely free of viable bacteria. This reinforces the difficulty faced when using chemical treatments alone in dealing with bacteria in the root canal environment.

The benefits of ultrasonic energy combined with ozone seen in this study are similar to the effects seen when physical agitation using laser-generated shockwaves are combined with irrigants such as EDTA and hydrogen peroxide (30, 31). The mechanical agitation of fluid appears to enhance smear layer removal and the disruption of biofilms. The latter effect is known in industrial settings, but the phenomenon requires more research to ensure that the optimal benefits are gained when it is applied to the clinical setting of endodontics.

TABLE 1. Group Comparisons Using the Dunn Multiple Comparisons Test

Comparison	P value
Saline vs NaOCl	<.001
Saline vs ozone with ultrasonic	<.001
Saline vs ozone	<.001
Saline vs ultrasonic	NS
NaOCl vs ozone with ultrasonic	NS
NaOCl vs ozone	<.05
NaOCl vs ultrasonic	<.001
Ozone vs ozone with ultrasonic	NS
Ultrasonic vs ozone with ultrasonic	<.001
Ozone vs ultrasonic	<.01

NS, not significant.

Conclusions

The results of this laboratory study provide support for the concept that ozone gas delivered into irrigating fluids in the root canal may be useful as an adjunct for endodontic disinfection. Further research should be undertaken to examine the efficacy of agitated ozone on bacteria other than *E. faecalis* and, in particular, on mixed biofilms. It would also be of interest to compare rinsing canals with ozonated water or other fluids as opposed to delivering ozone-enriched air as in the present study.

Acknowledgments

The authors deny any conflicts of interest related to this study.

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