

**ORIGINAL ARTICLE**

# ***In vitro* assessment of the recurrent doses of topical gaseous ozone in the removal of *Enterococcus faecalis* biofilms in root canals**

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## Abstract

**Aim:** To evaluate the potential antibacterial effect of recurrent doses of topical gaseous ozone on the *Enterococcus faecalis* biofilms growth in human root canals *in vitro*.

**Materials and Methods:** One hundred and thirty four human single-rooted mandibular premolars were enlarged to a size 35 K-File. Each root canal were inoculated with an overnight culture of *Enterococcus faecalis* ATCC 29212 in tryptic soy broth for 24 hours and incubated for 7 days at 37°C. At 7-day interval, 4 specimens were prepared for Scanning Electron Microscope (SEM) analysis to confirm the presence and purity of biofilms whilst the other contaminated root canals were irrigated and disinfected. One hundred root canals of total 134 specimens were selected to create the experimental groups and divided into 5 subgroups. In each experimental group ( $n = 20$  root canals), recurrent ozone doses were applied with different irrigation and disinfection protocols in 5 different time intervals. Bacterial growth was analyzed by counting viable *E. faecalis* on tryptic soy agar plates.

**Results:** According to intergroup comparison results observed in the final sample collection analysis, the amount of remaining bacteria in the positive control group were found to be significantly higher compared to Groups 1, 2, 3, 4, 5 and the material control group ( $P < 0.01$ ). The remaining amount of bacteria in the last count of Group 1 were found to be significantly higher compared to Group 2 ( $P < 0.05$ ), Group 4 ( $P < 0.01$ ), Group 5 ( $P < 0.05$ ) and the material control group ( $P < 0.01$ ).

**Conclusion:** The application of topical gaseous ozone in recurrent doses provides a positive effect in the removal of *E. faecalis* biofilm from root canals. However, during disinfection procedure, the combined use of recurrent doses of topical gaseous ozone with 2% NaOCl enhanced its antibacterial effect against *E. faecalis* biofilm.

**Key words:** Antibacterial effect, disinfection, *Enterococcus faecalis* biofilm, irrigation, recurrent doses, root canals, topical gaseous ozone

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## Introduction

Endodontic infections are based on multiple species of microorganisms which have to be treated considering the resistance of polymicrobial flora.<sup>[1]</sup> The success of root canal treatment mainly depends on the elimination of microorganisms from root canals by using effective

instrumentation methods besides irrigation regimen. Otherwise, reinfection due to the penetration of bacteria or by-products into dentinal tubules might occur. The prognosis of primary root canal treatment was detected to have better results than apical periodontitis.<sup>[2]</sup> Studies on

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persistent infections have shown the relevance between *Enterococcus faecalis*, which is a gram-positive facultative anaerobic bacteria and secondary apical periodontitis.<sup>[3]</sup> The resistance of *E. faecalis* is explained with the penetration potential into dentinal tubules,<sup>[4-6]</sup> prolonged survival capacity in root-filled teeth,<sup>[7]</sup> adhesion ability to collagen matrix existing in dentin<sup>[8,9]</sup> and inadequate response of antimicrobial irrigation solutions.<sup>[10]</sup>

Thus, researchers progressively attempt to improve some alternative methods or solutions which might provide coping with the persistent infections related with endodontics. For instance, calcium hydroxide [ $\text{Ca}(\text{OH})_2$ ] has been a medication of choice used for intracanal disinfection due to its high antibacterial effect for many years. Its antibacterial mechanism is explained by the hydroxyl ion release in an aqueous environment,<sup>[11]</sup> which prevents the microorganisms to survive in its highly alkaline environment.<sup>[12]</sup> However, functioning proton pump of *E. faecalis* enables the bacteria to survive due to its defusing capacity of  $\text{Ca}(\text{OH})_2$  alkalinity at/below pH 11.2.<sup>[13]</sup> Moreover, *E. faecalis* is detected to survive against  $\text{Ca}(\text{OH})_2$  by reason of its adhesion capability to collagen matrix that provides colonization on dentin.<sup>[8,9]</sup> As a further alternative, sodium hypochlorite (NaOCl) has been also routinely used in endodontics for its high antimicrobial and organic tissue removal efficacy for many years. However, because of its high cytotoxic results on oral cells and periapical tissues detected in previous studies,<sup>[14,15]</sup> NaOCl is preferred to be used in low concentrations which might result in failure<sup>[16,17]</sup> in the total elimination of *E. faecalis* biofilms in root canals. So, increasing its temperature, activation with sonic and ultrasonic devices or increasing its volume might be some proper techniques to obtain the eradication of persistent infections despite its low concentration. Nevertheless, along these methods, cytotoxic effects of NaOCl to periapical tissues in high concentrations,<sup>[15]</sup> inability of NaOCl to reach bacteria and potential inadequacy of  $\text{Ca}(\text{OH})_2$  to eliminate *E. faecalis*<sup>[18]</sup> prompted researchers to seek further reliable disinfectants/methods as an alternative to currently used ones.

