AN IN-VITRO ANALYSIS TO EVALUATE THE DISINFECTION EFFECTIVENESS OF COLD ATMOSPHERIC PRESSURE (CAP) PLASMA JET IN *ENTEROCOCCUS FAECALIS* INFECTED ROOT CANALS

THESIS

Submitted to

All India Institute of Medical Sciences, Jodhpur
In partial fulfilment of the requirement for the degree of
MASTER OF DENTAL SURGERY (MDS)
(CONSERVATIVE DENTISTRY AND ENDODONTICS)

JULY, 2020

AIIMS, JODHPUR **DR. P SOUNDHARRAJAN**

ALL INDIA INSTITUTE OF MEDICAL SCIENCES
JODHPUR

CERTIFICATE

This is to certify that thesis entitled "An in-vitro analysis to evaluate the disinfection effectiveness of Cold Atmospheric Pressure (CAP) Plasma Jet in *Enterococcus faecalis* infected root canals" is an original work of **Dr. P Soundharrajan** carried out under our direct supervision and guidance at Department of Dentistry, All India Institute of Medical Sciences, Jodhpur.

GUIDE

Dr. Pravin Kumar
Professor
Department of Dentistry
AIIMS, Jodhpur

CO-GUIDES

Dr. Ram Prakash

Associate Professor
Department of Physics
IIT, Jodhpur

Dr. Sarika Prabhakar Kombade
Associate Professor
Department of Microbiology
AIIMS, Jodhpur

Dr. Ankita Chugh

Additional Professor
Department of Dentistry
Oral and Maxillofacial Surgery
AIIMS, Jodhpur

ALL INDIA INSTITUTE OF MEDICAL SCIENCES, JODHPUR

DECLARATION

I, hereby declare that the work reported in the thesis entitled **"An in-vitro analysis to evaluate the disinfection effectiveness of Cold Atmospheric Pressure (CAP) Plasma Jet in *Enterococcus faecalis* infected root canals"** embodies the result of original research work carried out by me in the Department of Dentistry, All India Institute of Medical Sciences, Jodhpur.

I further state that no part of the thesis has been submitted either in part or in full for any other degree of All India Institute of Medical Sciences or any other Institution/University.

DR. P SOUNDHARRAJAN
Junior Resident
Department of Dentistry
Conservative Dentistry and Endodontics
All India Institute of Medical Sciences, Jodhpur.

ACKNOWLEDGEMENT

I wish to express my sincere appreciation to those who have contributed to this thesis and supported me in one way or the other during this amazing journey.

I would like to express my deepest gratitude to my revered, erudite teacher and **Guide Dr. Pravin Kumar,** Professor and Head, Department of Dentistry, AIIMS, Jodhpur**.** His warm-hearted guidance to this study is immeasurable. I am deeply indebted to his compassion and wisdom. With his tremendous indulgence, dynamism and exceptional commitment, this journey has been transformed into an extraordinary experience. He has been instrumental in bringing out the best of my contribution towards this project with his prompt and appropriate words of enlightenment. His constructive criticism and critical screening has helped me in my journey.

I also owe a great part of this project to my Co-Guides **Dr. Ram Prakash,** Associate Professor, Department of Physics, IIT Jodhpur, **Dr. Sarika Prabhakar Kombade,** Associate Professor, Department of Microbiology, AIIMS, Jodhpur, **Dr. Ankita Chugh,** Additional professor, Department of Dentistry, AIIMS, Jodhpur**.** Their in-depth knowledge and willingness to help me at any time to complete my thesis in time bound manner was tremendous. I am fortunate to be influenced by their vision and leadership. My vocabulary would never encompass enough words to express my gratitude towards them as teachers.

I am extremely thankful to **Dr. Arun Kumar Patnana**, Senior Resident, Pedodontics, for the marvelous support during this project. The vibrant and exuberant ideas and inputs provided by him helped me to carry on in the hard times.

