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## Antibacterial Effect of Various Irrigants Against Enterococcus Faecalis

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**Antibacterial Effect of Various Irrigants Against Enterococcus Faecalis**

Morgan Kesecker, DDS

Thesis submitted to the School of Dentistry at West Virginia University  
in partial fulfillment of the requirements for the degree of

Master of Science in Endodontics

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Morgantown, WV  
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## **Abstract**

### Antibacterial Effect of Various Irrigants Against *Enterococcus Faecalis*

**Morgan Kesecker, DDS**

#### **Introduction:**

One of the goals of endodontic therapy is to debride infected pulps of teeth and return the tooth to a state of health in preparation of restorative care and return to function. Chemically cleansing of the canal system augments mechanical debridement by flushing out debris, dissolving tissue, and disinfecting the root canal system. The purpose of this research is to compare the efficacy of five irrigants: 0.008% HOCl acid (Endocyn®), 0.03% HOCl (Vashe®), 6% and 8.25% sodium hypochlorite (NaOCl), and Ozone against a typical bacterial species found in infected pulps, *Enterococcus faecalis* (*E. faecalis*).

#### **Methods:**

After 24 hours of growth of *E. faecalis* onto an Innovatech 96 well hydroxyapatite coated peg plate, the biofilms were introduced to five different irrigants for 30 seconds of exposure, then immersed into a Difco D/E Neutralizing broth solution for 30 minutes. Each hydroxyapatite peg was placed into 1.5mL centrifuge tubes and diluted by a factor of 10. Dilution samples were transferred into experimental 5mL bottom round test tubes and the Invitrogen's Molecular Probes LIVE/DEAD BacLight Bacterial Viability Counting Kit was used to prepare the final samples for analysis at WVU's Flow Cytometry and Single Cell Core Facility.

#### **Results:**

There was a significant difference in antibacterial effectiveness among the five irrigant groups ( $p < 0.0001$ ). The greatest antibacterial efficacy was with 6% NaOCl and 8.25% NaOCl. Compared with each other, they were similar in antibacterial efficacy ( $p = 0.999$ ). Ozone had the least antimicrobial efficacy.

#### **Conclusions:**

All irrigants tested had antibacterial properties against *E. faecalis*. 6% NaOCl and 8.25% NaOCl demonstrated the greatest antibacterial effect against *E. faecalis* compared to other irrigants.

## Dedication:

- **To my parents, Jeff and Angie:** Thank you for always being there for me. Your tough love, words of wisdom, encouragement and support have shaped me to become who I am today. I am forever appreciative of everything that the two of you have done to raise me. I love you both.
- **Andrew:** I will forever be grateful God placed you in my life. You're the best life partner anyone can ask for. I look forward to our life together! You are my perfect complement and I can't thank you enough for being there for me through the highs and lows of residency. I love you so much!
- **My brother, Jeffery:** Ever since I can remember you were always the person I admired and looked up to growing up. You're not only my brother but my lifelong best friend. I look forward to chasing our career dreams together. I love you bro!

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- **Kathleen Brundage:** Thank you so much for your hours spent running the flow cytometry for me which contributed tremendously to this project. It was much appreciated.

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**List of Symbols, Abbreviations or Nomenclature**



American Association of Endodontists - AAE

Beckton Dickinson - BD

Blood Agar Plate - BAP

Chlorohexidine- CHX

Colony Forming Unit – CFU

Electrolyzed Oxidizing - EO-

Hypochlorous Acid- HOCl

Micro-computed tomography- MicroCT

Minimum Biofilm Eradication Concentration Assay - MBEC Assay

Ozone- O<sub>3</sub>

Sodium Hypochlorite- NaOCl

## **Introduction:**

### *Goal of Endodontics:*

The main goal of endodontic treatment is the prevention or treatment of apical periodontitis in which bacteria play a critical role (1-3). Thorough debridement of pulp tissue and disinfection of the canal space is essential in achieving this goal (2-3). Sterilization of the root canal system is virtually impossible to achieve with current instruments, substances, and techniques; thus, the major realistic microbiological goal of endodontic treatment is to reduce intracanal bacterial populations to levels that are compatible with periradicular tissue healing. Biomechanical use of instruments, as well as root canal cleansing, requires the use of a chemical solution. Inadequate accomplishment of these objectives risks the short-term as well as the long-term outcome of endodontic therapy (4). The root canal treatment success depends on correct chemomechanical disinfection to eliminate the pulp tissue, the remains of dentin, and microorganisms, thus eliminating the etiological factors that cause the endodontic infection (5). Therefore, the root canal instrumentation must always be accompanied by irrigation to remove the remains of pulp tissue and dentin (4-5). Without irrigation solutions, bacterial remains would accumulate causing the instruments to become ineffective (6-8).

### *Irrigation Methods:*

It is important to properly shape a root canal system to develop a conduit that can be effectively cleaned, irrigated, and filled (6,7,9). In addition to the proper shape of the root canal system; many irrigation methods and other factors play a role in proper disinfection of the root canal system (9-10). The intracanal medicament used, contact time, the concentration of the solution, and irrigation technique can all influence the disinfection of the root canal system (3).

The market has many different delivery systems (EndoVac®, GentleWave®, etc.) of available commercial solutions that can be introduced into the canal in various ways. The commercially available delivery systems are another method of attempting to enhance the antimicrobial effects of the irrigation solution used (10). For this study, we focused solely on the antibacterial effect that various irrigants have on one of the most commonly recovered species found within the root canal system, *Enterococcus faecalis* (*E. Faecalis*) (8). It is recognized as a limitation to focusing only on irrigants, that other variables can play a pivotal role in the disinfection of the root canal system (10).

*E. Faecalis:*

Primary infections of the endodontic space are mainly caused by obligate anaerobic species (15). Bacteria persisting within the root canal system are the major cause of endodontic treatment failures (8, 11-14). In failed endodontic cases, *E. faecalis* is the dominant species recovered in persistent endodontic infections and is most popularly studied in vitro (11-14). The inherent antimicrobial resistance and the ability to adapt to changing environments helps *E. faecalis* to persist in the root canal system. *E. faecalis* can adhere to the root canal walls, accumulate, and form communities organized in biofilm. These characteristics help it resist destruction by enabling the bacteria to become more resistant to phagocytosis, antibodies, and antimicrobials when compared to non-biofilm-producing organisms (12-14). The presence of isthmi and anastomosis can make the chemical cleansing of this microorganism within the root canal system very difficult since they can be filled with the smear layer (9). Additionally, as Sedgley et al in 2006, showed *E. faecalis* has the capacity to recover from a prolonged starvation state in the root canal treated teeth (13). These characteristics of *E. faecalis* in the root canal

system in addition to its ease of growth in the laboratory make it one of the more commonly used bacterial species in studies in the endodontic literature. There are still many other bacterial specimens that contribute to primary and secondary endodontic infections (14). For this bench-top study, we have solely used *E. Faecalis* as the micro-organism.

