ISSN Print: 2394-7489
ISSN Online: 2394-7497
IJADS 2021; 7(3): 292-296

www.oraljournal.com
Received: 04-05-2021
Accepted: 10-06-2021

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Ozonated water antimicrobial efficacy in comparison to antimicrobial activity of different irrigant solutions against *E. faecalis* and *E. coli*

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DOI: https://doi.org/10.22271/oral.2021.v7.i3e.1313

Abstract
Aim: To determine the antimicrobial efficacy of ozonated water and compare the antimicrobial activity of different irrigant solutions against *E. faecalis* and *E. coli*.
Material and Method: This study was conducted in the Department of Pedodontics and Preventive Dentistry, Jaipur Dental College, Jaipur in collaboration with Dr. B. Lal Institute of Biotechnology, Jaipur to test and compare the efficacy of Ozonated Water, 3% Sodium Hypochlorite and Normal Saline in relation to Enterococcus faecalis (MTCC 439) and Escherichia coli (ATCC 25922) micro-organisms. After incubation, 21 contaminated roots were divided into 3 groups according to the irrigation regimen used i.e. Group A: Ozonated Water (7 teeth), Group B: 3% NaOCl (7 teeth) and Group C: Saline (7 teeth). All the teeth were handled with sterile gloves and sterile tweezers to prevent contamination. A sterile 5 mL syringe with 26 gauge needle was used to deliver irrigant into the canal. All experimental teeth were then flushed with distilled water to prevent potential carry-over of irrigants.
Results: Results indicated that Group C showed highly significant difference from group A and Group B indicating that Group A and Group B were effective against both *E. faecalis* and *E. coli*. Results also show that there was no significant difference in efficacy of Group A and Group B.
Conclusion: The results found that ozonated water has proved to be an alternative antimicrobial agent.

Keywords: *E. faecalis*, *E. coli*, Ozonated Wate

Introduction
Naidorf in 1972 stated the necrotic pulp becomes a “privileged sanctuary” for clusters of bacteria [1]. Primary root canal infections are polymicrobial, typically dominated by obligatory anaerobic bacteria [2]. The most frequently isolated microorganisms before root canal treatment include gram-negative anaerobic rods, Gram-positive anaerobic cocci, Gram-positive anaerobic and facultative rods, Lactobacillus species, and Gram-positive facultative Streptococcus species [3].
Enterococcus faecalis is a facultative anaerobe and a persistent organism found in a high percentage of root canal failures and it is able to survive in the root canal as a single organism or as a major component of the flora [4]. The prevalence of *E. faecalis* in primary endodontic infection is 40% and in persistent endodontic infection 24 to 77% [5].
Instrumentation has a key role in the cascade of treatment procedures to eradicate microbes in the root canal system. Thus, root canal treatment of teeth with pulp necrosis and periapical lesions should not only sacrifice bacteria, but also remove the dead cells and/or promote the inactivation of the lipid A (the toxic portion of the endotoxin) [6]. The successful treatment of an infected root canal involves a combination of mechanical and chemical means. Using mechanical instrumentation alone might reduce the number of bacteria in the root canal system by 50%.
Several irrigating solutions, such as Chlorhexidine (CHX) and sodium hypochlorite, are used during endodontic treatment [7]. The effectiveness of irrigation depends on the working mechanism(s) of the irrigant and the ability to bring the irrigant in contact with the microorganisms and tissue debris in the root canal [8].

A negative side effect of sodium hypochlorite is the potential toxicity and high surface tension that reduces the possibility of entering the canal irregularities and dentinal tubules [9]. It also reduces the elasticity of dentin and makes it susceptible to fractures [10]. That is why many various combinations of disinfecting solutions and irrigation devices such as photo-activated disinfection [11], active root canal irrigation [12] and intracanal laser application [13] as well as ozone or plasma devices have been used [14]. All the irrigation solutions at our disposable have their share of limitations and the search for an ideal root canal irrigant continues with the development of newer materials and methods [15].

Broadwater *et al*. in 1973 reported that ozone at low concentration, 0.1 ppm, is sufficient to inactivate bacterial cells including their spores [16]. Ozone dissociates readily back into oxygen (O_2), thus liberating so called singlet oxygen (O_1), which is a strong oxidizing agent. It is this particular reactivity that provides the starting point for ozone's therapeutic effects on the body [17].