Undoubtedly, a special word of thanks for all my senior residents **Dr. Ashish Choudhary**, **Dr. Rajat Sharma, Dr. Pratibha Singh** and **Dr. Arun kumar D,** for their continuous motivation and everlasting support.

My heartfelt gratitude to Abhijit Mishra for his suggestions, immense support and kind assistance for this study.

I extend my sincere gratitude to **Dr. Pankaj Yadav** for guidance during statistical analysis.

I express my gratitude to my colleague **Dr. Ankita Kapoor,** and my juniors **Dr. Gajender Rawat, Dr. Sumit Kumar Yadav** who have always extended their helping hands and round the clock availability at all times.

I thank my parents **A. Palanisamy and P. Selvi** and my brother **P. Deepan** for their perpetual love and blessings during this endeavor. I would also like to thank my wife **S. Tamilarasi** for her constant support and encouragement during this endeavor.

I would also like to thank my friends **Dr. S Nithyaraj, Dr. Suganya, Dr. Elakkiyaselvi, Dr. P Vignesh, Dr. S Sathyagireeswaran, Dr. R Baskar** for their continued support and encouragement during this endeavor.

I would like to offer my sincerest gratitude to my institute; **All India Institute of Medical Sciences, Jodhpur** which provided a platform to carry out this research work.

CONTENTS

LIST OF ABBREVIATIONS

Ace : Collagen binding proteins of *E.faecalis*

ALX : Alexidine

APDT : Antimicrobial Photodynamic Therapy

APNP : Atmospheric Pressure Nonequilibrium Plasma

ATCC : American Type Culture Collection

BHI : Brain Heart Infusion

CAP : Cold Atmospheric Pressure

CFU : Colony Forming Units

CHX : Chlorhexidine

CMP : Chemo Mechanical Preparation

CP : Cold Plasma

CTR : Cetrimide

ECM : Extra Cellular Matrix

EDTA : Ethylene Diamine Tetraacedic Acid

EfLTA : Lipoteichoic acid purified from Enterococcus Faecalis

E.Faecalis : Enterococcus Faecalis

Er: YAG : Erbium- doped Yttrium Aluminium Garnet

FCM : Flow cytometry

HEBP : Etidronic acid

KV : Kilo Voltage

LTAPP : Low Temperature Atmospheric Pressure Plasma

MBEC : Minimal Bacterial Eradication Concentration

MTAD : Mixture of Tetracycline Acid and Detergent

MTT : Tetrazolium Salt

Nd: YAG : Neodymium- doped Yttrium Aluminium Garnet

Ni-Ti : Nickel Titanium

NTP : Non Thermal Plasma

OCT : Octenisept

OD : Optical Density

PAD : Photo Activated Disinfection

PDT : Photo Dynamic Therapy

pH : Potential of Hydrogen

PMJ : Plasma Jet

pscl : Political Science Computational Laboratory

PW : Pulse Width

RF : Reduction Factor

RCT : Root Canal Treatment

SS : Stainless Steel

Spr : Serine proteases

TLR2 : Toll Like Receptor 2

VBNC : Viable but Non-Cultivable

ZIP : Zero Inflated Poisson

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Bacterial infection has been long recognized as the primary etiologic factor for the development of pulp and peri-apical infections (1). In general, endodontic infection is a polymicrobial infection and the number of microorganisms within an infected root canal system may vary from 10^2 to 10^8 (2). The presence and growth of bacteria in the coronal dentin, root canal space, or radicular dentin is the most important etiologic factor in the development of endodontic disease (3). The root canal treatment aims to eliminate the complete polymicrobial infection from the root canal system and achieve a hermetic, bacteria tight or fluid impervious seal (4). Any failure in achieving the fluid tight seal, aids in the re-growth of microbes and further resulting in post treatment diseases. Hence, the success of root canal treatment depends on complete eradication of microorganisms and their byproducts from the root canal system.

