



RESEARCH ARTICLE

Evaluation of Synergetic Antibacterial Effect of Gaseous Ozone and Ozonated Water against *Enterococcus Faecalis* in Human Root Canals

Banu Uygun Can¹, Gizem Özbay², Hatice Altundal², Parla Meva Durmazpinar^{3*}, Burak Kitiki², Sertaç Peker², Tanju Kadir¹ and Betül Kargul⁴

¹Department of Microbiology, Marmara University, Turkey

²Private Practice, Istanbul, Turkey

³Department of Endodontics, Izmir Demokrasi University, Turkey

⁴Department of Pediatric Dentistry, Marmara University, Turkey

*Corresponding author: Dr. Parla Meva Durmazpinar, Department of Endodontics, Izmir Demokrasi University, Üçkuyular Mahallesi, Gürsel Aksel Bulvarı, No: 1435140 Karabağlar/İzmir, Turkey, Tel: +90-533-211-11-48, Fax: +90-232-260-1004



Abstract

Background: Elimination of the microorganisms in root canals is one of the most important stages for a successful endodontic treatment. Several techniques and agents have been tested to find an alternative option to sodium hypochloride for effective root canal disinfection. The aim of this *in vitro* study was to evaluate the antimicrobial efficacy of gaseous ozone and ozonated water in order to establish their potential as endodontic root canal disinfectant.

Methods: Eighty teeth with single canal were prepared and inoculated with *Enterococcus faecalis* suspension and incubated for a week. The contaminated root canals were exposed to irrigating solutions including gaseous ozone (44 µg ml⁻¹) with ozonated water (5 µg ml⁻¹), 5.25% sodium hypochlorite, 2% chlorhexidine for 3 min. Samples from the root canals were collected by paper points, before and after irrigation. The bacterial count was analyzed by serial decimal dilutions and the percentage of surviving microorganisms was statistically assessed.

Results: There was a significant reduction in the bacterial count after all treatments ($P < 0.001$) while having greater antimicrobial reduction was observed on samples of NaOCl irrigation. There was no significant difference between gaseous ozone and gaseous ozone + ozonated water groups.

Conclusion: Under limitations of this study, it could be concluded that the gaseous ozone, as an irrigant agent, significantly reduced the number of *E. faecalis* in root canals but was not able to eliminate it. However, ozonated water was insufficient to increase the antibacterial effectiveness of gaseous ozone.

Keywords

Chlorhexidine, *Enterococcus faecalis*, Gaseous ozone, Ozonated water

Introduction

An approving outcome of the endodontic treatment of teeth with apical periodontitis depends on effective control of the root canal infection [1,2]. Chemomechanical preparation root canals can reduce the number of microorganisms but not entirely get rid of them [3]. Instead of strict anaerobes, some facultative anaerobic bacteria, especially *E. faecalis* have been shown to be associated with failed root canal treatment [4,5]. Inadequate aseptic control and instrumentation of the root canals, leaking temporary or permanent cavity restorations are the main factors that may be critically important in post-treatment endodontic disease. Microbiological findings from filled root canals with periapical disease have shown a high proportion of enterococci, ranging from 24% to 77% [6]. *E. faecalis* has ability to grow in high salt concentrations, a wide temperature range, tolerance a broad pH range, as well as growth or persist in the presence of intracanal medicaments [4,7]. Thus, effective disinfecting processes and antimicrobial irrigants to remove *E. faecalis* are necessary for successful treatment of the infected root canals, especially

those with persistent apical periodontitis [8].

Sodium hypochlorite (NaOCl) has been widely used as an irrigant since its introduction in Endodontics by Walker in 1936, however it has cytotoxic and neurotoxic effects when infused into the periapical tissues [9]. It has been notified that the antibacterial and tissue-dissolution property of NaOCl increases with its concentration, but this is also accompanied by an increase in its toxicity [10]. To avoid the potential side effects of NaOCl, there is an interest in developing new disinfecting agents and methods.

