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EVALUATION OF ANTIMICROBIAL EFFICACY OF GASEOUS OZONE AND OZONIZED WATER AGAINST ENTEROCOCCUS FAECALIS BIOFILM

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ABSTRACT

40 single-canalled permanent teeth were instrumented then inoculated with *Enterococcus faecalis* and stored in incubator for one week. After biofilm model conformation by SEM, the samples were irrigated with 5% sodium hypochlorite, gaseous ozone (44 µg ml-1) and gaseous ozone with ozonized water (5 µg ml-1). Root canals were sampled before (S1) and immediately after irrigation (S2). The samples were taken by three dry sterile paper points placed to the full WL kept in the canal for 1 min then transferred to tubes containing .05 mL BHI broth solution. After obtaining 1:10 serial dilutions from each sample, aliquots were plated out on KF streptococcus agar plates. Then the plates were incubated anaerobically at 37 C for 2 days. The number of bacterial colonies of *E. faecalis* were counted and expressed as CFUs using a digital colony counter. All groups showed a significant reduction of CFUs ($P < 0.001$) while greater antimicrobial reduction was observed on samples of NaOCl irrigation. There was no significant difference between gaseous ozone and gaseous ozone + ozonized water groups. It can be concluded that the gaseous ozone reduced *E. faecalis* number significantly but was not able to eradicate it completely. While ozonized water failed to increase the antimicrobial efficacy of gaseous ozone.

INTRODUCTION

The main aim of root canal treatment is to eradicate bacteria which cause pulpal / periapical infection and obtaining optimal obturation to prevent reinfection after treatment[1]. Bacteria are considerably reduced by Chemo-mechanical preparation. However, there are limitations regarding its effectiveness, up to 60% of the radicular dentin is not touched during cleaning and shaping phase [2]. Furthermore, bacterial biofilms show high resistance against disinfecting materials[3]. Primary infections are associated with gram-negative, gram-positive and anaerobic bacteria[4]. Post-treatment endodontic infections are associated with a high level of anaerobic and gram-positive bacteria. Particularly, previous studies showed that E. faecalis has been associated with many cases of endodontic failures[4].

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Ozone (O3), a naturally very strong selective oxidant gas [5]. Ozone destroying bacterial cell walls by oxidation. In a previous in vitro study, Ozonized water reduced the number of E. faecalis significantly in infected dentinal tubules [6]. In another study, ozonized water was less effective in killing E. faecalis in a biofilm model compared to NaOCl solution [7]. As far as the antimicrobial power of NaOCl solution compared to ozone is concerned, the antimicrobial action of ozone has shown controversial findings [9–11].

Two of these investigations [10, 11] showed that gaseous and aqueous ozone is less effective in killing E. faecalis in the biofilm model compared to sodium hypochlorite solution, while in another study [11], aqueous and gaseous ozone were as effective as sodium hypochlorite being able to completely eradicate the suspended bacteria. The use of ozone can also be mandatory in cases of immature or resorbed root avoiding the risk associated with NaOCl irrigation.

Aim of the study

Evaluation of the additional antimicrobial effect of gaseous Ozone and Ozonized water against biofilms of E. faecalis.

MATERIALS AND METHODS

Teeth Preparation

40 extracted human single-canalled permanent teeth with mature closed apices were used. They were horizontally sectioned below the cement-enamel junction with a diamond disk to standardized 15 mm root length. The canals were then instrumented using rotary instruments with crown down technique (M-Pro IMD, Guangdong, China) up to apical size #25 taper 6 for each tooth sample. Sequential use of 3 mL of 5% NaOCL was used during the chemo-mechanical preparation. 5 ml of 17% EDTA was used to remove the smear layer for 3 minutes.

All specimens were inserted inside a 96 well cell culture plate, closed then placed in sterilization pack and autoclaved for 20 minutes at 121C°, and pressure 2 bars.

Preparation and Inoculation of *E. faecalis*

Cultures of *E.faecalis* ATCC 29212 (Strain bank, Microbiology Department, Cairo University) grown on KF streptococcus agar plates(Difco, USA) were used in this study after incubation at 37°C for 48 hours.

Isolated colonies of pure cultures of *E.faecalis* were scraped, aseptically suspended in brain heart infusion (BHI) broth, dispersed by vortexing under laminar air flow to adjust turbidity of the bacterial suspension visually to match the turbidity of 1.5 × 10^8 colony-forming units (CFUs)/mL (equivalent to 0.5 McFarland standard).