Recently, ozone ( $\text{O}_3$ ) has been introduced to dentistry for its antibacterial effect especially for the elimination of endodontal and periodontal pathogens.<sup>[19,20]</sup> It is thought to be an alternative to NaOCl in endodontics regarding its antimicrobial effect on the resistant/persistent microorganisms. Ozone is composed of three oxygen atoms and exists in nature as an oxidant which can react with organic compounds.<sup>[21,22]</sup> Ozone was shown to be effective to planctonic forms of *E. faecalis* rather than biofilms.<sup>[23]</sup> In addition, in the current literature, there are also studies regarding the use of gaseous  $\text{O}_3$  in terms of its effectiveness in the eradication of *E. faecalis* biofilms from root canals.<sup>[19]</sup>

However, there has been no study concerning the antimicrobial efficacy of topical gaseous  $\text{O}_3$  when applied

in recurrent doses to *E. faecalis* biofilm-coated root canals. Therefore, the aim of this study was to assess the antimicrobial effect of recurrent doses of topical ozonized gas in the presence and absence of NaOCl and  $\text{Ca}(\text{OH})_2$ . Ethylenediamine tetraacetic acid (EDTA) was included in this study as a part of current irrigation protocol due to its inorganic tissue removal effect rather than an alternative antimicrobial agent.

## Materials and Methods

### Specimen preparation

Human single-rooted mandibular premolars ( $n = 134$ ) with a single root canal and with no anatomical or pathological modifications were used. The teeth were stored in a sterile saline solution for seven days. The crown of each tooth was sectioned from the cement-enamel junction with IsoMet® 1000 Precision Sectioning Saw (Buehler, Lake Bluff, IL, USA) to standardize the root length to 15 mm. Root canals were enlarged with step-back technique using K-file instruments (Kerr-files, Maillefer, Ballaigues, Switzerland) and the master apical file for each sample was a size 35 K-file. During chemomechanical preparation, root canals were irrigated with side-perforated needles (Hawe irrigation probe, Bioggio, Switzerland) containing sterile saline solution at each instrument change.

The apical foramen of each root canal was sealed with light-polymerizing flowable composite (Aelite flo, Schaumburg, USA) and the specimens were embedded into cold acrylic (Imicryl, Konya, Turkey) exposing the cervical parts of the root canals which include the access cavities. The specimens were autoclaved at 121°C for 15 minutes.

### Bacteria and culture conditions

*Enterococcus faecalis* ATCC 29212 was subcultured on trypticase soy agar (TSA, Merck, Darmstadt, Germany) and incubated aerobically at 37°C for 24 h. To generate a stock inoculum, a single colony was then harvested, placed in tryptic soy broth (TSB, Merck, Darmstadt, Germany), following the same incubation conditions; the turbidity of *E. faecalis* culture was adjusted to No. 0.5 Mc Farland Standard.

### Specimen contamination and biofilm formation

Five micro liters of bacterial suspension (final concentration of about  $1.5 \times 10^8$  cfu/ml) were applied into the mechanically enlarged root canals with a sterile micropipette, except ten canals, which served as negative controls. The suspension was applied into the canal using a sterile endodontic file size ISO #20 (Kerr-files; Maillefer, Ballaigues, Switzerland) for the same period for each sample. The openings of the canals were sealed with a temporary filling material (Coltosol® Coltene, Whaledent). All samples were stored at 37°C for seven

days in a humidity atmosphere under aerobic conditions. Root canals were reinoculated with fresh stock culture at first, fourth and sixth day.