#### *History Of Various Irrigants Introduced To Dentistry:*

Countless compounds in aqueous solution have been suggested as root canal irrigants ranging from inert substances such as sodium chloride (saline) to highly toxic and allergenic biocides such as formaldehyde (formacresol). Most began as antiseptics for traumatic wounds that occurred during war. For example, hypochlorous acid was identified in 1834 by a French chemist, Antoine Jérôme Balard and was used as an antiseptic for traumatic wounds in World Wars I and II. During 1915, in World War I, Dakin introduced sodium hypochlorite (NaOCl) solution for disinfection of open or infected wounds. In 1915, Barret spread the use of Dakin's solution in dentistry, especially for root canal irrigation (16-18). In 1930, Fisch started using ozone on a regular basis in his dental practice in Switzerland. The Swiss dentist was one of the first people credited with using ozone as an antibacterial agent in infected teeth and seeing its effects (19). Walker, in 1936, introduced the use of 5% NaOCl solution as a root canal irrigant in endodontic practice (20).

Several, less toxic irrigation solutions have been used over the past decades like Chlorhexidine (CHX), glutaraldehyde, citric and lactic acid to enhance the disinfection of the root canal system. CHX was developed in the late 1940s in the research laboratories of Imperial Chemical Industries Ltd. in England and was synthesized as an antiviral. However, it had little antiviral efficacy and was put aside, only to be re-discovered some years later as an antibacterial agent (2).

NaOCl if used improperly can result in detrimental complications. Therefore, in recent decades antibacterial solutions like Ozone and HOCl, have been used in healthcare and evolved in quality with less toxic properties (3,21). In this study the antibacterial efficacy of the most commonly used root canal irrigants were compared:

0.008% HOCl (Endocyn®),

0.03% HOCl (Vashe®),

Ozone (O<sub>3</sub>),

6% NaOCl, and

8.25% NaOCl

against one of the most commonly recovered species in endodontically treated teeth, *E. faecalis*.

The purpose of this study was to evaluate and compare the antibacterial activity of the aforementioned antibacterial irrigants against *E. faecalis*, the most commonly recovered bacterial species in infected pulps, after 30 seconds of exposure to the irrigant.

### **Research Hypothesis**

The research hypothesis is that there is a significant difference amongst the various solutions samples. The null hypothesis is that there is no statistically significant difference amongst the various solutions samples.

### **Assumptions**

1. The Multiple equivalent biofilms for antibiotic and biocide susceptibility test (MBEC Assay®) is a quantitative and reproducible method for determining the efficacy of antibacterial agents against biofilms.

2. The operator of the flow cytometer was knowledgeable and well versed in properly handling the instrument.

### **Limitations**

1. This was an *in vitro* experiment and the results may or may not replicate the clinical environment.

2. Operator error may have occurred during the experimentation process.

3. Endodontic infections comprise biofilms that are difficult to eradicate in the root canal system. The responses of different bacteria to dental materials may vary depending on the environment and time they are exposed to.

### **Delimitations**

1. Bacteria enumeration was conducted via flow cytometry analysis.

2. All samples were prepared by the principal investigator.

3. The following experiment tested only one species of bacteria in its planktonic form. *E. faecalis* was the bacteria chosen due to its ability to survive the harsh conditions of the root canal system.

## **Review of Literature:**

### *Role of Irrigants:*

Irrigants play a pivotal role in the cleaning and disinfection of the root canal system. A study by O. A. Peters, in 2004, showed how rotary instrumentation alone left 35% of the root canal space untouched. Micro-computed tomography (MicroCT) evaluation of the teeth in Peters experimental study showed biofilms of bacteria undisrupted that will likely lead to post-treatment disease (22).

Rotary instrumentation and chemical solutions of irrigants synergistically work together to enhance the disinfection of the root canal system. Success in endodontic treatment depends to a great extent on chemo-mechanical debridement of the canals. Although instruments remove most of the canal contents in the main root canal area, irrigation plays an indispensable role in all areas of the root canal system, in particular those parts that are inaccessible for instrumentation (22-24).

Over the decades, many different irrigation solutions have been tested. Siqueira et al studied the antibacterial effect of endodontic irrigants in anaerobic Gram-negative bacteria and facultative bacteria. The solutions used were: 0.5%, 2.5%, and 4.0% NaOCl; 0.2% and 2.0% chlorhexidine; 10.0% citric acid and 17.0% EDTA. All solutions inhibited the tested bacteria. The 4.0% NaOCl was the most effective antibacterial agent (23). Giardino et al evaluated the antimicrobial efficacy of 5.25% NaOCl; Tetraclean® (a mixture of doxycycline, citric acid and detergents); and, MTAD® (a mixture of doxycycline, citric acid and detergents). The study confirmed the supremacy of NaOCl as it eliminated the biofilm in 5 minutes (25).

### *Ozone (O<sub>3</sub>):*

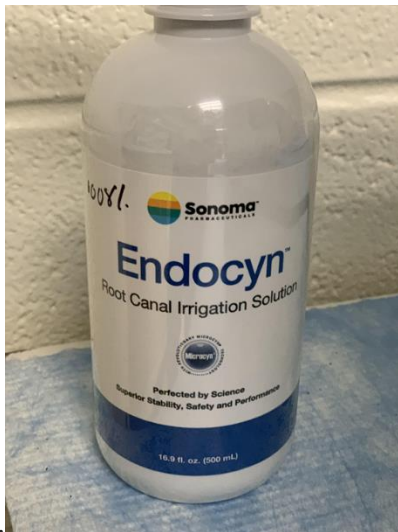
Ozone has been introduced as a disinfectant into endodontic practice. The antibacterial effect of the ozone is a result of its action on cells by damaging the cytoplasm membrane (26). Ozone (O<sub>3</sub>) at low concentration, 0.1 ppm, is sufficient to inactivate bacterial cell membranes and bacterial spores (27). It can be easily produced by an ozone generator. Most of the endodontic literature on ozone relates to its antimicrobial activity (28) and not its safety and storage. Kaptan et al reported positive effects of topical gaseous ozone in recurrent doses in eradication of *E. faecalis* biofilm from the root canals. They noticed that ozone had a greater antimicrobial effect if combined with 2% NaOCl (29). Nagayoshi et al found that ozonated water was effective in killing both gram-positive and negative micro-organisms. Gram-negative bacteria, such as *Porphyromonas*, *P. endodontalis* and *P. gingivalis* were more sensitive to ozonated water than gram-positive oral *streptococci* and *C. albicans* in pure culture (30). Hems et al evaluated the potential of ozone as an antibacterial agent using *E. faecalis* as a test species. Ozone was used both as gasiform (produced by Pure zone device), and aqueous (optimal concentration 0.68 mg/L) (31). They concluded that ozone in solution was antibacterial against planktonic *E. faecalis*. A study by Cardoso et al evaluated the effectiveness of ozonated water in the elimination of *Candida (C.) albicans*, *E. faecalis*, and endotoxins from root canals. Findings revealed that ozonated water was effective against both *C. albicans* and *E. faecalis* immediately after treatment; however, it did not have substantivity (27).



*Hypochlorous acid (HOCl):*

HOCl is a highly effective antimicrobial agent that is generated by human cells as a defensive agent. Under most conditions HOCl is unstable, however technology has evolved and introduced various irrigation solutions with a stabilized form of HOCl (**Figure 1, Figure 2**). In this present experiment Vashe® and Endocyn® were the two solutions used containing two different concentrations of HOCl acid.