Although ozonated water is a powerful antimicrobial agent against bacteria, fungi, protozoa and viruses, less attention has been paid to the antibacterial activity of ozonated water in bacterial biofilm and hence in root canal infection [18]. Therefore the aim of this study was to determine the antimicrobial efficacy of ozonated water and compare the antimicrobial activity of different irrigant solutions against *E. faecalis* and *E. coli*.

Materials and Method

This study was conducted in the Department of Pedodontics and Preventive Dentistry, Jaipur Dental College, Jaipur in collaboration with Dr. B. Lal Institute of Biotechnology, Jaipur to test and compare the efficacy of Ozonated Water, 3% Sodium Hypochlorite and Normal Saline in relation to *Enterococcus faecalis* (MTCC 439) and *Escherichia coli* (ATCC 25922) micro-organisms. The microbiological media used:

1) Brain Heart Infusion Broth
2) Brain Heart Infusion Agar Media
3) Macconkey Agar Media

Inclusion Criteria for Selection of Teeth

1. Single rooted anterior teeth
2. Teeth With complete root formation
3. Teeth With patent canals
4. Teeth Without anatomic variation

Exclusion Criteria

1. Teeth With open apices
2. Calcified canals
3. Multi rooted teeth
4. Variations in the radicular anatomy
5. Teeth With previous root canal treatment

The armamentarium used for the present study was 30 extracted non-carious single rooted anterior teeth, Ozonated Water generating device (Kent Ozone Dental Jet, Kent technology, India), 3% Sodium Hypochlorite (Prime Dental Products Pvt. Ltd), Normal Saline, Carborundum disk, Straight Handpiece, Distilled Water, Kerr files (MANI Inc.), Type II GIC (GC Fuji), Nail Varnish, Paper points, Sterile gloves, 26 gauge needle and syringes, Micropipette, Sterile Appendorf tubes, Petridishes, Digital colony counter, Vortex agitator and Incubator.

Preparation of Samples

- 30 extracted non-carious, single rooted human incisors which were extracted for periodontal reasons were used in this study.
- Calculus and tissue tags were removed using hand and Ultrasonic scaling.
- The teeth were soaked in 5% NaOCl for 30 minutes to remove any remaining residual loose tissue and debris from the root surface.
- The teeth were stored in gauze soaked sterile saline till use to prevent dehydration.
- All the teeth were marked and then sectioned 14 mm from the apex with a carborundum disc using a low speed straight hand piece, so as to standardize roots of all the teeth approximately to the same length.

Instrumentation

- An ISO #15 K file was used to determine the working length.
- The root segments were mounted in wax bases for ease of instrumentation.
- All root canals were instrumented, using the step back technique and the circumferential filing motion, to K file #60.
- During cleaning and shaping, sterile distilled water was used after each instrument size.
- The segments were then removed from the wax bases.

Sealing of Root Apices

- Finally, the canals were flushed with 5 mL of distilled water to remove any debris.
- The root apices were sealed with type II GIC and coated with two coats of nail varnish to prevent bacterial leakage.

Sterilization of Samples: Each tooth was sterilized in steam autoclave for 30 minutes under 15 psi pressure at 121°C.

Inoculation

- The bacterial strains used in this study are *Enterococcus faecalis* (MTCC 439) and *Escherichia coli* (ATCC 25922).
- The primary culture was raised by inoculating *Enterococcus faecalis* (MTCC 439) and *Escherichia coli* (ATCC 25922) in the Brain heart infusion (BHI) broth, after incubation at 37°C for 24 hrs.
- The canals of the experimental teeth were cautiously inoculated using a micropipette with 20 µL of the freshly prepared suspension of both the organisms and sterile #15 K file was used to carry the bacterial suspension to the entire root canal length.
- The teeth were then incubated at 37°C for 72 hours.

Root Canal Irrigation

After incubation, 30 contaminated roots were divided into 3 groups according to the irrigation regimen used i.e Group A: Ozonated Water (10 teeth), Group B: 3% NaOCl (10 teeth) and Group C: Saline (10 teeth). All the teeth were handled with sterile gloves and sterile tweezers to prevent contamination. A sterile 5 mL syringe with 26 gauge needle was used to deliver irrigant into the canal. All experimental teeth were then flushed with distilled water to prevent potential carry-over of irrigants.