Root canal infection

Using a sterile micropipette (Eppendorf, Germany), 20 μl of the bacterial suspension was injected into each root canal until the entire canal space was filled with fluid then sealed with glue wax. Roots inoculated with *E.faecalis* were incubated at 37°C for 7 days. Fresh bacterial suspension was added to the root canals at 1, 3, and 5 days after the initial inoculation. After 7 days, two roots were longitudinally split, dried in ascending ethanol concentrations and subject to scanning electron microscope (SEM) examination.

Experimental design

The incubated samples were randomly divided into 4 groups (n: 10);

Group 1, NaOCl group: samples were irrigated with 5 ml 5.25% NaOCl (Clorox,Egypt) for 3 min using 30-gauge needle (FANTA ,China), applied 2 mm short of the working length.

Group 2, Ozone group: by the aid of ozone-generating device, gaseous ozone was applied

(Ozonytron XP, Germany) to deliver ozone in air at the rate of 44 μg ml-1 at 1 atm 20°C through the KPX probe for 4 min.

Group 3, Gaseous ozone + ozonized water group: Gaseous ozone (as applied in group 2) and ozonized water (2 ml, 4 min) were used together subsequently. Ozonized water (5 μg ml-1) was freshly prepared using an ozone gas generator machine (Ozomed Basic) (Kastner-Praxisbedarf GmbH Co, Ltd).

Group 4, normal saline (El Nasr company, Egypt) (positive control- 5 samples): The contaminated samples were irrigated with 3 ml of 0.9% sterile normal saline solution.

Five samples received no treatment as negative control. The positive control was used to check bacterial viability and the negative control was used to test sterility throughout the experiment.

Root canals were sampled before (Sample1) and immediately after the treatment (Sample2). Root canals were filled with sterile saline and the samples were taken by three dry sterile standardized paper points (Dia Dent, Korea). Paper points were kept in the canal for 1 min to absorb the fluid then transferred to tubes containing .05 mL BHI broth solution (Oxoid ltd, UK) and agitated in vortex (Vortex mixer, Milano, Italy) for 1 minute. After obtaining 1:10 serial dilutions from each sample, aliquots were plated out on KF streptococcus agar plates and spread using sterile platinum loop. Then the plates were incubated anaerobically at 37° C for 2 days. The number of bacterial colonies of *E. faecalis* were counted and expressed as CFUs using a digital colony counter (Stuart, Bibby Scientific Limited, UK).

After irrigation, two samples of each group were evaluated using SEM. Two longitudinal grooves were prepared in each root then carefully split into two longitudinal halves with a hammer and chisel. Thereafter each half was dehydrated in graded ethanol series (70, 80, 90, and 99%), attached to coded stubs, coated with gold and examined under SEM at 30 KV. The root canal of each specimen was examined at different magnifications to investigate the presence of bacterial colonies and compare the scanning results with agar plates counting.

Numerical data were presented as mean and standard deviation (SD) values. Logarithmic transformation (Log_{10} transformation) was applied to bacterial counts (CFU) data due to the high variation in counts.

After logarithmic transformation, numerical data were explored for normality by checking the distribution of data, calculating the mean and median values as well as using the tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests.

For % reduction data (parametric data); one-way ANOVA test followed by Bonferroni's post-hoc test was used for comparisons between the groups.

RESULTS

Comparison between Log_{10} CFU of bacterial counts in the four irrigating solutions

Before antibacterial treatment, there was no statistically significant difference between the groups. While after treatment; there was a statistically significant difference. Although NaOCl group (2.56±2.74) showed the lowest mean Log_{10} of bacterial counts, it was non-statistically significant different from Gaseous ozone group (3.83±2.37) and Gaseous ozone + ozonized water group (4.62±1.88).

Saline group showed the highest mean Log_{10} of bacterial counts (6.39 ± 0.30). It showed statistically significant difference from all groups.

TABLE (1): Mean Log_{10}, standard deviation (SD) values and results of comparison between Log_{10} CFU of bacterial counts in the four groups after antibacterial treatment (In vitro)

	Mean Log_{10}	*SD*	*P*-value
NaOCl	2.56 [c]	2.14	
Gaseous ozone	3.83 [bc]	2.37	
Gaseous ozone and ozonized water	4.62 [bc]	1.88	**<0.001***
Saline	6.39 [a]	0.30	

: Significant at P ≤ 0.05, Different superscripts are statistically significantly different

Changes after chemomechanical preparation within each group

There was a statistically significant decrease in the mean Log_{10} CFU of bacterial counts after treatment for all tested irrigation solutions.