**Figure 1:** 0.008% Hypochlorous solution (Endocyn®)



**Figure 2:** 0.03% Hypochlorous solution (Vashe®)



NaOCl's antibacterial effects are considered to be due to the chlorine moiety in the molecule. The chlorine is available to act as a strong oxidizing agent. The chlorine irreversibly oxidizes essential bacterial enzymes, and disrupts bacterial metabolic functions (32). In a study by Hsieh et al, the researchers concluded that both EO- (electrolyzed oxidizing) waters containing 0.0125% and 0.0250% HOCl revealed a remarkable but similar bactericidal effect (> 99.9%) to that of conventional NaOCl against *E. faecalis* and *S. mutans* (33). Another study found HOCl is effective for cleaning biofilm-contaminated implant surfaces and has the potential to be an antiseptic for peri-implantitis treatment. These results suggest that the efficacy of HOCl may be equivalent to NaOCl and CHX (34). Nevertheless, there are few experimental studies in the literature comparing HOCl and other endodontic irrigants.

#### *Sodium Hypochlorite NaOCl:*

Sodium hypochlorite is the most frequently used antimicrobial endodontic irrigant for its activity speed, effectiveness as an antimicrobial agent and tissue-dissolving capabilities. It has

low viscosity allowing easy introduction into the canal architecture, an acceptable shelf life, is easily available and inexpensive. In a survey by Dutner to members of the American Association of Endodontists (AAE), >90% of respondents used NaOCl as an irrigant (35). Giardino et al compared the efficacy of NaOCl and MTAD against *E. Faecalis* biofilm. A low, 5.25% solution of NaOCl disintegrated and removed the biofilm at all time periods studied (25). The antimicrobial effectiveness of NaOCl, based on its high pH, is similar to the mechanism of action of calcium hydroxide.

The high pH of NaOCl interferes in the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation observed in lipidic peroxidation (2). Siqueria et al compared the antibacterial activity of various irrigants against four black pigmented anaerobic bacteria and four facultative bacteria through agar diffusion tests. The antibacterial effectiveness of 4% NaOCl and 2.5% NaOCl was remarkably greater than other agents which were tested (36).

In 1970 Shih studied *in vitro* antibacterial action of 5.25% sodium hypochlorite on *E. faecalis* and *S. aureus*. Shih used the commercial brand, Clorox® since this product possesses a 5.25% NaOCl concentration. Shih concluded 5.25% was necessary to use for the most immediate antimicrobial effectiveness (37). A study by Harrison and Hand showed that 5.25% NaOCl was better than lower diluted concentrations (38).

In 2002, Gomes et al evaluated the effectiveness of five concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of chlorhexidine gluconate (CHX) (gel and liquid) in three concentrations (0.2%, 1% and 2%). They found that all irrigants were effective in killing *E.*

*faecalis*, but required different times to do so (39). Cullen et al found the pulp dissolution property of 8.25% NaOCl was significantly faster than any other tested concentration of NaOCl (40). Currently, for the cleaning and disinfection of the root canals, 8.25% NaOCl is the most frequently used (35). It has a high antimicrobial action and capacity to dissolve organic material.

### **Methods:**

*Ethical Statement:* This study was approved by the Institutional Biosafety Committee (#22-01-01) and the Institutional Review Board as a non-human subject project (protocol number 2201503138) at the West Virginia University, Morgantown, WV.

#### *E. faecalis*

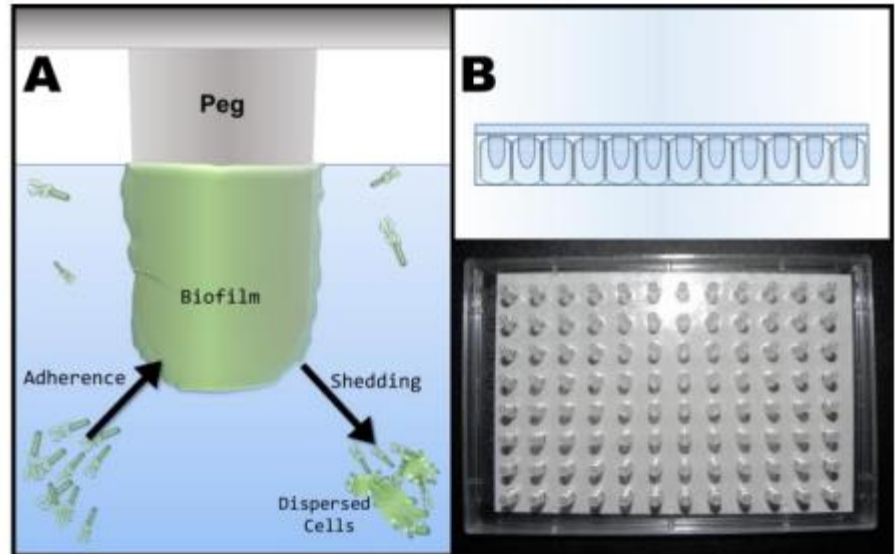
*E. faecalis* (ATCC 29212, Manassas, VA) was used in this experiment. Following the protocol of Sedgley, a concentration of  $1.0 \times 10^7$  CFU/ml was used (41). The handling of bacteria and bacterial inoculum was performed inside a Biological Safety Cabinet II. *E. faecalis* strain was taken from  $-80^\circ\text{C}$  stock and plated on blood agar plates (BAP) (Thermo Fisher Scientific, Waltham, MA) and incubated aerobically for 24 hours at  $37^\circ\text{C}$  (**Figure 3**). A stock solution was created using the freshly grown bacteria from one of the BAPs. A Mcfarland Densitometer was used to obtain a concentration of  $1.0 \times 10^7$  CFU/mL of stock solution.

**Figure 3.** Incubator with *E. faecalis* growing at 37°C.



**Growth of *E. Faecalis*:**

A MBEC® (Minimum Biofilm Eradication Concentration) Assay was used to aid in biofilm growth of *E. faecalis*. The MBEC Assay Biofilm Inoculator consisted of a plastic lid with 96 hydroxyapatite pegs and corresponding wells. The individual wells allowed for the growth of *E. faecalis* on the hydroxyapatite peg. A total of 50 wells were inoculated with stock solution of *E. faecalis*. The remaining wells served as positive and negative controls. Using a multichannel pipet, 200uL of the  $1.0 \times 10^7$  CFU/mL stock solution was pipetted into each well of the Innovatech 96 well hydroxyapatite coated peg plate. The 96 well peg plate with *E. faecalis* was placed on an orbital platform shaker in a 37°C incubator for 24 hours (**Figure 4**).



**Figure 4.** MBEC Assay®

**Note:** A) Biofilms form on the hydroxyapatite pegs. B) The peg lid has 96 identical pegs with a corresponding well. C) This lid fits into a standard 96-well microtiter plate or a trough plate with channels that are set up to contain an inoculated growth medium.