Microbes in the root canal system can occur either in a planktonic state or in an organized biofilm. Biofilms are more resistant to phagocytosis, antibodies and antimicrobial agents than planktonic bacteria and are therefore a major cause of recurrent endodontic infections (5). Bacterial species that persist even after routine endodontic treatment procedures are gram-positive facultative anaerobes (*Parvimonas micra, Propionibacterium species, Pseudoramibacter alactolyticus, Actinomyces species, Lactobacilli, Enterococcus faecalis and Olsenella uli*) and some gram negative bacteria (*Fusobacterium nucleatum, Prevotella and Campylobacter species*) (6).

Researchers began using microbiological culture techniques to study the relationship between *E. faecalis* and intra-canal infection in the 1960s (7). A distinctive feature of *E. faecalis* is its ability to survive and grow in an environment that would be toxic to many bacteria (8). The ability of *E. faecalis* to grow as a biofilm on root canal

walls and as a monoinfection in treated canals without synergistic support from other bacteria makes it highly resistant to antimicrobial agents. *E. faecalis* invades the dentinal tubules however, the mechanism of invasion is not fully understood. The attachment of bacteria to the walls of the dentinal tubules is considered to be the initial step in this process. Collagen is widely considered to be the primary substrate for the specific binding of *E. faecalis* to dentin. Collagen-binding proteins of *E. faecalis* (Ace) and serine proteases (Spr) are believed to play important roles in binding of *E. faecalis* to root canal dentin (9).

Chemomechanical preparation of the root canal system includes mechanical instrumentation, irrigation and inter-appointment intracanal medication of the root canals. Mechanical instrumentation cannot produce a complete bacteria free root canal system as there are chances of leaving some residual necrosed or vital pulp tissue within the root canals (10). Furthermore, canals anatomical intricacies ensure that certain areas though small, remain beyond mechanical manipulation. Therefore, irrigation or disinfection systems are required to remove the residual pulp tissue from the root canal even after comprehensive mechanical instrumentation. Thus, amongst all the methods of endodontic disinfection, irrigation plays the most important role in eliminating microorganisms from the root canal system.

Sodium hypochlorite (NaOCl) has been used as an endodontic irrigant for more than 70 years. NaOCl has been used for wound irrigation since 1915 and as an endodontic irrigating solution since the 1920s. It still remains the preferred solution for endodontic irrigation and disinfection as it fulfils most of the criteria defined for an ideal irrigating solution (11). Hypochlorite is generally a strong oxidizing, hydrolytic agent and it has bactericidal and proteolytic properties. Several studies have reported that NaOCl has a wide spectrum of antimicrobial activity and can rapidly kill vegetative

and spore-forming bacteria, fungi, protozoa, and viruses (12). The bactericidal effect of NaOCl results from the formation of hypochlorous acid (HOCl) on contact with organic matter. HOCl develops its bactericidal effect by oxidizing sulfhydryl groups in bacterial enzymes, thereby disrupting the metabolism of microorganisms (13), further leading to the destruction of bacterial cells. NaOCl is also capable of dissolving organic tissue containing fatty acids and lipids through saponification reactions (14).

Though it has been proved that NaOCl has an efficient antibacterial property for endodontic disinfection, its inability to effectively remove the smear layer from dentinal walls still remains its main limitation. Thus, an additional solution [Ethylenediamine tetraacetic acid (EDTA)] needs to be used for smear layer removal even after the use of irrigating solutions such as NaOCl or Chlorhexidine (CHX). It has been reported that the interaction of CHX and EDTA forms a white precipitate which was not a desired outcome of an irrigating regimen.

Qmix is a relatively new one step endodontic irrigant containing a mixture of bisbiguanide antiseptic (CHX 2%), calcium chelating agents (EDTA) and surfactants (15). Though Qmix includes both CHX and EDTA, the formation of white colour precipitate is avoided because of its chemical design (16). The presence of both CHX and EDTA in Qmix makes it both an antiseptic as well as smear layer removal agent. It is also reported that Qmix is as effective as 17% ethylenediaminetetraacetic acid (EDTA) in reducing the smear layer (17) and has strong antimicrobial activity in disinfecting hydroxyapatite discs infected with *E. faecalis* (18). Further, some studies have shown that NaOCl is more effective in biofilm removal than Qmix (19).