Another antimicrobial agent is Chlorhexidine gluconate (CHX) which has been recommended for the root canal irrigation [11]. CHX is a cationic molecule, which has a wide range of antimicrobial activity [12]. On the other hand, CHX may cause mucosal desquamation and a high cytotoxic effect on epithelial cells [13]. Therefore, many recent studies have focused on an alternative disinfectant irrigant with high effective as well as relative lack of toxicity against sound apical tissue [14,15].

Ozone is a natural allotrope of oxygen found in upper layer of atmosphere protecting living organisms on earth from Ultraviolet (U.V) radiation. It is a powerful oxidizing agent and currently being discussed as a possible alternative antiseptic in dentistry due to its high antimicrobial effect without the development of drug resistance [16]. Ozone damages the bacterial cell membranes by ozonolysis and oxidates intracellular proteins leading to loss of organelle function [17]. This action is selective to microbial cells and thus does not affect human body cell. Ozone is a good antioxidative and very effective in antibiotic resistant strains with accelerated efficiency in acidic pH environment [18]. It is administered on patients for therapeutic purposes in various forms like gaseous ozone, as an aqueous solution, oil or as ozonated water [16,19]. Kist, et al. suggested that ozone gas might be a possible alternative disinfection agent within the root canal treatment of apical periodontitis based on the results of a clinical trial [20]. Furthermore, It has been shown that ozonated water (up to 20 gm L⁻¹) is non-toxic against oral cells *in vitro* and the ozone gas (4 gm³) is slightly less toxic than 2.5% NaOCl [21]. In the light of this information, the aim of this *in vitro* study was to evaluate antimicrobial efficiency of gaseous ozone with additional use of ozonated water against *E. faecalis* in root canals and compare with traditional endodontic irrigants such as NaOCl and CHX. The null hypothesis of the present study was that there would be no difference in elimination of *E. faecalis* among gaseous ozone, gaseous ozone + ozonated water and 2.5% NaOCl groups.

Methods

Tooth preparation

The study consisted of eighty single-rooted human permanent teeth which extracted because of periodon-

tal disease or extensive carious lesions. The investigation was obtained by the Ethics Committee of the Health Sciences Institute of University of Marmara, Turkey (No.153/2015). The external root surface of the teeth was cleaned with curettes to remove calculus and periodontal soft tissues. A #10 K-type file (K-Files, Mani Inc., Utsunomiya, Japan) was used to confirm patency of the root canal and to establish a clinical working length 1 mm short of the apical foramen. Root canal preparation commenced with Gates Glidden Burs 4-3-2 (Dentsply Maillefer, Ballaigues, Switzerland) in a crown-down mode [22]. Biomechanical root-canal preparation was done by step-back technique up to a size 70 K-File (taper 0.02) (Dentsply Maillefer, Ballaigues, Switzerland) [23]. Canals were irrigated with 1 ml of 2.5% NaOCl (Wizard, Rehber Chemistry, Istanbul, Turkey) between each instrument. After the mechanic preparation of the root canals, they were filled with 1 ml 15% Ethylenediaminetetraacetic acid (EDTA) for 1 min followed by a final rinse with 1 mL 2.5% NaOCl and 1 ml saline solution for smear layer removal [24]. Then the teeth were decoronated to a constant root canal length of 16 mm with a rotary diamond saw (BUEHLER, Isomet low speed saw, IL) at 700 rpm under water-cooling [25]. Apical foramina were sealed with a restorative material (Z100; 3M ESPE, Seefeld, Germany) and outer surfaces of the roots were covered with two layers of nail varnish. Teeth were embedded into epoxy resin (Clássico, Artigos Odontológicos S/A, São Paulo, SP, Brazil) to prevent bacterial leakage. All samples were sterilized by autoclaving (120 °C for 30 min).

Tested organism

A reference strain of Gram-positive facultative anaerobic coccus (*E. faecalis* ATCC 29212) obtained from our laboratory stock collection was used. Bacteria were plated onto Brain-heart infusion (BHI) agar (Sigma-Aldrich, Co., St. Louis, USA) and incubated aerobically at 37 °C for 24 hours, then suspended in BHI broth (Sigma-Aldrich St., Louis, USA) and the optical density was adjusted to optical turbidity of McFarland 0.5 to obtain approximately 1 × 10⁸ colony forming units (CFU/ml).

