

Effects of ozone and photo-activated disinfection against *Enterococcus faecalis* biofilms *in vitro*

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Abstract

Objectives: The purpose of this study was to compare the antibacterial effects of gaseous ozone (O₃) and photo-activated disinfection (PAD) methods against *Enterococcus faecalis* (*E. faecalis*) biofilms.

Materials and Methods: Sixty-five human mandibular premolars with straight root canals were selected. After root canal preparation, the samples were sterilized and placed into eppendorf tubes with 1 mL brain heart infusion broth containing 1.5 × 10⁸ colony-forming units (CFUs)/mL of *E. faecalis*. The contaminated samples were then divided into four groups (*n* = 15) according to the disinfection method used: Group 1, Saline (positive control); Group 2, NaOCl (negative control); Group 3, Gaseous O₃; and Group 4, PAD. Three non-contaminated teeth were used to control the infection and sterilization process. The CFUs were counted and the data were analyzed statistically.

Results: There was a statistically significant difference between the experimental and control groups (*P* < 0.05). The saline group had the highest number of remaining microorganisms. Complete sterilization was achieved in the 2.5% NaOCl group. There were no statistically differences between PAD and gaseous O₃ (*P* > 0.05).

Conclusion: Both PAD and gaseous O₃ have a significant antibacterial effect on infected root canals. However, 2.5% NaOCl was superior in terms of its antimicrobial abilities compared with the other disinfection procedures.

Key words: Biofilm, ozone, photo-activated disinfection, root canal disinfection

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Introduction

Complete disinfection of the root canals with a combination of chemical agents and root canal files plays an important role in the success of root canal treatment.^[1] Although, treatment is adequate, failure may occur given the presence of pathogenic residual bacteria in dentinal walls of the canal.^[2]

Complex anatomy of root canal systems, with a variety of dentin tubules, apical canal ramifications, isthmuses and irregularities where bacteria may exist in the form of biofilms, makes the elimination of the microbial environment difficult.^[3] These microorganisms may

reinfect the root canals if they cannot be eliminated. Among the microorganisms, commonly isolated from root canals, *Enterococcus faecalis* (*E. faecalis*) has the ability to penetrate the dentinal tubules,^[2,4,5] exhibits strong adhesion to collagen^[5] and shows resistance to the irrigation solutions usually used during the instrumentation of root canals.^[6,7] Because, it is the representative bacteria of secondary infections in its planktonic and biofilm forms,^[8] the studies related to the elimination of root canal infections have been designed using *E. faecalis* biofilm models.^[9,10]

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A number of agents have been used as alternative irrigation solution: NaOCl, ethylenediaminetetraacetic acid (EDTA), chlorhexidine and other agents facilitating the elimination of microorganisms, and necrotic tissues. Of these, NaOCl is currently the most widely used endodontic irrigant because of its ability to dissolve necrotic tissues and its antibacterial properties.^[11-13] The main disadvantages of NaOCl are its unpleasant taste, cytotoxic effect on periradicular tissues,^[14] inability to remove the smear layer,^[15] and incomplete eradication of all bacteria from infected root canals.^[6]

Many researchers^[10,21] are still searching for new disinfection processes that will be effective for all bacteria and biocompatible with periradicular tissues in order to eliminate infection from root canal systems. Ozone (O₃) - a natural pale blue gas that can be found in the atmosphere, is a powerful oxidizing agent that can be produced by generators.^[16] As an antimicrobial agent, O₃ causes cellular lysis by affecting the osmotic stability of cell membranes with oxidated radicals in the presence of liquid, depending on the proceeding of reaction time.^[17] In dentistry, it has been used to promote soft tissue healing after surgical procedures, the treatment of root caries, and endodontic procedures.^[18,19] The research on O₃ systems continues to develop, allowing them to be used more effectively in root canals.^[20,21]

Recently, a new method of disinfection called photo-activated disinfection (PAD) has come to be used in endodontics. In this system, a photosensitizer agent attaches to the membrane of a microorganism, absorbs energy from the light and then releases this energy to oxygen (O₂), which is transformed into highly reactive O₂ ions and radicals. This reactive O₂ reacts and destroys the microorganisms effectively.^[22] Using the principles described above, a system involving a lamp (FotoSan; CMS Dental APS, Copenhagen, Denmark) has been developed for endodontic use as an alternative for high-power lasers function by dose-dependent heat generation. This PAD method is a treatment based on the combination of a photo-sensitizer (toluidine blue O [TBO]) and a powerful light in the red spectrum with a power peak at 628 nm. It is claimed that the light activated disinfection (LAD) principle is not only effective against bacteria, but also against other microorganisms including viruses, fungi, and protozoa.^[23,24] It has been concluded from toxicological tests that LAD has no negative side effect.^[25]

Considering the role of *E. faecalis* and its byproducts in the etiology of persistent pulpal and periapical pathogenesis, the aim of this study was to evaluate the effectiveness of O₃ and LAD as new disinfection strategies in the elimination of *E. faecalis* in root canals.