Recently, Cold Atmospheric Pressure (CAP) plasma has emerged as a novel tool with encouraging prospects in endodontic root canal disinfection (20). Plasma, the

fourth state of matter, a partially ionized gas containing charged particles of negative ions, positive ions, electrons, photons, and free radicals may be considered a new agent for antimicrobial intervention (21). CAP plasma jet at atmospheric pressure can be created by the discharge of a dielectric barrier (DBD) using an inert gas such as helium or argon and a high frequency energy source, which generates reactive oxygen and nitrogen species and ultraviolet radiation, energized ions and charged particles (22). CAP plasma jet applications in medical fields, such as sterilization, promotion of blood coagulation, wound healing, induction of tumour cell apoptosis, have been proven (23). It was reported that CAP plasma jet did not cause significant thermal burns or damage and the possibility of applying it to the oral environment was also proved (24). CAP plasma jet, which can penetrate the root canals of instrumented and cleaned teeth, have made it possible to use plasma to remove microorganisms associated with infected root canals (25). Thus, considering the recent developments in the irrigation protocols, the present in-vitro study was planned to evaluate the disinfection effectiveness of CAP plasma jet in comparison to irrigation with 5.25% NaOCl and Qmix in *E. faecalis* infected root canals at different time intervals. The null hypothesis stating that, there will be no difference in the disinfection effectiveness of CAP plasma jet, NaOCl and Qmix in *E. faecalis* infected root canals.

REVIEW OF LITERATURE

1. **Siqueira JF et al.,**(26) in 2000 evaluated the in-vitro intracanal bacterial reduction produced by instrumentation and irrigation with 1, 3 and 5.25% NaOCl or saline solution. All test solutions significantly reduced the number of bacterial cells in the root canal. There was no significant difference between the three NaOCl solutions tested. All NaOCl solutions were significantly more effective than saline solution in reducing the number of bacterial cells within the root canal.

2. **Gomes BPFA et al.,**(27) in 2001 assessed the effectiveness of several concentrations of NaOCl (0.5%, 1%, 2.5%, 4% and 5.25%) and two forms of CHX (gel and liquid) in three concentrations (0.2%, 1% and 2%) in the elimination of *E. faecalis.* A broth dilution test using 24-well cell culture plates was performed and the time taken for the irrigants to kill bacterial cells was recorded. CHX in the liquid form at all concentrations tested (0.2%, 1% and 2%) and NaOCl (5.25%) were the most effective irrigants. However, the time required by 0.2% CHX liquid and 2% CHX gel to promote negative cultures was only 30 s and 1 min, respectively. Even though all tested irrigants possessed antibacterial activity, the time required to eliminate *E. faecalis* depended on the concentration and type of irrigant used.

3. **Siqueira JF et al.,**(13) in 2003 tested the effectiveness of 4.0% NaOCl used with three irrigation methods in the elimination of *E. faecalis* from the root canal. Root canals contaminated with *E. faecalis* were treated as follows: (i) irrigation with 2 mL of NaOCl solution and agitation with hand files; (ii)

irrigation with 2 mL of NaOCl solution and ultrasonic agitation; (iii) irrigation with NaOCl alternated with hydrogen peroxide. Contaminated canals irrigated with sterile saline solution served as the control. There were no statistically significant differences between the experimental groups. However, NaOCl applied by the three methods tested, was significantly more effective than the saline solution (control group) in disinfecting the root canal.