Sampling technique (Pre and Post irrigation)

- A small amount of distilled water was introduced into the canal, and an endodontic hand file was used in a filing motion to a level approximately 1 mm short of the root apex.
- The canal contents were aspirated with and then placed into appendorf tubes containing 1 mL of sterile saline.
- #50 paper point was then placed into the canal at the working length for 30 seconds each and also used to soak up the canal contents.
- Paper points were transferred to the same tubes containing 1 mL saline and agitated in vortex for 1 minute.
- Aliquots of 500 µl of the appropriate dilutions were cultured into BHI agar and MacConkey agar plates.
- All plates were cultivated at 37°C in a micro-aerophilic environment in 5% CO2 for 48 hours. The colonies were identified on the basis of their morphology and counted using a digital colony counter.
- Confirmation of NA was performed under light microscopy after staining a heat fixed smear slide. Microbial counts were expressed as colony-forming units (CFU) per ml of sample. The laboratory staff and clinicians evaluating the culture plates were blinded to the subject's group assignment.

$$\textbf{Colony forming unit /ml} = \frac{\text{Number of colonies obtained X Dilution Factor}}{\text{Volume of sample inoculated}}$$

Results

Table 1indicates the distribution of study and control groups. Table 2 shows Pre and post irrigation comparison within group using paired t test, whose results indicate that there was a significant reduction in post irrigation values in all the three groups.

Table 3 indicates pairwise comparison between three groups using Post Hoc analysis with respect to percentage reduction in CFU post irrigation. Results indicate that Group C showed highly significant difference from group A and Group B indicating that Group A and Group B were effective against both *E. faecalis* and *E. coli*. Results also show that there was no significant difference in efficacy of Group A and Group B.

Table 1: Description of the study groups

Group A	Ozonated Water
Group B	3% Sodium Hypochlorite
Group C	Positive Control – Saline

Table 2: Pre and post irrigation comparison within group using paired t test

Group Name			N	Mean	SD	P-Value
Group A	Pair 1	*E. faecalis* Pre-Irrigation	10	9.19	3.25	0.0002
		E. faecalis Post-irrigation	10	1.60	1.56	
	Pair 2	*E. coli* Pre-Irrigation	10	4.29	4.09	0.022
		E. coli Post-irrigation	10	0.89	1.00	
Group B	Pair 1	*E. faecalis* Pre-Irrigation	10	2.00	0.84	0.0004
		E. faecalis Post irrigation	10	0.28	0.27	
	Pair 2	*E. coli* Pre Irrigation	10	0.50	0.21	0.0002
		E. coli Post-irrigation	10	0.11	0.07	
Group C	Pair 1	*E. faecalis* Pre-Irrigation	10	4.07	1.60	0.0006
		E. faecalis Post-irrigation	10	3.18	1.22	
	Pair 2	*E. coli* Pre-Irrigation	10	0.63	0.28	0.0005
		E. coli Post-irrigation	10	0.52	0.27	

$p<0.05$ statistically significant

Table 3: Comparison of mean percentage reduction among three different groups using Post Hoc test.

Dependent variable	Group Name (I)	Group Name (J)	P value
E. faecalis % age reduction	Group A	Group B	0.7637
		Group C	0.0000
	Group B	Group A	0.7637
		Group C	0.0000
	Group C	Group A	0.0000
		Group B	0.0000
E. coli % age reduction	Group A	Group B	0.8074
		Group C	0.0000
	Group B	Group A	0.8074
		Group C	0.0000
	Group C	Group A	0.0000
		Group B	0.0000

$p<0.05$ statistically significant

Discussion

The main cause of endodontic failure is the persisting infection in the root canal system. About 92% of teeth without apical periodontitis heal, whereas only 74% of teeth with apical periodontitis heal with endodontic treatment [19]. This depends on different factors. Although there is an array of potential factors that influence the outcome of endodontic treatment, success is most significantly dependent upon the elimination of root canal infection present when treatment starts and the prevention of contamination during treatment [20]. We are constantly searching for new antimicrobial agents that can provide us with a cleaner root canal and thereby

improve the success rate of endodontically treated teeth.