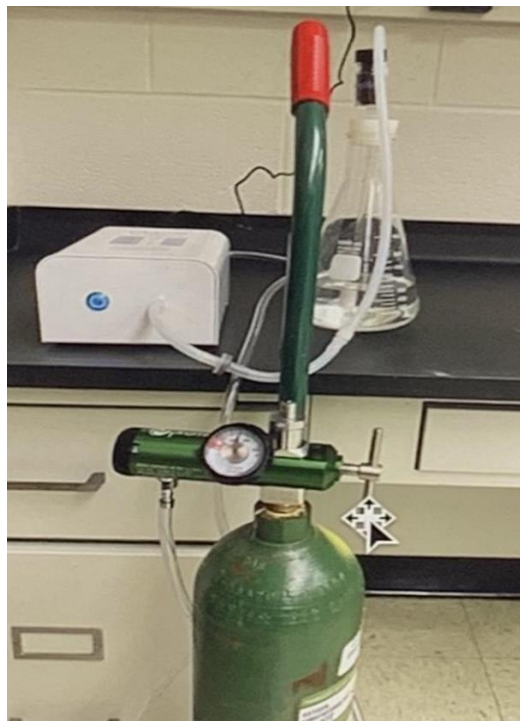
**Exposure of Irrigants Against *E. Faecalis*:**

A total of five irrigants were prepared in solution bottles and used in the experiment:

- 0.008% hypochlorous acid (HOCl; Endocyn®),
- Ozone (O<sub>3</sub>) water,
- 0.03% HOCl (Vashe®),
- 6% Sodium hypochlorite (NaOCl),
- 8.25% NaOCl

O<sub>3</sub> water was prepared using the Promolife O<sub>3</sub> Elite mini ozone generator (**Figure 5**).

**Figure 5.** Ozone preparation



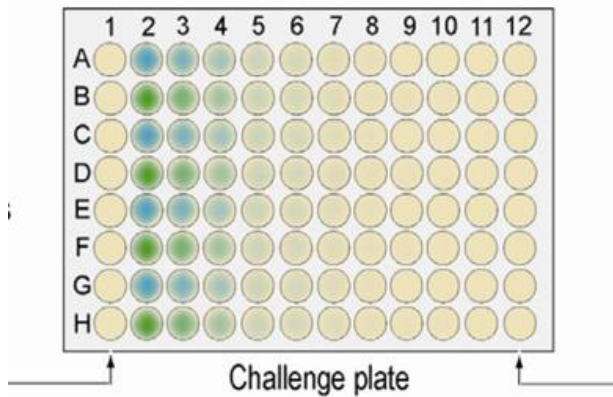
**Note:** Ozone preparation took 500mL of distilled water and was filled in an ozone water bubbler flask. Promolife 03 Elite mini ozone generator was connected to a medical grade oxygen tank with regulator turned to 1/32 oxygen output to generate 65 ug/mL (gamma) ozone output. It ran for 30 minutes to make fully saturated ozone water.

Positive and negative controls were used for comparison. Positive controls had no exposure to an irrigant. A challenger well plate was used for exposure of hydroxyapatite pegs (**Figure 6**). Negative controls were not exposed to *E.faecalis*.

After 24 hours of growth, the Innovatech 96 well hydroxyapatite coated peg plate with biofilm was removed from the platform shaker in the 37°C incubator. The hydroxyapatite pegged biofilm plate lid was introduced to new sterile 96 well flat-bottomed cell culture plate containing 200uL of each irrigant (0.008% HOCl, Ozone, 0.03% HOCl, 6% NaOCl, and 8.25% NaOCl), 1 row containing 200uL of fresh BHI broth for positive control, and 2 columns of solutions only with no *E. faecalis* present to serve as negative controls. The Challenge Plate with biofilm peg

lid was placed into the 37°C incubator for a total exposure time of 30 seconds. The period of exposure to irrigating solutions was selected according to the protocol suggested by Gomes et al (37).

**Figure 6.** 96-well plate example



**Note:** The diagram depicts an example of the 96 well plate of 200 uL of each irrigant for exposure to *E. faecalis*. Row A1-A10 contained 0.008% HOCl. Row B1-B10 contained Ozone. Row C1-C10 contained 0.03% HOCl. Row D1-D10 contained 6% NaOCl. Row E1-E10 contained 8.25% NaOCl. Rows F and G were empty and not used. Row H1-H10 was positive controls containing BHI broth. Columns 11 and 12 served as negative controls containing only the irrigant.

### Neutralization of Irrigants

The Challenge plate with hydroxyapatite pegged biofilm lid was removed from the 37°C incubator (**Figure 7**). The pegged biofilm plate lid was immediately placed into a new sterile 96 well flat-bottomed cell culture plate containing 200uL of Difco D/E Neutralizing broth solution for a total time of 30 minutes to prevent further bacterial death (**Figure 8**). After 30 minutes in the incubator exposed to Difco D/E Neutralizing broth solution the hydroxyapatite pegs were broken off and transferred to 1.5ml centrifuge tubes containing 800mL of saline. The remaining Difco D/E Neutralizing broth from each corresponding well was transferred to the 1.5ml centrifuge tubes to make a total of 1 ml of solution containing 200uLD/E Neutralizing



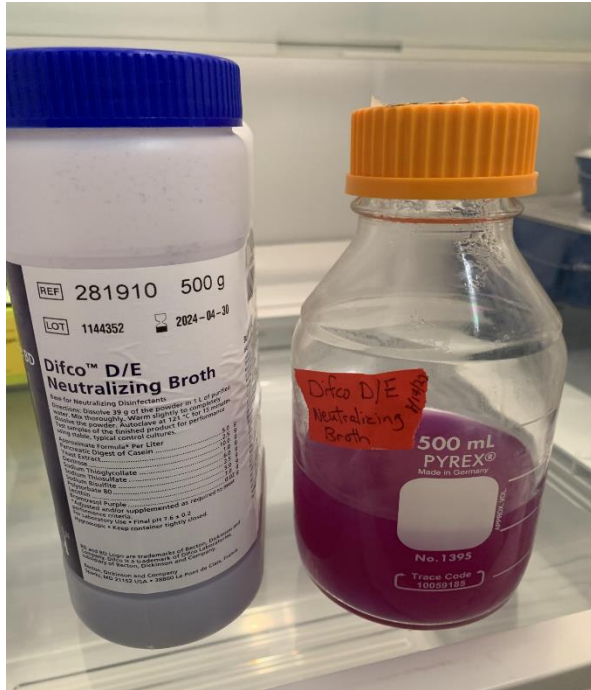
broth/peg/800uLsaline. The samples were sonicated for 30 minutes and vortexed to dislodge the biofilm from the peg.

**Figure 7.** 96-well plate photograph



**Note:** The photograph depicts the 96 well plate. In the experiment one was filled with 200uLD/E Neutralizing broth (recovery plate) and the other was filled with the different irrigation solutions and controls (challenge plate).

**Figure 8.** Depicts the Difco D/E Neutralizing Broth



### **Dilution of Samples**

All positive, negative and experimental samples were diluted by a factor of 10. 100 $\mu$ L was taken from the initial 1mL sample peg/saline/DE mix and placed in a new-labelled 1.5ml microcentrifuge tube containing 900 $\mu$ L of 0.85% sterile saline.