4. **Kayaoglu G et al**.,(28) in 2004 reported that up to 90% of Enterococcal infections in humans are caused by *E. faecalis.* Enterococci can grow at 10°C and 45°C, at pH 9.6, in 6.5% NaCl broth, and survive at 60°C for 30 minutes. *E. faecalis* can adapt to adverse conditions. It becomes less sensitive to normally lethal levels of sodium dodecyl sulfate, bile salts, hyperosmolarity, heat, ethanol, hydrogen peroxide, acidity, and alkalinity. Starving *E. faecalis*, can enter the viable but non-cultivable state, a survival mechanism adopted by a group of bacteria when exposed to environmental stress, and resuscitate upon returning to favourable conditions. Aggregating Substance in *E. faecalis* was found to mediate binding to extracellular matrix proteins, including type I collagen.

5. **Radcliffe CE et al**.,(29) in 2004 determined the resistance of microorganisms associated with refractory endodontic infections to NaOCl used as a root canal irrigant. Two strains each of Actinomyces naeslundii, Candida albicans and *E. faecalis* were tested as late logarithmic phase inocula, against NaOCl adjusted to 0.5, 1.0, 2.5 and 5.25% w/v. Contact times used were 0, 10, 20, 30, 60 and 120 s. In the case of *E. faecalis*, additional experiments used contact times of 1.0, 2.0, 5.0, 10.0 and 30.0 min. All concentrations of NaOCl lowered CFU below the limit of detection after 10 s in the case of A. naeslundii and C.

albicans. However, *E. faecalis* proved to be more resistant to NaOCl. Using 0.5% NaOCl for 30 min reduced CFU to zero for both strains tested. This compares with 10 min for 1.0%, 5 min for 2.5% and 2 min for 5.25%. The published association of *E. faecalis* with refractory endodontic infection may result, at least partially, from high resistance of this species to NaOCl.

6. **Sena NT et al**., (30) in 2006 investigated the antimicrobial activity of 2.5% and 5.25% NaOCl and 2.0% CHX gel and liquid as endodontic-irrigating substances against selected single-species biofilms. Saline did not inhibit the growth of any of the tested micro-organisms, with or without agitation, being statistically different from NaOCl and CHX. Mechanical agitation improved the antimicrobial properties of the chemical substances tested using a biofilm model, favouring the agents in liquid presentation, especially 5.25% NaOCl and 2% CHX.

7. **Stuart C et al**.,(31) in 2006 reported that the prevalence of *E. faecalis* in persistent endodontic infections ranges from 24% to 77%. It has been shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells, and alter host responses. *E. faecalis* is able to suppress the action of lymphocytes, potentially contributing to endodontic failure. *E. faecalis* is not limited to its possession of various virulence factors. It is also able to share these virulence traits among species, further contributing to its survival and ability to cause disease. *E. faecalis* overcomes the challenges of survival within the root canal system in several ways. It exhibits widespread genetic polymorphisms and possesses serine protease, gelatinase, and collagen-binding protein (Ace), which help it bind to dentin. It is small enough to proficiently invade and live within dentinal tubules. It has the capacity to endure prolonged periods of starvation

until an adequate nutritional supply becomes available. Once available, the starved cells are able to recover by utilizing serum as a nutritional source. Serum, which originates from alveolar bone and the periodontal ligament, also helps *E. faecalis* bind to type I collagen.

8. **Berber VB et al.,**(32) in 2006 conducted an invitro study to evaluate the efficacy of various concentrations of NaOCl and instrumentation techniques in reducing E. faecalis within root canals and dentinal tubules. At all depths and thirds of the root canals and for all techniques used, 5.25% NaOCl was shown to be the most effective irrigant solution tested when dentinal tubules were analysed, followed by 2.5% NaOCl. No differences among concentrations in cleaning the canals were found. Especially at higher concentrations, NaOCl, was able to disinfect the dentinal tubules, independent of the canal preparation technique used.