TABLE (3): Mean Log_{10}, standard deviation (SD) values and results of comparison between Log_{10} CFU of bacterial counts before and after treatment within each group

	Before treatment		After treatment		P-value
	Mean Log_{10}	SD	Mean Log_{10}	SD	
NaOCl	7.21	0.08	2.56	2.14	0.012*
Gaseous ozone	7.15	0.16	3.83	2.37	0.012*
Gaseous ozone and ozonized water	7.08	0.18	4.62	1.88	0.012*
Saline	7.02	0.44	6.39	0.30	0.012*

: Significant at P ≤ 0.05

Comparison between % reductions in Log_{10} CFU of bacterial counts in the six groups.

There was a statistically significant difference between the groups. NaOCl group showed the highest mean % reduction in Log_{10} of bacterial counts (99.57 ± 0.53). NaOCl group showed non-statistically significant difference from gaseous ozone (98.67 ± 0.68) and **Gaseous ozone and ozonized water** (97.48 ± 1.44). Saline group showed the lowest statistically significant mean % reduction in Log_{10} of bacterial counts (70.45 ± 20.31).

TABLE (4): Mean %, standard deviation (SD) values and results of comparison between % reductions in Log_{10} CFU of bacterial counts in the six groups (In vitro study)

	Mean %	SD	P-value
NaOCl	99.57 [a]	0.53	
Gaseous ozone	98.67 [a]	0.44	
Gaseous ozone and ozonized water	97.48 [a]	0.68	<0.001*
Saline	70.45 [b]	20.31	

: Significant at P ≤ 0.05, Different superscripts are statistically significantly different

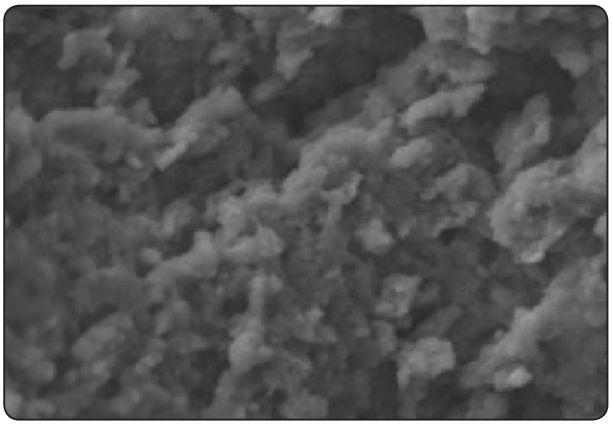

Fig. (1) : E Faecalis biofilm

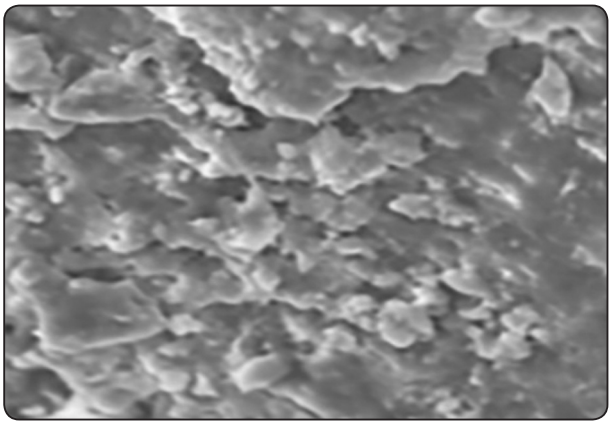

Fig. (2): Treatment with normal saline

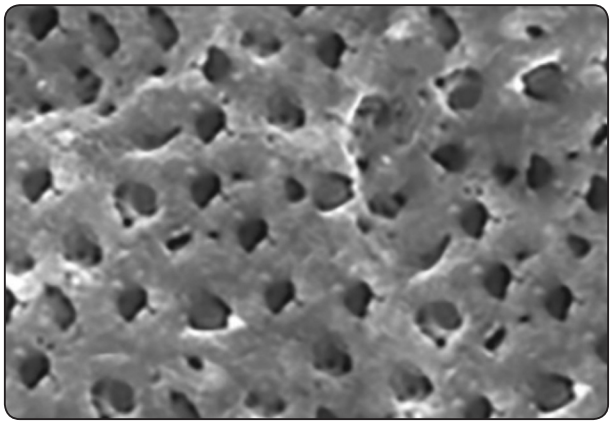

Fig. (3): Treatment with 5% sodium hypochlorite (1,000x)