### Scanning electron microscopy

At 7-day interval, 4 specimens were prepared to confirm the biofilm formation by scanning electron microscopy (SEM) analysis. Four roots were immersed in a fixative solution containing 4% buffered paraformaldehyde for 24 hours. The roots were dehydrated in ascending degrees of ethanol series following the separation with a diamond bur (Acurata GmbH and Co. KG, LOS 4209061801, Thurmansbang, Germany) longitudinally. Samples were air-dried at room temperature for 24-hours and coated with a gold layer (5 nm thick) with a sputter coater (Model BAL-TEC SCD 005 Sputter Coater, Balzers, Liechtenstein) to impart electrical conductivity. The accelerating voltage was 5 kV for all experiments. SEM images were obtained from the whole length of the separated root canals, but especially including specific areas of interest for apical region at various magnifications (20000 × and 15000 ×).

### Experimental endodontic procedures

Following the chemomechanical preparation with the use of step-back technique and sterile-saline solution irrigation, 100 root canals from the total of 134 specimens were selected to create the experimental groups due to the materials used for the irrigation and disinfection protocols. Experimental groups were further divided into 5 subgroups ( $n = 20$ ), whilst 30 root canals of total 134 specimens were divided into 3 subgroups ( $n = 10$ ) as the negative control, positive control and material control groups. Following instrumentation and *E. faecalis* contamination procedures, disinfection and irrigation protocols were applied at 5 different time intervals as shown in Table 1. Seven days after the first application, initial sample collection was done. Recurrent  $O_3$  applications were performed every 3 days and the treatment protocol is shown in Table 1. 2% (Chlorax, lot no. 29-12-2011) and 5.25% NaOCl (Chlorax, lot no. 22.10.2012, PPH Cerkamed, Stalowa Wola, Poland) irrigations were applied for 2 minutes whilst EDTA (Endo-Solution, 15%, lot no. 27-08-2012/1, PPH Cerkamed, Stalowa Wola, Poland) irrigation was done for 1 minute. Topical gaseous  $O_3$  (OzonytronX, MYMED, Germany) was applied 5 times during a 19-day period according to the manufacturer's instructions. In the beginning of the study, the numbers per all experimental groups 1, 2, 3, 4 and 5 were 20 ( $n = 20$ ), which were decreased in number to 10 ( $n = 10$ ) after the initial sample collection.

### Quantification of initial and final *E. faecalis* contamination

The canal cultures were collected in two different periods from the root canals of the test groups and positive control

group. The initial sample collection ( $n = 10$ ) was after 7 day of contamination, by scrapping a size 30 sterilized Hedstrom-File to the root canal walls and immersing a size 25-absorbent paper inside the root canal for 1 minute. The final culture collection ( $n = 10$ ) was done in the same way 2 weeks later from the first application of irrigation and disinfection protocols. Dentin chips isolated from root canal walls were transferred into polypropylene flasks containing 1 ml of sterile saline solution, by means of H-File and absorbent papers. All collected samples were vortexed for 10 seconds and 10-fold dilutions were prepared. Aliquots of 0.1ml suspensions were inoculated on TSA plates and incubated at 37°C for 24 h. Colony forming units per ml (cfu/ml) were enumerated for per root canal sample.

### Statistical analyses

Statistical analysis was performed using the SPSS (Statistical package for social sciences) program for Windows 15.0. The intergroup comparisons of parameters were analyzed by Kruskal-Wallis test whereas Mann-Whitney U test was used for determining the group, causing the difference. The intragroup comparisons of parameters were analyzed by Wilcoxon signed rank test. Statistical significance level was set at  $P < 0.05$ .

## Results

In intragroup comparisons, the decrease in the last bacterial count in Groups 1 and 2 compared to the initial count was found to be statistically significant ( $P < 0.01$ ). In Group 3, no significant change was observed in the last bacterial count compared to the initial count. ( $P > 0.05$ ). In Group 4, the amount of decrease between the first and last bacterial counts were found to be statistically significant ( $P < 0.05$ ) [Table 2].

In intergroup comparison, the amount of remaining bacteria in the last count of the control group were found to be significantly higher compared to Groups 1, 2, 3, 4, 5 and the material control group ( $P < 0.01$ ). Also, according to the analysis of the samples recovered from the last step, no significant difference was observed between group 2 and the other tested groups 3, 4, 5 and material control group ( $P > 0.05$ ) except group 1 ( $P < 0.05$ ). The amount of remaining bacteria in the root canals in Groups 3 and 4 were found to be significantly higher compared to the material control group ( $P < 0.05$ ) [Table 3].