### **LIVE/DEAD BacLight™ Bacterial Viability and Counting Kit (L34856®):**

To prepare for flow cytometry analysis, Invitrogen's Molecular Probes LIVE/Dead BacLight Bacterial Viability Counting Kit® was used. 10 $\mu$ L of each dilution sample was transferred into a 5mL polystyrene round bottom conical tube containing 977 $\mu$ L of saline. The instructions were followed using the kit according to the manufacturer's instructions. The LIVE/DEAD BacLight™ Bacterial Viability and Counting Kit® uses flow cytometry to

quantify dead and alive bacteria. The kit has a mixture of two nucleic acid stains green-fluorescent SYTO- 9 dye and red-fluorescent propidium iodide for viability determinations (42). For accurate sample volume measurements, the kit includes calibrated suspensions of microspheres. With the aid of a flow cytometer operator, accurate enumeration of bacteria was performed at the WVU Flow Cytometry and Single Cell Core Facility for analysis on the Becton Dickinson (BD) LSRFortessa cell analyser using the FSC (Forward Scatter) photo-multiplying tube (PMT). Approximate fluorescence and emission maxima was 480/500 nm for SYTO BC stain bound to DNA. All samples were run at medium speed for 60 seconds. Data was compiled in FACSDiva 8.0 software from BD Biosciences.

**Statistical Analysis:**

Using the plot of forward scatter versus fluorescence, the number of bacteria was calculated according to the following equation:

$$(\text{Syto (+) events} / \text{Beads (+) events}) \times \text{dilution factor} = \# \text{ of bacteria} \times 10^6/\text{mL}$$

A one-way ANOVA analysis was conducted. Significance was set, *a priori*, at  $P < 0.05$ .

## Results

**Table 1.** Pairwise comparison of bacterial growth

Tukey Comparisons	Estimated Difference in Bacterial growth	Standard Error	p-value
8.25% NaOCl versus Endocyn	- 9,560,637	10,073,900	0.9313
8.25% NaOCl versus Ozone	- 44,253,697	9,613,659	0.0004
8.25% NaOCl versus 6.0% NaOCl	- 1,429,838	8,724,253	0.999
8.25% NaOCl versus Vashe	- 14,753,052	8,963,318	0.5733
Endocyn versus Ozone	- 34,693,059	10,853,262	0.0285
Endocyn versus 6.0% NaOCl	8,130,799	10,073,900	0.9648
Endocyn versus Vashe	- 5,192,414	10,281,631	0.9957
Ozone versus 6.0% NaOCl	42,823,858	9,613,659	0.0007
Ozone versus Vashe	29,500,645	9,831,120	0.0467
6.0% NaOCl versus Vashe	- 13,323,213	8,963,318	0.6744

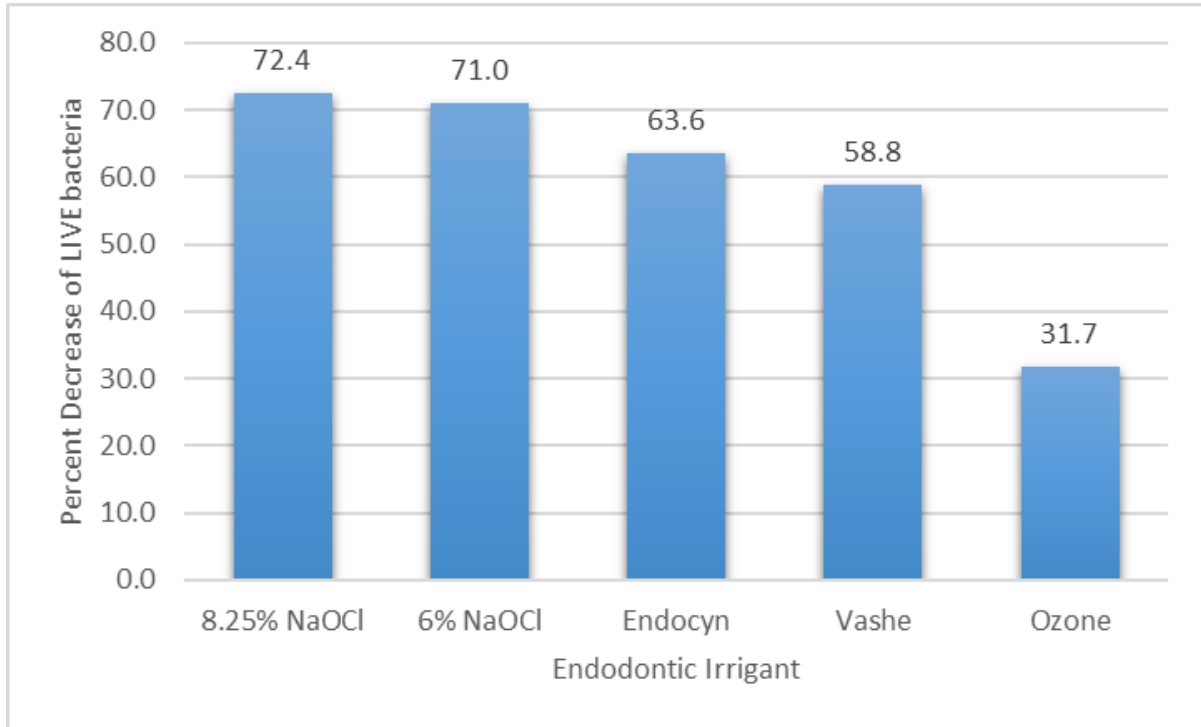
All analyses were conducted with R4.2.2 (R Core Team, Vienna Austria).

**In comparisons involving 8.25% NaOCl,** 8.25% NaOCl was significantly better in killing *E. Faecalis* than ozone ( $p = 0.0004$ ); however, it had similar efficacies as Endocyn® ( $p= 0.9313$ ), Vashe ( $p=0.5733$ ), and 6% NaOCl ( $p=0.999$ ).

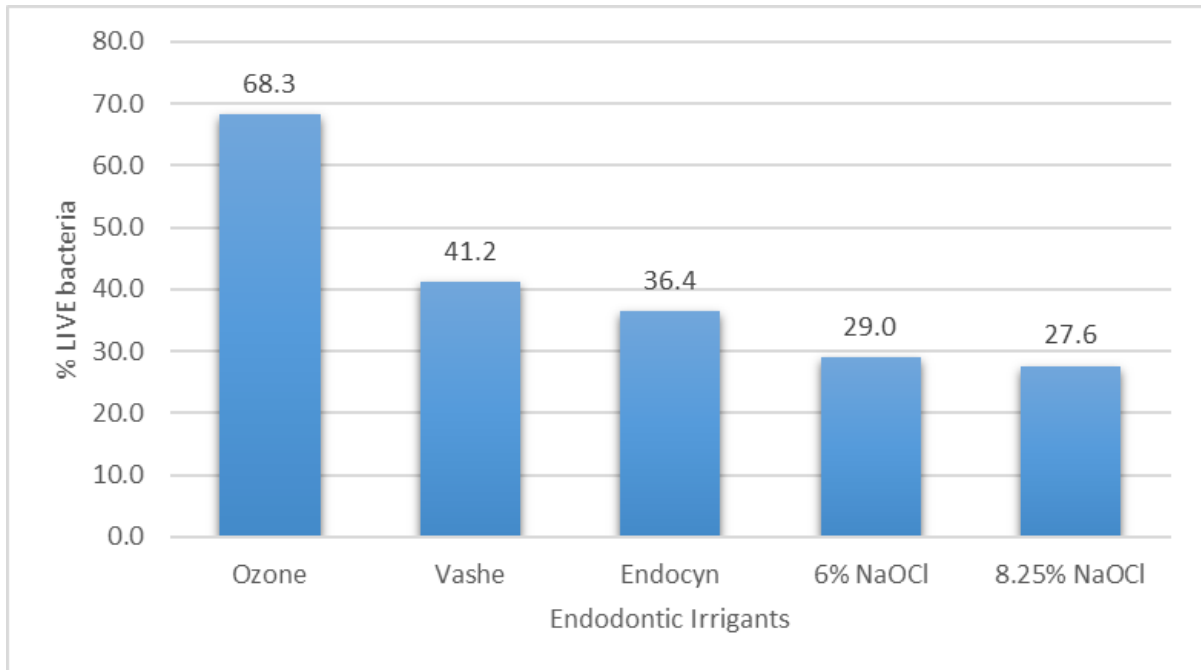
**In comparisons involving 6.0% NaOCl,** 6.0% NaOCl was significantly better in killing *E. Faecalis* than ozone ( $p = 0.0007$ ); however, it had similar efficacies as Endocyn® ( $p=0.9648$ ), Vashe ( $p=0.6744$ ), and 8.25% NaOCl ( $0.999$ ).