The experimental groups were inoculated with *E. faecalis* suspension (1 × 10⁸ CFU/ml), using sterile 2 ml tuberculin syringes of sufficient volume to fill the root canal. Then the samples were incubated for 7 days aerobically at 37 °C. The inoculum was renewed every 3 days, always using 24 hours pure culture prepared and adjusted to the same turbidity of the first suspension.

Experimental design

After incubation of the samples, they were randomly divided into five experimental groups (n : 15);

Group 1, NaOCl group: The root canals were irrigated with 5 ml 5.25% NaOCl (Sultan Chemists, Inc., Englewood, NJ, USA) for 3 min using 30-gauge needle (NaviTip FX; Ultradent Products Inc, South. Jordan, UT),

Table 1: Counts of *E. faecalis* CFUs before and after disinfection protocols.

	Before treatment			After treatment			p
	Mean	Median	Range	Mean	Median	Range	
5.25% NaOCl	1.34×10^8	1×10^8	$6 \times 10^7 - 3 \times 10^8$	2.1×10^5	2×10^5	$6 \times 10^2 - 4 \times 10^5$	0.001*
2% CHX	4.8×10^7	5×10^7	$1 \times 10^7 - 1 \times 10^8$	2×10^4	2×10^4	$1 \times 10^4 - 9 \times 10^4$	0.001*
Gaseous O ₃ + O ₃ water	6.68×10^7	3×10^7	$4 \times 10^4 - 4 \times 10^8$	1.77×10^6	1×10^6	$1 \times 10^3 - 9 \times 10^6$	0.001*
Gaseous O ₃	2.38×10^6	2×10^7	$3 \times 10^5 - 8 \times 10^6$	2.2×10^5	2×10^5	$0.01 - 1 \times 10^6$	0.001*
Saline	1.49×10^6	1×10^6	$1 \times 10^5 - 3 \times 10^6$	1.49×10^6	1×10^6	$0.1 \times 10^6 - 3 \times 10^6$	1.000

Wilcoxon signed ranks test *p < 0.01.

which was placed 2 mm short of the working length.

Group 2, CHX group: The root canals were irrigated with 2 ml 2% CHX (Cerkamed, Poland) for 3 min using 30-G needle, which was placed 2 mm short of the working length.

Group 3, Ozone group: Gaseous ozone was applied with an ozone-generating device (Ozonytron XP, MIO International Ozonytron GmbH Co., Germany) to deliver ozone in air at the rate of $44 \mu\text{g ml}^{-1}$ at 1 atm 20 °C through the KPX probe for 3 min.

Group 4, Gaseous ozone + ozonated water group: Gaseous ozone (as applied in group 3) and ozonated water (2 ml, 3 min) were used together subsequently. Ozonated water ($5 \mu\text{g ml}^{-1}$) was freshly prepared using an ozone gas generator machine (Ozomed Basic) (Kastner-Praxisbedarf GmbH Co, Ltd).

Group 5, Saline (positive control) group: The contaminated root canals were irrigated with 2 ml of 0.9% sterile 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.

After carrying out the disinfection protocols, the root canals were irrigated with sterile saline (2 ml) for 1 min and sampled by using 3 sterile paper points. Then the paper points were aseptically transferred to tubes containing 1 ml of BHI broth and immediately homogenized using a vortex mixer at maximal setting for 30 s. Following 10-fold serial dilutions, aliquots of 0.1 ml were plated onto BHI medium agar in triplicate, incubated at 37 °C aerobically for 48 hours and colonies were counted. The mean Colony forming units (CFUs) per unit determined for test cultures before and after treatment (mean \pm SD; n = 15) procedures for each experimental group were calculated and within the group.

Analyses were performed using IBM SPSS Statistics 22 (IBM SPSS, Türkiye). Kolmogorov-Smirnov test was applied to verify the distribution of the parameters between research groups. The resulting data were nonparametric and hence Kruskal-Wallis test was used to test for differences in bacterial reduction rate among groups and for intergroup comparative analysis. This global test was followed up with pair-wise comparisons

Table 2: Percentage reduction among groups.