Non-significant difference was observed between material control group and Group 2 ( $P > 0.05$ ) according to the final sample collection results [Table 3 and Figure 1].

SEM images obtained from the visualization of *E. faecalis* biofilm growth in root canals at 7-day interval are shown

**Table 1: Treatment protocol showing the use of recurrent ozone doses with different disinfectants against *E. faecalis* biofilms**

	Group 1	Group 2	Group 3	Group 4	Group 5	Material control group
1 <sup>st</sup> application	O3	O3	O3	O3	%2 NaOCl Ca (OH) 2	%5.25 NaOCl Ca (OH) 2
7 <sup>th</sup> day	Initial sample					
2 <sup>nd</sup> application-10 <sup>th</sup> day	O3	%2 NaOCl %15EDTA O3	Distilled water O3	%2 NaOCl %15EDTA O3	%2 NaOCl %15EDTA O3	%5.25 NaOCl %15 EDTA
3 <sup>rd</sup> application-13 <sup>th</sup> day	O3	O3	O3	O3	O3	-
4 <sup>th</sup> application-16 <sup>th</sup> day	O3	O3	O3	O3	O3	-
5 <sup>th</sup> application-19 <sup>th</sup> day	O3	O3	O3	O3	O3	-
	Final sample collection					

EDTA=Ethylenediamine tetraacetic acid

**Table 2: P values of intragroup comparisons obtained from the analysis of both initial and final counts**

The amount of remaining bacteria in root canals	Average $\pm$ SD (median)		+ + P
	Initial sample collection results	Final sample collection results	
Positive control group	148750 $\pm$ 154068,4 (90000)	15347,5 $\pm$ 15928,3 (9000)	0,012*
Group 1	27200 $\pm$ 19723,3 (19500)	130 $\pm$ 188,6 (40)	0,005**
Group 2	27200 $\pm$ 19723,3 (19500)	120 $\pm$ 379,5 (0)	0,005**
Group 3	81,0 $\pm$ 143,1 (15)	48,0 $\pm$ 117,5 (10)	0.4
Group 4	81,0 $\pm$ 143,1 (15)	0,00 $\pm$ 0,00 (0)	0,042*
Group 5	140 $\pm$ 442,7 (0)	4,00 $\pm$ 8,43 (0)	0.655
Material control group	0,00 $\pm$ 0,00 (0)	0,00 $\pm$ 0,00 (0)	1
+P	0,001**	0,001**	

SD=Standard deviation, +Kruskal Wallis test, ++Wilcoxon sign test, \*P&lt;0.05, \*\*P&lt;0.01

in Figure 2, in which the connection between the bacteria and surface and to each other was noticeable suggesting the biofilm phenotype of *E. faecalis*.

## Discussion

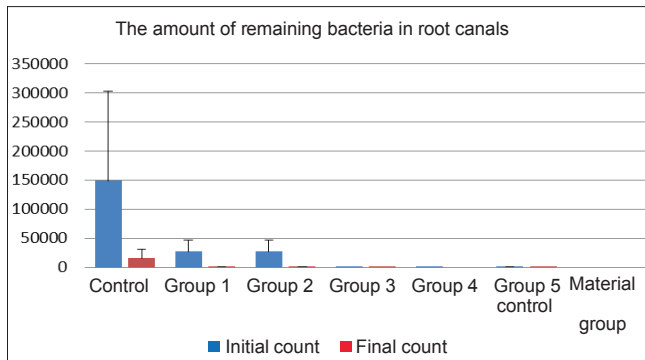
Primary apical periodontitis can be defined briefly as the infection of necrotic pulp tissue. The main cause of treatment failure in teeth with primary endodontic infections can be summarized as the inadequate elimination of intracanal microbiota and incomplete implementation of treatment procedures. In addition, persistent/secondary apical periodontitis is the consequence resulting in the failure of root canal treatment of teeth with primary apical periodontitis.<sup>[24]</sup> Persistent apical periodontitis is characterized by the persistent radiolucent lesion, which provides the progressive destruction of peri-radicular tissues by the aid of specific microorganisms. Ability to survive in the poor environmental conditions<sup>[7]</sup> and penetration capacity into dentinal tubules, isthmuses and lateral canals<sup>[25]</sup> might be the most prominent features that enable the microorganisms to provide infection persistence. Thus, different endodontic pathogens were detected to be responsible for primary and secondary apical periodontitis.<sup>[26]</sup>