**In comparisons involving Vashe®**, Vashe® was significantly better in killing E. Faecalis than ozone (p=0.0467); however, it had similar efficacies as Endocyn® (p=0.9957), 8.25% NaOCl (p=0.5733) and 6.0% NaOCl (p = 0.6744).

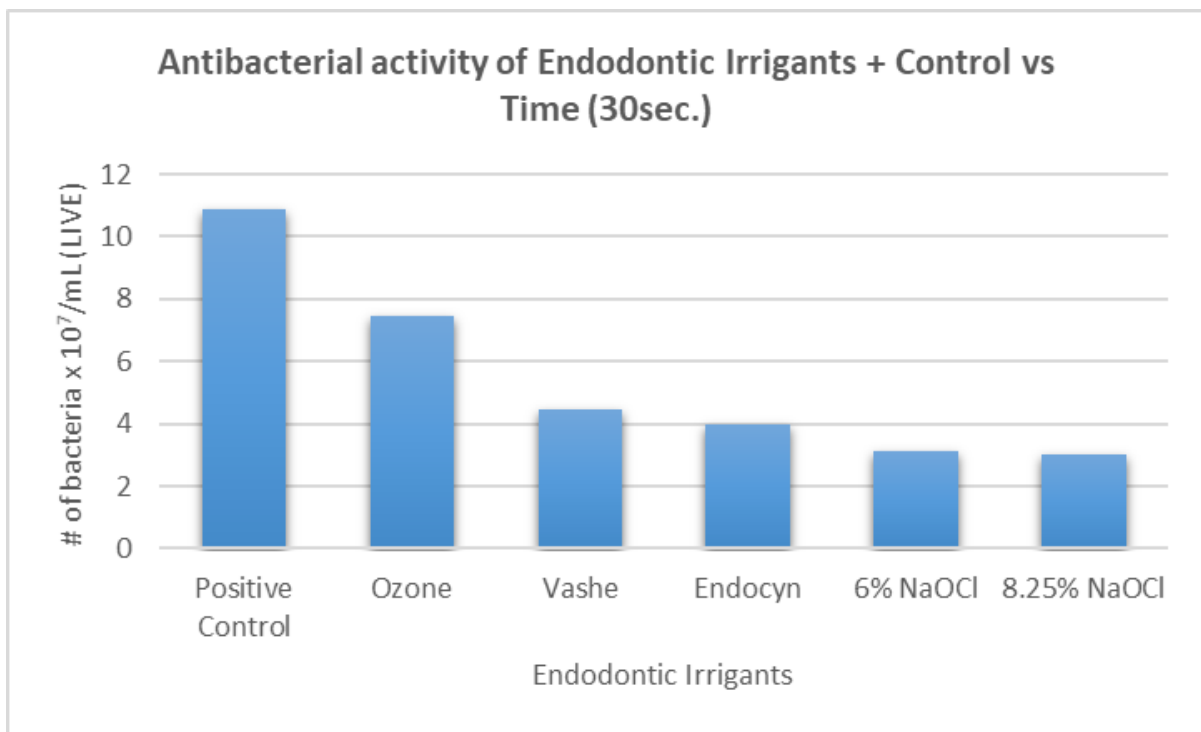
**Ozone** was significantly worse in killing E. Faecalis than the other tested irrigants. It did have a significant difference from the positive control, indicating it was bactericidal (p = 0.01).



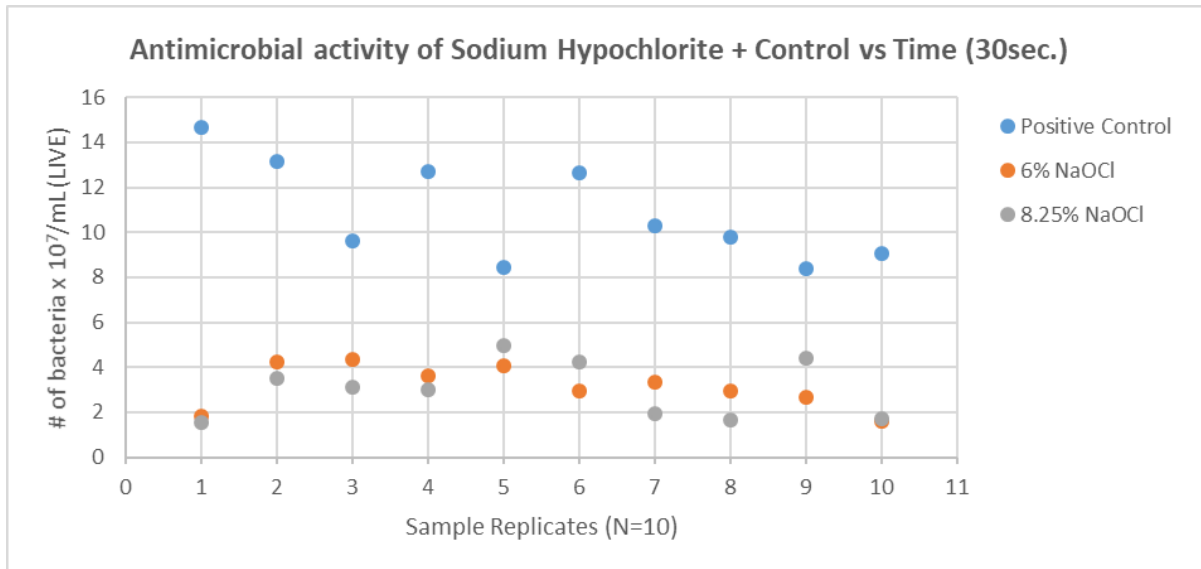
**Figure 9.** Percent decrease in live bacteria after 30 second exposure to each irrigant.