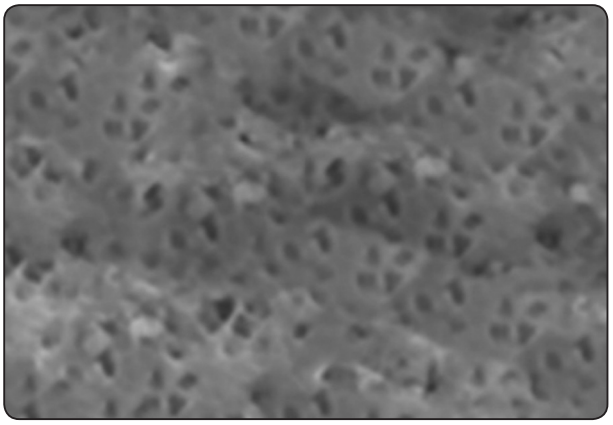

Fig. (4): Treatment with 5% sodium hypochlorite (1,500x)

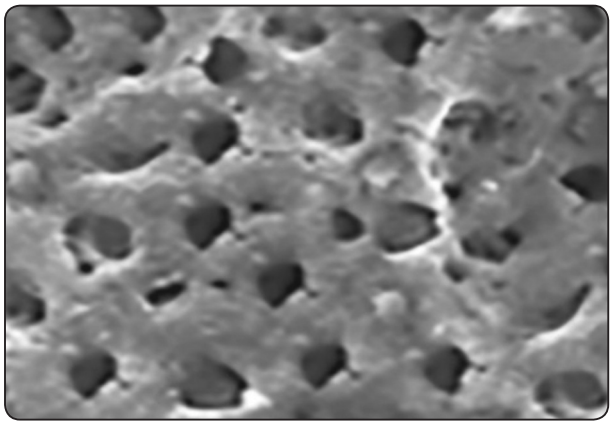

Fig. (5): Treatment with gaseous ozone and ozonized water (1,500x)

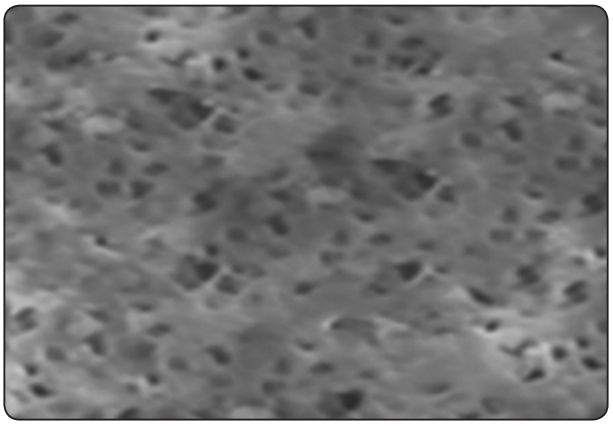

Fig. (6): Treatment with gaseous ozone and ozonized water (1,000x)

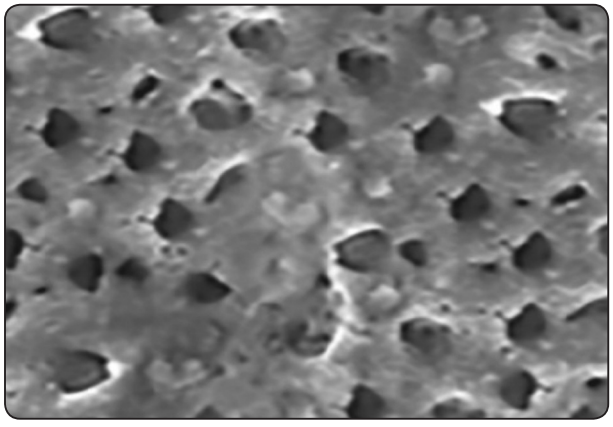

Fig. (7): Treatment with gaseous ozone for 4 minutes (1,500x)

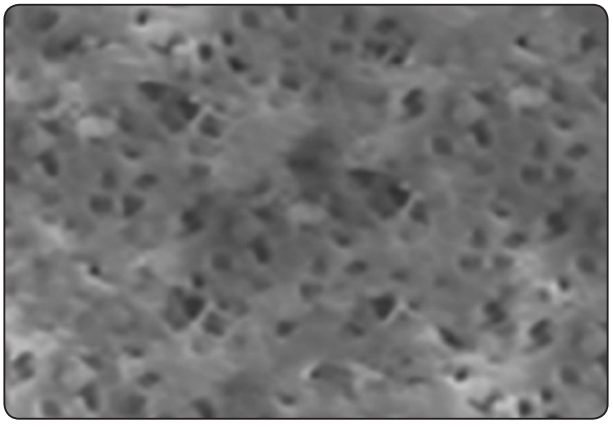

Fig. (8): Treatment with gaseous ozonefor 4 minutes (1,000x)