9. **Kho P et al.,**(33) in 2006 compared the antimicrobial efficacy of irrigation with 1.3% NaOCl/ Biopure MTAD versus irrigation with 5.25% NaOCl/ 15% EDTA in the apical 5 mm of roots infected with E. faecalis. Bilaterally matched human teeth were sterilized and inoculated with *E. faecalis*. After chemomechanical root canal preparation, the root-ends were resected and pulverized in liquid nitrogen to expose *E. faecalis* in dentinal tubules or other recesses away from the main root canal system. No significant differences were seen between the number of colony forming units of *E. faecalis* for teeth irrigated with 5.25% NaOCl/15% EDTA versus those teeth irrigated with 1.3% NaOCl/Biopure MTAD. This study demonstrated that there is no difference in antimicrobial efficacy for irrigation with 5.25% NaOCl/15% EDTA versus irrigation with

1.3% NaOCl/Biopure MTAD in the apical 5 mm of roots infected with *E. faecalis*.

10. **Krause TA et al.,**(34) in 2007**,** compared the antimicrobial effect of MTAD, two of its components, doxycycline and citric acid, and NaOCl in two in vitro models on *E. faecalis*. In the bovine tooth model, the lumens of 30 bovine dentin discs were infected with *E. faecalis* for 2 weeks before treating with either one of the experimental irrigants or saline. Bacteria in the shavings were collected with two sizes of burs and enumerated after overnight culturing. Zones of inhibition were recorded in the agar diffusion model for each irrigant. In the tooth model, NaOCl and doxycycline were more effective than control in killing E. faecalis at the shallow bur depth, but at the deeper bur depth only NaOCl was superior. In the agar diffusion model, NaOCl produced less inhibition than MTAD or doxycycline.

11. **Giardino L et al.,**(35) in 2007 compared the antimicrobial efficacy of 5.25% NaOCl, BioPure MTAD and Tetraclean against *E. faecalis* biofilm generated on cellulose nitrate membrane filters. After incubation, the membrane filters were transferred into tubes containing 5 mL of the selected antimicrobial solution test agent or NaCl 0.9% (positive control) and incubated for 5, 30, and 60 minutes at 20°C. Statistical analysis showed that only 5.25% NaOCl can disintegrate and remove the biofilm at every time, however, treatment with Tetraclean caused a high degree of biofilm disintegration in every considered time intervals as compared with MTAD.

12. **Baumgartner JC et al.,**(36) in 2007 compared the antimicrobial efficacy of 1.3% NaOCl/BioPure MTAD to 5.25% NaOCl/15% EDTA for root canal

irrigation. Twenty-six bilaterally matched pairs of human teeth were collected and incubated with *E. faecalis* for 4 weeks. The teeth were divided into two experimental groups and one positive control group. The canals were instrumented and irrigated with either 5.25% NaOCl/15% EDTA or 1.3% NaOCl/BioPure MTAD. The first bacterial samples revealed growth in 0 of 20 samples with 5.25% NaOCl/15% EDTA irrigation and in 8 of 20 samples with 1.3% NaOCl/BioPure MTAD irrigation. Samples taken after additional canal enlargement revealed growth in 0 of 20 samples in 5.25% NaOCl/15% EDTA and in 10 of 20 samples in 1.3% NaOCl/BioPure MTAD group. This investigation showed consistent disinfection of infected root canals with 5.25% NaOCl/15% EDTA. The combination of 1.3% NaOCl/BioPure MTAD left nearly 50% of the canals contaminated with *E. faecalis*.

13. **Xinpei LU et al.,**(20) in 2009 conducted an invitro study that used a CAP plasma jet device, which can generate plasma inside the root canal. When He/O_2(20%) is used as working gas, the preliminary inactivation experiment results show that it can efficiently kill *E. faecalis*, one of the main types of bacterium causing failure of root canal treatment in several minutes.

14. **Arias-Moliz MT et al.,**(37) in 2009 conducted an invitro study to evaluate the minimal biofilm eradication concentration (MBEC) of NaOCl, CHX, EDTA, and citric and phosphoric acids after 1, 5, and 10 minutes of exposure to biofilms of *E. faecalis*. The biofilms grew in the MBEC high-throughput device for 24 hours at 37 degree C and were exposed to 10 serial two-fold dilutions of each irrigating solution. The viable cell counts were log10 transformed, and a concentration of an irrigant was considered to eradicate the biofilms when it produced a reduction of 5 logarithmic units. NaOCl was the most effective

agent, capable of eradicating the biofilms after 1 minute at a concentration of 0.00625%. CHX eradicated biofilm after 5 minutes at 2%. EDTA and citric and phosphoric acid solutions were not effective against the biofilms at any concentration or time tested.