% Reduction	Median (Minimum-Maximum/Range)	p
5.25% NaOCl	99.87 (99.50 - 99.95)	
2% CHX	99.97 (99.55 - 99.99) [‡]	
Gaseous O ₃ + O ₃ water	95.00 (86.67 - 99.75) ^{‡*}	0.001*
Gaseous ozone	90 (70 - 98) ^{‡*}	
Saline	0 (0 - 0) [†]	

Kruskal wallis test [†]p < 0.01, Mann whitney U test p < 0.05; [†]The group that showed less bacterial reduction percentage than the other tested groups (p < 0.05); [‡]The group that showed less bacterial reduction percentage than CHX group (p < 0.05); ^{*}The group that showed less bacterial reduction percentage than NaOCl group (p < 0.05).

with Mann-Whitney U tests. Wilcoxon Signed Ranks test was used for the intragroup comparative analysis. The level of statistical significance was set at P < 0.05.

Results

Table 1 presents the distribution of the results (mean, median, range, reduction rate of CFUs) before and after disinfection protocols. The reduction in the number of CFUs after the treatment protocol was highly significant for all groups (P < 0.01) except saline group (positive control) (p > 0.05). All experimental groups were significantly superior over the positive control (p: 0.001; p < 0.01).

Bacteria were viable in the positive control group, whilst the negative control group was free of microorganisms under the experimental conditions.

There was significantly different percentage reduction between the tested groups (p: 0.001; p < 0.01) (Table 2). The results showed that there was statistically a higher bacterial reduction in NaOCl group than gaseous ozone, gaseous ozone + ozonated water and CHX groups (p: 0.001; p < 0.01). The bacterial reduction percentage in CHX group (99.87%) was significantly superior than in gaseous ozone group (90%) and gaseous ozone + ozonated water group (95%). Although combined disinfection with gaseous ozone and ozonated water group achieved higher bacterial reduction percentage than gaseous ozone group, there was no statistically significant difference among them (p: 0.076; p > 0.05).

Discussion

According to the results of the present study, all experimental groups were successful in decreasing the bacterial count (except saline-positive control),

while non of them was able to eliminate *E. faecalis* completely. This result may be due to the type of organism which is the most resistant phase to medication. Also, *E. faecalis* that found in the starvation phase produce intracellularly organic products, which may play a role in the inactivation of the antibacterial activity of the medicament [4]. Moreover, *E. faecalis* was chosen as the test organism in this study because it is the most often associated with persistent endodontic infections [5] and can be seen in primary endodontic infections [26]. Null hypothesis was rejected as there was difference in the antibacterial efficacy of gaseous ozone, gaseous ozone + ozonated water, CHX, and 5.25% NaOCl. The percentages of bacterial reduction of the experimental groups were 90%, 95%, 99.87% and 99.97%, respectively. The current results showed that 5.25% NaOCl achieved the best antibacterial results among the tested groups. Consistent with our findings, Noites, et al. [12] reported that NaOCl, CHX and gaseous ozone alone were ineffective in totally eliminating *Candida albicans* and *Enterococcus faecalis*.

They also stated that the association of CHX at 2% followed by ozone gas for 24 seconds promoted the elimination of all microorganisms. Huth, et al. [27] found that ozonated water and gaseous ozone eliminated over 96% of *E. faecalis* in biofilm when used in maximum concentrations (20 $\mu\text{g ml}^{-1}$ and 53 gm^{-1} , respectively), whereas lower concentrations reduced substantially but did not eradicated them.