Materials and Methods

Selection and preparation of tooth specimens

Sixty-five freshly extracted human mandibular premolars

with straight root canals that were extracted for orthodontic reasons were selected. These teeth were anatomically similar, has fully developed apices and were free of cracks, caries, and fractures. Soft tissues and calculus were mechanically removed from the root surfaces with a periodontal scaler. Both buccolingual and mesiodistal preoperative digital radiographs were taken for each tooth to confirm the canal anatomy. The teeth were then stored in distilled water at room temperature until use.

Teeth were decoronated to obtain roots 15 mm in length. Canal patency was determined by passing a size 15 K-file (Mani Co., Tokyo, Japan) through the apical foramen. The specimens with apical diameters smaller than 15 K-file or >20 K-file were excluded. Gates–Glidden burs #4 (Dentsply Maillefer, Ballaigus, Switzerland) were used to prepare the 3 mm of coronal root canal orifices. The working length (WL) was determined by subtracting 1 mm from the point that the 15 K-file was seen at the major foramen. For the standardization of the root canal diameter, root canals were prepared using a reciproc R40, followed by an R50 (VDW, Munich, Germany). Irrigation was performed using 3 mL of 2.5% NaOCl after every 3 pecking motions and between files. After preparation, apical patency was controlled via a size 15 K-file. Finally, all root canals were irrigated with 2.5% NaOCl, 17% EDTA, and distilled water for 3 min each in an ultrasonic bath (Elmasonic S, Singen, Germany).

After preparation, 2 coats of nail varnish were applied to the external surface of all roots to prevent bacterial microleakage through lateral canals. Then, each specimen was submerged in 1 mL saline in a 2 mL eppendorf tube for sterilization by autoclaving at 121°C for 20 min. After sterilization, the roots were incubated in brain heart infusion (BHI) broth (Acumedia; Neogen Co., Lansing, MI, USA) for 24 h at 37°C to ensure that there is no bacterial contamination. Three noninoculated teeth were kept incubated at 37°C as an aseptic control during the 7-day contamination period.

Preparation and inoculation of *Enterococcus faecalis*

Enterococcus faecalis (ATCC 29212) was inoculated using BHI agar (Acumedia) and grown overnight at 37°C in an incubator. Then, a colony of the pure-cultured strain was collected and suspended in BHI broth. The concentration of the bacterial suspension was adjusted to an optical density (OD₆₀₀) of 0.1 and McFarland standard number of 0.5 to provide a suspension of approximately 1.5×10^8 colony-forming units (CFU)/mL.

Dentin infection

Sixty-two root canal specimens were placed in eppendorf tubes with 1 mL BHI broth containing 1.5×10^8 CFUs/mL of *E. faecalis*. Each specimen was incubated for 7 days at 37°C, and the 1 mL BHI broth was refreshed every 2 days to ensure the viability of the bacteria.^[10] 7 days after the inoculation,

control of *E. faecalis* contamination was performed using a size 40 sterile paper points (Dentsply/Maillefer) that were placed inside the root canal for 1 min and then transferred to eppendorf tubes containing 1 mL saline. The tubes were vortexed for 30 s (MRC Vortex mixer, HaGavish 3 Holon, Israel). Aliquots of 0.1 mL were plated on BHI agar in duplicate. After 24 h of incubation at 37°C, microbial growth was verified, confirming the existence of *E. faecalis* in root canals. Furthermore, 2 samples were separated and prepared for scanning electron microscopy (Leo 440; Oxford Microscopy Ltd., Cambridge, England) to confirm the existence of bacteria and biofilms.

Sixty infected samples were divided into two experimental groups, one negative control; and one positive control groups ($n = 15$).

Disinfection procedures

Root canals were irrigated with 5 mL sterile saline in the positive control group and with 5 mL 2.5% NaOCl in the negative control group using a 27-gauge dental injector with peristaltic motion that was placed 2 mm away from the WL for 2 min.