In this study, *Enterococcus faecalis* and *Escherichia coli*, were chosen as test organisms because they are well recognized endopathogenic microorganisms. *E. faecalis* is more associated with secondary infection, whereas *E. coli* and its endotoxin is more prevalent in primary endodontic infections. *E. faecalis* has been used in previous studies testing the efficacy of irrigant solutions [21]. The prevalence of Enterococci, has been a conspicuous finding in a high percentage of root-canal failures which has been attributed to its high resistance and its ability to survive as a single organism in monocultures.

E. faecalis and *E. coli* have been found suitable for experimental penetration into dentinal tubules. In the present study, *E. faecalis* and *E. coli* Were incubated for 72 hours to ensure adequate penetration of microbes, however this time is not sufficient enough to form an organized *in vitro* biofilm but we have taken this time to ensure a suitable microbiological environment is created.

In this study, the apices of all sampled teeth were sealed with glass ionomer cement followed by nail varnish application to prevent any contamination from the outer tooth surface during the sampling procedure. To eliminate the variable effects of mechanical instrumentation and smear layer removal in reducing bacterial count, both were accomplished before sterilization and inoculation of sample.

Treatment with ozone is a relatively new concept in dentistry. Ozone is a very powerful bactericide that can kill microorganisms effectively even at a concentration as low as 0.1ppm [16]. There are several known actions of ozone on human body, such as anti-microbial, immunostimulating, antihypoxic, analgesic, detoxicating, bioenergetic and biosynthetic (activation of the metabolism of carbohydrates, proteins & lipids) etc. The anti-microbial effect of ozone as a result of its action on cells by damaging its cytoplasmic membrane due to ozonolysis of dual bonds and also ozone-induced modification of intracellular contents because of secondary oxidants effects. This action is non-specific and selective to microbial cells; it does not damage human body cells because of their major antioxidative ability [22]. Its antimicrobial activity increases in liquid environment of the acidic pH. Ozone causes the synthesis of biologically active substances such as interleukins, leukotrienes and prostaglandins which is beneficial in reducing inflammation and wound healing.

Our goal was to evaluate if additional ozone gas during endodontic treatment resulted in a cleaner root canal. In this study we used bacterial sampling to indicate the presence of infection in the canal. All the teeth treated with ozonated water showed a positive reduction in bacterial growth. This study showed that there was no significant difference in reduction of infection if ozonated water or 3% sodium hypochlorite was used during irrigation. The difference between the groups is small to show a significant difference. Nagayoshi *et al.* (2004) [23] also showed that ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl during irrigation, especially when combined with sonication, and showed a low level of toxicity against cultured cells.

This study shows that ozone treatment has visible effects on bacterial strains of gram negative and gram positive bacteria. In this study *E. coli* and *E. faecalis* were representing the two bacterial strains and the ozone treatment was executed on agar plates. It must be considered that beside these two bacteria, the infected root canal has other bacterial species as well. Although ozone has one of the strongest bactericidal, antifungal and viral characteristics it has shown to have a little effect on bacteria in a biofilm *in vitro* [24].

Teeth treated without ozone i.e., with 3% sodium hypochlorite which showed equally good results. In fact some samples of this group have shown 100% reduction in bacterial count. However, Hypochlorite is acutely operator sensitive, requiring careful application during root canal cleaning to prevent inoculation through the apex into bone or soft tissue, which can cause alarming and destructive oedema, pain and tissue damage, whereas ozone has therapeutic effect on oral tissues. Nagayoshi *et al.* in 2004 [23] along with antimicrobial activity also compared the cytotoxicity against L-929 mouse fibroblasts between ozonated water and NaOCl. They found that the metabolic activity of fibroblasts was high when the cells were treated with ozonated water, whereas that of fibroblasts significantly decreased when the cells were treated with 2.5% NaOCl.

Another element that may have had an effect on the results is the fact that concentration of ozone (0.1 mg/mL) used was less as compared to other ozonators such as Heal Ozone. Out of all the resources that were available Kent Ozone dental jet was the available ozonator. Keeping in mind the limitations ozonated water has proved to be an alternative antimicrobial agent. Probably increasing the ozone concentration will prove to be a better root canal irrigant without any significant side effects.

Conclusion

The results found that ozonated water has proved to be an alternative antimicrobial agent.

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