Contrary to our results, Abdullah, et al. [28] concluded that 3% NaOCl achieved 100% kill for all presentations of *E. faecalis* after a 2 min contact time. In our study, Total elimination of *E. faecalis* could not be achieved even with higher concentration of NaOCl (5.25%) after 3 min contact. Within the methodology of the present study, we tested the efficacy of gaseous ozone plus ozonated water with the aim of providing to reduce *E. faecalis* biofilm *in vitro*. Based on the results of our study, ozonated water did not increase the antibacterial effect of gaseous ozone. Furthermore, gaseous ozone + ozonated water combination showed significantly less antimicrobial effect when compared with NaOCl and CHX. Nagayoshi, et al. [21] reported that the ozonated water had the same activity with 2.5% NaOCl especially, in specimens irrigated with sonication in an *in vitro* study. The differences between the results of the mentioned studies and our results may be due to differences on the methodological design of each investigation: Standardization of tooth type and sample size, number of bacteria, incubation time, bacterial invasion of root dentinal tubules, quality control of the chemical irrigants and variation in their concentration. On the other hand, the irrigant concentration, contact time and the study model notwithstanding, the results of the present investigation are in accordance with those of previous studies [29,30].

It is well known that NaOCl and CHX are most common antimicrobial agents used in the treatment of endodontic and periodontal infections [1,28]. In the study of Gomes, et al. [1], *in vitro* effectiveness of several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of CHX in three concentrations (0.2%, 1% and 2%) in the elimination of *E. faecalis* were assessed. It was shown that all irrigants were effective in killing the bacteria tested. Similarly, we found that 5.25% NaOCl and 2% CHX were effective in reducing *E. faecalis*. 5.25% NaOCl showed superior antimicrobial effect than CHX, whereas CHX showed better antibacterial effect compared with gaseous ozone, ozonated water and gaseous ozone + ozonated water in our study. Boch, et al. [31] reported that reduction of *E. faecalis* inside the root canals with gaseous ozone (2100 ppm-60 sn) was 85%, while it was 90%, in our study. The reason of the difference with the study of Botch, et al. [31]. Might be the longer application time of ozone gas (3 min) despite less concentration of ozone that we applied. Using different ozone-generating devices also might have been caused the different results. We used Ozonytron XP (Ozonytron XP, MIO International Ozonytron GmbH Co., Germany) while they used HealOzone® 2130 C (Kavo, Biberach, Germany) for endodontic disinfection.

Ozonated water and gaseous ozone were evaluated and compared with traditional endodontic irrigants as alternative disinfectants. It was recently shown that even in large root canals the efficacy of instrumentation is surprisingly low [32]. Moreover, a lot of other areas cannot be reached with instrumentation such as interconnecting canals. Thus, we focused on the antimicrobial effect of only disinfecting agents. As a source of impreciseness in the present study, no neutralizers were used to arrest the action of the agents. Thus, the contact times for irrigants, which are known for their substantivity might be prolonged similar as in the clinical situation.

There is still no consensus respecting the application manner, dosages and time of O_3 to achieve significant results. Hems, et al. [29] reported that ozone had significant bacterial reduction but only applied for 240 s. However, Estrela, et al. [33] found that both ozonated water and gaseous ozone over 20 min was not sufficient to inactivate of *E. faecalis*. In the current study, the concentration of ozone gas and ozonated water were chosen according to manufacturer instructions. We were tested different time periods in a pilot study but no significant difference was achieved, thus so we decided to choose the application time as 3 minutes. We thought the time should be proper long for clinical use. It may be more effective if higher concentrations could be used with the ozone machines we have used. To the best of our knowledge, this is the first study investigating the antibacterial effectiveness of gaseous ozone (44 $\mu\text{g ml}^{-1}$) and ozonated water (5 $\mu\text{g ml}^{-1}$) with the indicated concentration and time (3 min).

Limitation of this study was to use only culture method by assessing the reduction in bacterial count. Biomolcular methods as realtime PCR or FISH (Rrna 16 s) techniques could be used to determine bacterial reduction. It is stated that these techniques are more sensitive than culture technique in several recent studies [34,35].

Conclusion

Under the tested conditions and within the limitations of this study, it may be concluded that NaOCl is still the most efficient disinfection agent in terms of eliminating endodontic pathogens. The results of the current study could guide to redirect researches towards new protocols and disinfection agents for reducing bacteria in infected root canals. Gaseous ozone might be used as an adjunctive agent but not an alternative to NaOCl. Although *E. faecalis* is the major pathogen in secondary endodontic infections, further studies should be carried out to investigate the treatment protocol of ozone for endodontic infections in different clinical situations and on different bacterial species.

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