Ozone group

A dental O₃ device (Ozonytron XL-Bioozonix, Munich, Germany) was used by attaching a sterile endodontic cannula (KP probe; Ozonytron XL-Bioozonix). A KP probe was inserted 2 mm short of the apex. O₃ was delivered at 100% for 40 s with peristaltic motions, as recommended by the manufacturer. The probe was removed from the canal after each 40 s cycle to prevent room air being delivered during the system purging. The canal was then irrigated with 1 mL sterile saline and removed between each cycle. The O₃ treatment was repeated 3 times, giving a total ozonation time of 2 min.

Photo-activated disinfection Group

Teeth were disinfected with a PAD device (FotoSan). The LAD solution (FotoSan Agent photosensitizer; low viscosity, containing 100 µg/ml TBO) was injected into the canal using a 27-gauge endodontic microneedle (Ultradent Inc., South Jordan, ABD), and the liquid was then agitated in each canal for 90 s using size 40 K-files. The emitter was inserted 2 mm short of the apex and light-cured for 30 s (15 W light-emitting diode [LED], output intensity of 2000 mW/cm², wavelength of 625–635 nm). The entire procedure was finished in 2 min.

All procedures were carried out in a laminar flow chamber (Atlas Clean Air, Preston England), using files. Root canals were sampled after the treatment protocols a smooth broach was inserted into the root canal and agitated for 1 min,^[10] and then the size 40 paper point was immersed in the root canal for 60 s to complete bacterial sampling. Following each sampling, the paper points were

transferred to eppendorf tubes containing 1 mL of freshly prepared BHI broth and vortexed for 1 min. After 10-fold serial dilutions, aliquots of 0.1 mL were plated onto BHI agar plates and incubated at 37°C for 24 h. The CFU were counted and recorded.

Statistical analysis

Statistical tests were performed using Sigmastat Software package (Systat; Chicago, IL, USA). The data were statistically analyzed using the one-way ANOVA, Kruskal–Wallis and Mann–Whitney U-tests. The level of statistical significance was set at $P < 0.05$.

Results

After 7 days of incubation, the initial total CFU count in a specimen reached 10⁸. The *E. faecalis* biofilms could not be destroyed successfully in the root canals at the positive control group [Figure 1a-b]. Many layers of bacteria still accumulated around the opening of the dentinal tubules. After the use of NaOCl, O₃ and LAD, it was observed that the regular structure of the biofilm was destroyed and replaced with ruptured bacteria [Figure 1c-d, e-f, g-h].

All treated groups revealed reduced CFU of *E. faecalis* as compared with the positive control group ($P < 0.05$). The

Table 1: Log CFU mean numbers of remaining bacteria

Groups	Total (n)	Mean (CFU mL ⁻¹)	SD (CFU mL ⁻¹)	Minimum-maximum (CFU mL ⁻¹)
Saline ^a (positive control)	15	42933.3	10905.2	20000-56000
NaOCl ^b (negative control)	15	0	0	0-0
Ozone ^c	15	4833.3	2181.6	1000-8200
LAD ^{c,d}	15	4090.6	2077.8	1160-8400

The same supercripted letters indicate no significant differences ($P > 0.05$). CFU=Colony-forming unit; SD=Standard deviation; LAD=Light activated disinfection

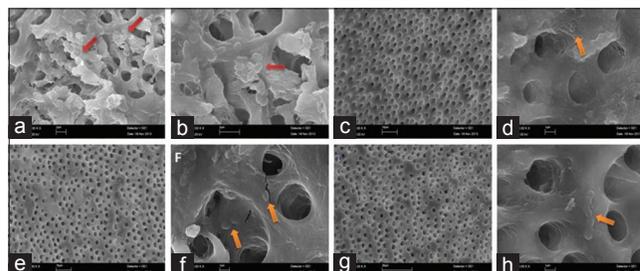


Figure 1: A scanning electron microscopy scan of *Enterococcus faecalis* (*E. faecalis*) biofilm in the positive control group (a; original $\times 10000$ and b; $\times 20000$). Individual cocci can be seen embedded in a dense matrix (red arrows). Bacteria, original $\times 3000$ and $\times 20000$, respectively; (c and d) NaOCl, (e and f) ozone, and (g and h) light activated disinfection group. Residual *E. faecalis* attached to the dentine after disinfection procedures at $\times 20000$ (orange arrows)

highest level of inactivation was obtained with NaOCl and statistically different compared with other treated groups ($P < 0.05$). There were no statistically differences between the O_3 and LAD groups ($P > 0.05$).

Discussion

In this study, an *in vitro* infected dentine model developed by Haapasalo and Ørstavik^[26] was used with some modifications. This model can be used to evaluate the antimicrobial effects of root canal disinfectants, medicaments, and sealers.^[27] The study simulated clinical conditions by using human teeth and microorganisms that were grown in a liquid medium containing a tooth, which are similar to those found in the oral environment.