**Table 3: P values of intergroup comparisons obtained from the analysis of both initial and final counts**

	p	
	Initial sample collection results	Final sample collection results
Control/Group 1	0,033*	0,001**
Control/Group 2	0,033*	0,001**
Control/Group 3	0,001**	0,001**
Control/Group 4	0,001**	0,001**
Control/Group 5	0,001**	0,001**
Control/Material control	0,001**	0,001**
Group 1/Group 2	1	0,024*
Group 1/Group 3	0,001**	0.194
Group 1/Group 4	0,001**	0,002**
Group 1/Group 5	0,001**	0,013*
Group 1/Material control	0,001**	0,002**
Group 2/Group 3	0,001**	0.102
Group 2/Group 4	0,001**	0.317
Group 2/Group 5	0,001**	0.626
Group 2/Material control	0,001**	0.317
Group 3/Group 4	1	0,013*
Group 3/Group 5	0.103	0.128
Group 3/Material control	0,013*	0,013*
Group 4/Group 5	0.103	0.146
Group 4/Material control	0,013*	1
Group 5/Material control	0.317	0.146

\*Mann Whitney U test, \*P&lt;0.05, \*\*P&lt;0.01. Control indicates positive control group

For instance, *E. faecalis* was found to be related in the secondary apical periodontitis<sup>[5,27]</sup> which is a Gram-positive facultative anaerobic commensal bacterium inhabiting in the gastrointestinal system of humans. It is supposed to be existed temporarily in the oral cavity and introduced into root canal system during/after root canal treatment of teeth with apical periodontitis.<sup>[28]</sup> The ability of *E. faecalis* to form aggregates or biofilms increases its resistance against intracanal disinfection. Since biofilms are formed when some bacteria species generate mucopolysaccharide structure to increase their survival rate and persistence compared to own planktonic types,<sup>[29]</sup> the use of biofilm-grown

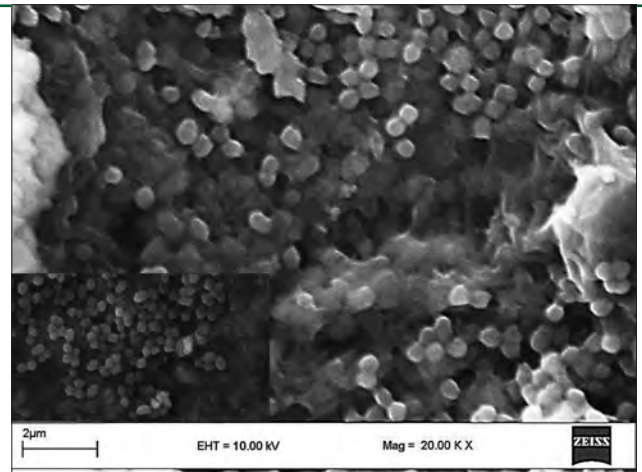


**Figure 1:** Graphic showing the amount of remaining bacteria in root canals

bacteria enables the study design to put forth better clinical simulation in the detection of effective irrigation and disinfection regimens.

Ca(OH)<sub>2</sub> and NaOCl have been routinely used for the elimination of endodontic infections based on their proven antimicrobial effects. However, irrigation and disinfection regimens are shown to be insufficient in the case of eradication of *E. faecalis* from the root canals.<sup>[10,18]</sup> Evans *et al.* (2002), reported the serious amount of reduction in cell survival when Ca(OH)<sub>2</sub> at pH 11.5 was introduced to *E. faecalis* strains.<sup>[13]</sup> But recently, Ca(OH)<sub>2</sub> was found to be ineffective in the removal of *E. faecalis* biofilm,<sup>[9]</sup> and even the increase in the amount of bacterial counts was detected when cultures were taken immediately following the removal of 4-week medication of root canals with Ca(OH)<sub>2</sub>.<sup>[30]</sup> In the present study, Ca(OH)<sub>2</sub> had reducing effect compared with single dose O<sub>3</sub> application; however, based on the results of final sample collection observed in comparison with group 2 and Ca(OH)<sub>2</sub> included group 4, Ca(OH)<sub>2</sub> was found to be ineffective in the eradication of *E. faecalis* biofilm from root canals. Additionally, following the removal of Ca(OH)<sub>2</sub> from root canals, the application of topical gaseous O<sub>3</sub> in recurrent doses might have reduced the growth of bacteria from the initial to final collection periods of the study. Furthermore, recurrent O<sub>3</sub> doses when used with/without Ca(OH)<sub>2</sub> and distilled water was also detected to be insufficient in the total elimination of *E. faecalis* biofilm from 1-week old root canals. On the other hand, recurrent O<sub>3</sub> doses exhibited eradication of *E. faecalis* biofilm from the root canals when used in combination with 2% NaOCl.