DISCUSSION

Except saline-positive control, all the experimental groups were successfully reducing the bacterial count, while all of them were able to completely kill *E. faecalis*. This type of bacteria is the most resistant one to irrigation and intracanal medication. Under starvation conditions, *E. faecalis* produce intracellular products, which may neutralize the medicament applied [12]. *E.* faecalis, which is an opportunistic, facultative anaerobe, was chosen as a biological marker because it is well recognized as a pathogen associated with persistent apical periodontitis in endodontically treated teeth and is highly prevalent in failed root canal filled teeth. It is non-fastidious and easy to culture [13, 14]. Null hypothesis was rejected as there was a difference in the antimicrobial efficacy of the tested groups. The % of bacterial reduction of the experimental groups were 90% for gaseous ozone group and ozonized water, 95% for gaseous ozone group, and 99.9% for sodium hypochlorite one. These results showed that NaOCl was the best antibacterial agent. In accordance with our results, Noites, et al. [15] found that complete elimination of *E. faecalis* cannot be achieved by sodium hypochlorite and gaseous ozone alone. Huth, et al. [16] reported that gaseous ozone and ozonized water when used in high concentrations (53 gm-1, 20 μg ml-1) eliminated more than 96% of *E. faecalis* in the biofilm model.

In contrast to our results, Abdullah, et al. [17] reported that total elimination (100%) of E. faecalis was obtained after two minutes contact time with 3% sodium hypochlorite. In this paper, complete elimination of the bacterium could not be obtained even with (5.25%) concentration of NaOCl and after three minutes contact time.

Based on our results, ozonized water did not have a synergistic effect of the gaseous ozone. In contrast, sodium hypochlorite showed significantly more antimicrobial effect when compared with ozonized water and gaseous ozone combination. In an *in vitro* study, the ozonized water had the same antimicrobial efficacy as 2.5% NaOCl particularly, in roots sonically irrigated [18]. The differences between the results of these studies and our results may be due to differences on the methodology of each study: specimen size and type, number of bacteria , time of incubaion, depth of bacterial invasion, irrigant concentrations and irrigant quality.

On the other hand, irrigant concentration, study model and the contact time notwithstanding, the results of our study are in accordance with the results of previous investigations [19, 20].

It is well known that the gold standard antibacterial irrigant used in the most endodontic treatments is sodium hypochlorite [17].

In a study by Gomes et al., all the experimental irrigants were effective in eliminating the tested bacteria [21]. Similarly, our study found that *E. faecalis reduced effectively by* 5.25%. 5.25% NaOCl showed higher antibacterial efficacy when compared with gaseous ozone and gaseous ozone plus ozonized water in this investigation. Boch, et al. [22] found that gaseous ozone reduced *E. faecalis to about* 85%, while in our study it was 90% .this may be due to the longer time of application of gaseous ozone (4 min) but the lesser concentration we used. This Difference in results also may be due to different ozone-generating machines. We used Ozonytron XP while Boch, et al. used HealOzone® 2130 C.

Recently it was shown that the instrumentation efficacy is greatly low even in wide root canals, [23]. Furthermore, a lot of radicular dentin cannot be touched like interconnecting canals.

Significant results may be depending on time of ozone application, manner, dosage and protocol, theses parameters still not standardized. Hems, et al. [19] found that ozone when applied for 4 minutes had a significant antimicrobial effect. In contrast, another study [24] reported that over 20 minutes application of ozonized water and gaseous ozone was insufficient to totally kill *E. faecalis*. In this paper, the ozone concentration was selected according to instructions of manufacturer. No significant difference was

achieved when we tested different application time periods in a pilot study, so we selected 3 minutes as a time applied. We thought the applied time should be enough and suitable regarding the clinical work.

Limitations of our study was to use only the scanning electron microscope and culture method to assess the reduction in bacterial count. Real-time PCR or FISH (Rrna 16 s) methods might be used. These parameters are highly accurate than culture method in many recent researches [25, 26].

CONCLUSION

Sodium hypochlorite is still the golden standard irrigant regarding disinfection of endodontic bacteria. Our results could be a guide for the next papers toward new disinfection techniques and irrigants. Gaseous ozone could be used as a synergistic disinfectant but not an alternative to sodium hypochlorite. Despite *E. faecalis* is the master pathogen in secondary root canal infections; further studies should investigate different bacterial types, different clinical situations and different treatment protocols of ozone for root canal disinfections.

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