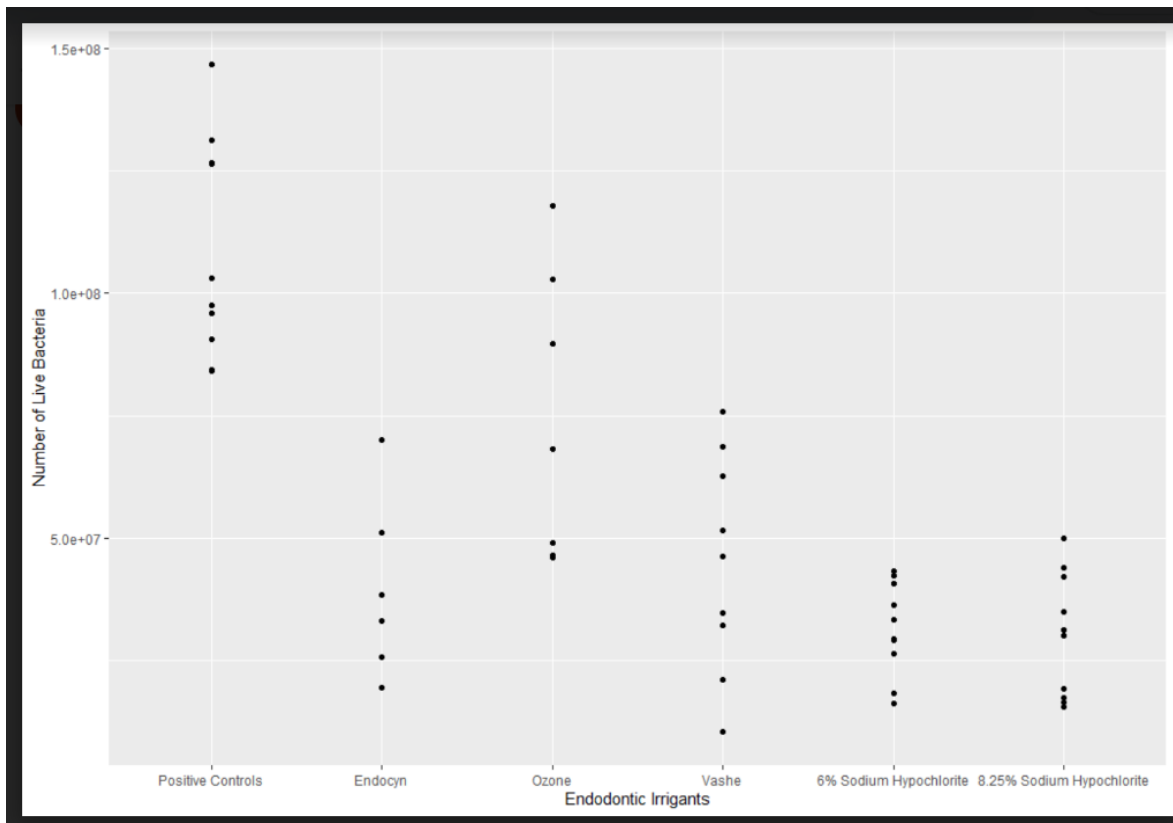
**Figure 10.** Percent of live bacteria remaining after 30 second exposure to each irrigant.



**Figure 11.** Average total number of live bacteria remaining after 30 second exposure to each endodontic irrigant (N=10).



**Figure 12.** Antimicrobial activity of 6 % and 8.25% sodium hypochlorite and the control after 30 seconds.



**Figure 13.** Depicts the number of live bacteria after exposure to each of the endodontic irrigants.

## Discussion

The intent of the current study was to evaluate and compare the antibacterial effect of Ozone, HOCl, and NaOCl in their antibacterial effect on *E. faecalis* biofilm. Our design allowed direct comparison of different irrigation solutions at a specific time frame of 30 seconds while eliminating other confounding variables such as root canal system complexities. This investigation showed all irrigants possessed antibacterial properties against *E. faecalis*, which is expected, as all are used in endodontic treatment. Six percent and 8.25% NaOCl demonstrated the greatest (and similar) antibacterial effect against *E. faecalis* compared to other irrigants at 30 seconds of contact time.

Successful endodontic treatment or retreatment is based on the combination of adequate instrumentation, irrigation, and obturation of the canal system. A great number of studies have focused on the tissue-dissolving ability of NaOCl (44). It has been found that its solvent capability depends not only on concentration but also time, volume, direct contact, and irrigation delivery (45). In this study, time, volume, direct contact and irrigation delivery were constant across the samples. A limitation of the current *in vitro* investigation was the use of a biofilm of only one bacteria, *E. faecalis*. Endodontic infections are polymicrobial in nature. However, the use of *E. faecalis* as a biofilm is common in endodontic studies.

Dunavant et al compared the efficacy of 1% or 6% NaOCl and 2% CHX, among other irrigants, against *E. faecalis* biofilms in a novel *in vitro* model system. Their model consisted of biofilms grown in a flow cell system. Biofilms were immersed in test irrigants for 1 or 5 minutes. They indicated that both concentrations of NaOCl provided statistically significantly better biofilm destruction than any other tested agents. Although the results of the Dunavant study are



consistent with our findings, direct comparisons cannot be made because of the differences in the biofilm model systems (46).

*Strengths and Limitations:*

The results of this study are limited in that we used an *in vitro* model for tooth structure which may not have accurately reproduced biofilm activity in an infected tooth. Also, *E. faecalis* was the sole bacteria and an infected tooth would have a community of bacteria in its biofilm. A study strength is the ability to reduce variance among replicates and provide a greater surface area for the biofilms to be exposed to the irrigant solutions with the use of the 96-well plates. We were also able to adequately control for time, volume, and irrigation delivery.

*Future research:*

The future direction for a study involving specific irrigant effects on biofilms should include standardization of biofilm models and methods that mimic the confines of a natural tooth. Additionally, of interest would be comparing the depth of penetration into the biofilm by specific irrigants and the effect of penetration depth on biofilm kill.

### **Conclusion**

The results of this investigation showed all irrigants possessed antibacterial properties against *E. faecalis*. 6% NaOCl and 8.25% NaOCl demonstrated the greatest antibacterial effect against *E. faecalis* compared to other irrigants.