In the study of Meire *et al.* (2012), irrigation with 2.5% NaOCl solution for 5 minutes or longer provided total remove of biofilm from dentin discs. Also, it is stated that higher concentrations are required to achieve the expected results in the *in vitro* conditions.<sup>[31]</sup> In addition, such a disinfection regimen needs to be applied that inactivates *E. faecalis* biofilm as well as removes completely from the root canals.<sup>[17]</sup> The application time,<sup>[30]</sup> intervals and the concentration rate<sup>[32]</sup> of NaOCl indicate its effectiveness



**Figure 2:** Visualization of *Enterococcus faecalis* biofilm in a control root canal by scanning electron microscope. (20,000 × and 15,000 ×)

of removing *E. faecalis* biofilm from root canals. The 6% concentration of NaOCl was detected to be effective in an independent manner of the implementation duration in the complete elimination of *E. faecalis* biofilms grown in a flow cell system.<sup>[32]</sup> Moreover, Clegg *et al.* (2006) stated that 6% NaOCl was the only disinfectant, which provides *E. faecalis* eradication from the apical part of the root canals.<sup>[17]</sup> Consistently, this present study revealed that 5.25% NaOCl, which was used in the material control group had eradication effect on *E. faecalis* biofilm in root canals. However, the application of NaOCl in high concentrations is an inconsistent regimen with the common expectation in endodontics, which requires the non-toxic effect of endodontic irrigants to periapical area. In the study of Huth *et al.* (2006), O<sub>3</sub> gas (4 g/m<sup>3</sup>) has been found to be less cytotoxic than NaOCl (2.5%) to oral cells.<sup>[15]</sup> In addition, eradication of 3-week-old *E. faecalis* biofilm has found to be possible when gaseous O<sub>3</sub> applied at a concentration of 32 g/m<sup>3</sup> for 1 minute or 4 g/m<sup>3</sup> for at least 2.5 minutes.<sup>[15]</sup> However, gaseous O<sub>3</sub> and NaOCl (2.5%) with 20 minutes contact time was found to be ineffective against 60-day contaminated of root canals with *E. faecalis* biofilm.<sup>[10]</sup>

Consequently, in the present study, the significantly higher toxic results observed in 2% NaOCl, Ca(OH)<sub>2</sub>, 17% EDTA included groups<sup>[4,5]</sup> in comparison with only O<sub>3</sub> applied group (group 1) was already expected. However, the application of recurrent O<sub>3</sub> doses alone showed a significantly higher positive and increasing effect in the elimination of bacteria from initial to final sample collection stages compared with positive control group. The only disadvantage of topical gaseous O<sub>3</sub> application in recurrent doses might be the increase in the number of sessions. However, in the case of persistent infections, debriding, shaping and disinfection procedures are also repeated several times to ensure the success of root canal treatment due to the eradication of microorganisms in root

canals. So, if orthograde O<sub>3</sub> application in recurrent doses provides a chance for the success of root canal treatment with persistent infections, the increase in the number of sessions may be disregarded. However, further studies should be performed to investigate the antimicrobial effect of topical gaseous O<sub>3</sub> in the persistent root canal infections.

## Conclusion

Within the limitations of this study, the application of recurrent O<sub>3</sub> doses with the combination of 2% NaOCl for 2 minutes, was totally eliminated *E. faecalis* biofilm from root canals. This result has a significance suggesting that the possibility of reducing NaOCl toxic effect by the combination of its lower concentrations with recurrent O<sub>3</sub> doses.

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