In the present study, the root canal system was contaminated with *E. faecalis*, a Gram-positive facultative bacteria. It has been reported to be the most commonly identified species in root canals that result in failed endodontic treatment.^[5] Because of the hardy nature of this bacteria, it can grow and survive as a monoculture under diverse conditions, including in nutrition-depleted root canals. It seems to be the one most able to penetrate into dentinal tubules, leading to gross infection.^[28]

NaOCl is a widely used irrigant in root canal treatment. It can create large zones of inhibition against *E. faecalis*.^[29,30] However, the data regarding the inhibitory concentration and application time of NaOCl against *E. faecalis* are not clear enough in the literature.^[29,32] In the present study, a significant effect was achieved with 2.5% NaOCl irrigation in only 2 min, much like in previous studies.^[21,22]

The bactericidal efficacy of O_3 is based on forming oxidated radicals in aqueous solutions, as a result of which the cell membranes become damaged due to altered osmotic stability and permeability.^[20] However, there is no consensus regarding the application manner, time, and dosages of O_3 needed to achieve significant results. Estrela *et al.*^[31] investigated the antimicrobial efficacy of ozonated water, gaseous O_3 , NaOCl and chlorhexidine in human root canals infected by *E. faecalis*. They reported that in both sample collection intervals (immediately and after 72 h), none of the irrigants had an antimicrobial effect against *E. faecalis* over a 20 min contact time in infected root canals. Hems *et al.*^[33] reported that when *E. faecalis* cells were treated with O_3 over time periods of 30–240 s using nutrient broth as the medium, there was a 1-to 2-log¹⁰ reduction of bacterial counts and that a significant reduction could be achieved only after 240 s application. In the present study, O_3 had an important antibacterial effect eliminating approximately 91% of *E. faecalis* biofilms in 2 min. This positive result, which was in contrast with the studies mentioned, was caused by differences in the contamination period, the delivery system of gaseous O_3 and the sample collection.

In this study, performed O_3 delivery system use cold plasma therapy technology to transform O_2 to O_3 . At the push of a button, the plasma gas nozzle allows plasma flooding at a high concentration of 22.000 ppm into the root canals. This technology could support the efficacy of O_3 in root canals. This antibacterial effect of gaseous O_3 can also be explained by the methodology used: Gaseous O_3 was applied for 120 s with a peristaltic motion to root canals irrigated with saline between cycles. The duration and methodology of these actions were important considerations in terms of the antibacterial effect of O_3 . There were significant reductions of *E. faecalis* in the O_3 group ($P < 0.05$), but it was not superior than 2.5% NaOCl. Case *et al.*^[34] reported that exposure to O_3 -enriched air for a total period of 2 min resulted in a 71.6% reduction in viable CFU as compared with the control group, but this was not superior than 1% NaOCl. Üreyan *et al.*^[21] investigated the effect of gaseous O_3 and low-temperature atmospheric pressure plasma (LTAPP) as an alternative for NaOCl. According the results of the study, a total gaseous O_3 application time of 2 min had no greater effect than LTAPP and 2.5% NaOCl. Our findings supports the results of these studies.^[21,34]

High-power lasers used for photodynamic therapy generate heat that may injure periapical tissues. Advanced noninvasive PAD using a photosensitizer formulation containing an oxidizer and an O_2 carrier has been demonstrated to disrupt the biofilm matrix and to facilitate the comprehensive inactivation and disinfection of mature endodontic biofilms.^[35] LED light serves as a safer alternative light source because it does not generate significant heat.^[36] FotoSan has been recently developed for this reason and has a slightly toxic effect and excellent biocompatibility.^[37] Its effectiveness against *E. faecalis* biofilm as compared with conventional canal disinfection protocols and O_3 has not been studied. In a previous study, FotoSan showed 97% bacterial elimination against to *E. faecalis* but had no superficial effect than 6% NaOCl.^[38] Our results are similar with this study that root canals treated with FotoSan exhibited a 91% reduction of *E. faecalis* viability that was better than the antibacterial efficacy of O_3 , whereas the 2.5% NaOCl has provided complete bacterial elimination. As well as, in the mentioned study,^[38] the combined use of NaOCl and the PAD had provided the most strong antibacterial efficacy.

Conclusion

Under limitations of this study, it can be concluded that NaOCl is still the most effective irrigating solution in terms of killing endodontic pathogens. O_3 and PAD with FotoSan could be considered ideal devices if used at the end of canal treatment to improve the effect of conventional irrigating solutions.

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