## REFERENCES

1. Baumgartner JC, Ibay AC. The chemical reactions of irrigants used for root canal debridement. *J Endodon* 1987;13:47-51.
2. Haapasalo M, Shen Y, Wang Z, Gao, Y. Irrigation in endodontics. *Br Dent J* 2014;216:299–303.
3. Zehnder M. Root canal irrigants. *J Endod* 2006;32:389–98.
4. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surg* 1998;85:86-93.
5. Cheung GS, Stock C.J. In vitro cleaning ability of root canal irrigants with and without endodontics. *Int Endod J.* 1993;26:334-43.
6. Navabi AA, Khademi AA, Khabiri M, Zarean P. Comparative evaluation of *Enterococcus faecalis* counts in different tapers of rotary system and irrigation fluids: an ex vivo study. *Journal of Dental Research.* 2018;15:173–79
7. Svensäter G, Bergenholtz G. Biofilms in endodontic infections. *Endodontic topics.* 2004;9:27-36.
8. Siqueira JF Jr, Rôças IN. Clinical implications and microbiology of bacterial persistence after treatment procedures. *J Endodon.* 2008;34:1291-1301.
9. Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implications of the smear layer in endodontics: A review. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2002;94:658–66.
10. Dioguardi M, Di Gioia G, Illuzzi G, Laneve E, Cocco A, Troiano G. Endodontic Irrigants: Different Methods to Improve Efficacy and Related Problems. *Eur. J. Dent.* 2018;12:459–66.
11. Williamson AE, Cardon JW, Drake DR. Antimicrobial Susceptibility of Monoculture Biofilms of Clinical Isolate of *Enterococcus faecalis*. *J Endodon.* 2009;35;95-97.
12. Dennis D, Farahanny W, Prasetya W, Batubara FY. Biological and mechanical principles of chemomechanical preparation in root canal therapy: A Review. 2021;14:187-98.
13. Sedgley C, Buck G, Appelbe O. Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR. *J Endod* 2006;23:104 –9.
14. Hancock H, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2001;91:579–86.
15. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposure of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* 1965;18:340–8.
16. Akbulut MB, Eldeniz AU. In vitro antimicrobial activity of different electrochemically-activated solutions on *enterococcus faecalis*. *Eur Oral Res.* 2019;53;44-50.
17. Barret MT. The Dakin-carrel antiseptic solution. *Dent Cosmos* 1917; 59: 446-448.
18. Dakin HD. On the use of certain antiseptic substances in treatment of infected wounds. *Br Med J* 1915;2:318–20.

19. Fisch, E A. Über die Anwendung von Ozon in der Stomatologie als wirksames Mittel einer neuzeitlichen Therapie, Dtsch. Zahnärztl. Wschr 1936. 40/41: 953- 946 u. 971- 974
20. Walker A. Definite and dependable therapy for pulpless teeth. J Am Dent Assoc 1936 23: 1418-24.
21. Chowdhury S, Mall S, Singh HP. Versatility of Ozone Therapy in Dentistry: A Literature Review. J Dent Sci Oral Rehab. 2015;6:20–3.
22. Peters OA, Barbakow F, Peters CI. An analysis of endodontic treatment with three nickel-titanium rotary root canal preparation techniques. Int Endodon J. 2004;37:849-59.
23. Siqueira JF, Rôças IN, Simone SM, Pinto TG, Magalhães KM, Lima KC. Bacteriologic investigation of the effects of sodium hypochlorite and chlorhexidine during the endodontic treatment of teeth with apical periodontitis. 2007;104: 122–30.
24. Hasselgren G, Olsson B, Cvek M. Effects of calcium hydroxide and sodium hypochlorite on the dissolution of necrotic porcine muscle tissue. J Endod 1988;14:125-7.
25. Giardino L, Savoldi E, Ambu E, Rimondini R, Palezona A, Debbia E. Antimicrobial effect of MTDA, Tetraclean, Cloreximid and sodium hypochlorite on three common endodontic pathogens. Indian J Dent Res. 2009;20:391–7.
26. Stoll R, Venne L, Jablonski-Momeni A, Mutters R, Stachniss V. The disinfecting effect of ozonized oxygen in an infected root canal: an in vitro study. Quintessence Int. 2008;39:231–6.
27. Cardoso MG, de Oliveira LD, Koga-Ito CY, Jorge AO. Effectiveness of ozonated water on *Candida albicans*, *Enterococcus faecalis*, and endotoxins in root canals. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2008;105:85–91.
28. Hems RS, Gulabivala K, Ng Y-L, Ready D, Spratt DA. An in vitro evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. Int Endodon J. 2005;38:22–9.
29. Kaptan F, Güven EP, Topcuoglu N, Yazici M, Külekçi G. In vitro assessment of the recurrent doses of topical gaseous ozone in the removal of *Enterococcus faecalis* biofilms in root canals. Niger J Clin Pract. 2014;17:573-78.
30. Nagayoshi M, Fukuizumi T, Kitamura C, Yano J, Terashita M, Nishihara T. Efficacy of ozone on survival and permeability of oral microorganisms. Oral Microbiol Immunol. 2004;19(4):240-246.
31. Hems RS, Gulabivala K, Ng YL, Ready D, Spratt DA. An in vitro evaluation of the ability of ozone to kill a strain of *Enterococcus faecalis*. Int Endod J. 2005;38:22–9.
32. Estrela C, Estrela CR, Barbin EL et al. Mechanism of action of sodium hypochlorite. Braz Dent J 2002;13:113-17.
33. Hsieh SC, Teng NC, Chu CC, Chu YT, Chen CH, Chang LY, Hsu CH, Huang CS, Hsiao GY, Yang JC. The antibacterial efficacy and in vivo toxicity of sodium hypochlorite and electrolyzed oxidizing (EO) water-based endodontic irrigating solutions. Materials 2020;13:260.
34. Chen CJ, Chen CC, Ding SJ. Effectiveness of Hypochlorous Acid to Reduce the Biofilms on Titanium Alloy Surfaces in Vitro. Int J Mol Sci. 2016;17:1161.
35. Dutner J, Pete M, Alfred A. "Irrigation Trends among American Association of Endodontists Members: A Web-Based Survey. J Endodon 2012;38:37-40.

36. Siqueira JF, Batista MM, Fraga RC, et al. Antibacterial effects of endodontic irrigants on black pigmented gram-negative anaerobes and facultative bacteria. *J Endodon* 1998;24:414-6.
37. Shih M, Marshall FJ, Rosen S. The bactericidal efficiency of sodium hypochlorite as an endodontic irrigant. *Oral Surg Oral Med Oral Pathol* 1970; 29: 613-19.
38. Harrison JW, Hand RE. The effect of dilution and organic matter on the antibacterial property of 5.25% sodium hypochlorite. *J Endodon* 1981;7:128-32.
39. Gomes BP, Ferraz CC, Vianna ME et al. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of *Enterococcus faecalis*. *Int Endodon J* 2001 34: 424-428
40. Cullen JK, Wealleans JA, Kirkpatrick TC, Yaccino JM. The effect of 8.25% sodium hypochlorite on dental pulp dissolution and dentin flexural strength and modulus. *J Endod.* 2015;41:920-4.
41. Sedgley C, Buck G, Appelbe O. Prevalence of *Enterococcus faecalis* at multiple oral sites in endodontic patients using culture and PCR. *J Endodon* 2006;23:104-9
42. Invitrogen Molecular Probes. *Bacteria Counting Kit Manual* 2007:4.
43. Bystron A, Sundqvist G. Bacteriologic evaluation of the effect of 0.5 percent sodium hypochlorite in endodontic therapy. *Oral Surg Oral Med Oral Pathol* 1983;55:307-312.
44. Shih M, Marshall FJ, Rosen S. The bactericidal efficiency of sodium hypochlorite as an endodontic irrigant. *Oral Surg Oral Med Oral Pathol* 1970;29:613-19.
45. Clarkson RM, Moule AJ. Sodium hypochlorite and its use as an endodontic irrigant. *Aust Dent J* 1998;43:250-56.
46. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against *Enterococcus faecalis* biofilms. *J Endodon* 2006;32:527-31.
47. Endodontists American Association of. *Glossary of endodontic terms*. American Association of Endodontists; 2003.