15. **Zhou X et al.,**(38) in 2010 investigated the antimicrobial activity of an atmospheric pressure room-temperature CAP plasma jet on simulated root canals infected with *E. faecalis*. The samples are divided randomly into 12 experimental groups and one control group. All experimental groups exhibited a significant reduction in viable bacteria compared with the control group ($P < 0.01$). The largest reductions were obtained in Group 9 (with CAP plasma jet containing 5.25% NaOCl sterilization for 12 min after irrigating the root canals with 1-ml sterile physiologic saline) and Group 12 (with plasma-jet sterilization for 12 min after irrigating the root canals with 1-ml sterile physiologic saline), with 6.21 and 5.62 log reductions, respectively. It was concluded that CAP plasma jet containing 5.25% NaOCl as well as CAP plasma jet only can effectively sterilize the simulated root canals.

16. **Ozdemir HO et al.,**(39) in 2010, aimed to evaluate the effects of ethylenediaminetetraacetic acid (EDTA) and NaOCl on *E. faecalis* biofilm growth in root canal dentin of young and old individuals. The root canals of extracted young (<30 years) and old (>60 years) single-rooted human teeth were sectioned at the crown and the apical parts. The root canals of the mid-root sections were enlarged with #2 Gates-Glidden burs. After treatment with 17% EDTA + 2.5% NaOCl, 17% EDTA alone, 2.5% NaOCl alone, or saline, the samples were incubated in *E. faecalis* suspension for 24 hours. Combination of EDTA and NaOCl significantly reduced the amount of intracanal biofilm in

both age groups. However, the bacterial counts of *E. faecalis* in the old group were still higher. It might be suggested that root canals from elderly population are more susceptible to canal infection. However, combined application of EDTA and NaOCl significantly reduces the amount of intracanal biofilm.

17. **Pasqualini D et al.,**(40) in 2010 evaluated the efficacy of subsonic agitation of NaOCl in reducing bacterial load in the root canal. Root canals of 112 extracted human single-root teeth were preflared using K-Flexofiles (Dentsply Maillefer, Ballaigues, Switzerland) up to #20 and then shaped using ProTaper S1-S2-F1-F2-F3 (Dentsply Maillefer) at the working length. Irrigation was performed with 33 mL of 5% NaOCl, alternating with 10 mL of 10% EDTA. After ethylene oxide sterilization, the root canals were infected with 30 mL of *E. faecalis* culture and randomly assigned to four groups (n = 25) of different irrigation regimens plus positive and negative controls. Irrigation was performed with 2 mL of 5% NaOCl. The standard irrigation groups (NaOCl 15 and 30) showed higher microbial counts than the Endo activator 30 group. Thiry seconds of NaOCl subsonic agitation with Endo activator appears to be slightly more effective in reducing bacterial load in the root canal compared with NaOCl irrigation alone.

18. **Prabhakar J et al.,**(41) in 2010 evaluated the antimicrobial efficacy of Triphala, green tea polyphenolsc(GTP), MTAD, and 5% NaOCl against *E. faecalis* biofilm formed on tooth substrate. Extracted human teeth were biomechanically prepared, vertically sectioned, placed in the tissue culture wells exposing the root canal surface to *E. faecalis* to form a biofilm. At the end of the 3rd and 6^{th} weeks all groups were treated for 10 minutes with the test solutions and control and were analyzed qualitatively and quantitatively.

Qualitative assay with 3-week biofilm showed complete inhibition of bacterial growth with Triphala, MTAD and NaOCl, except GTP and saline, which showed presence of bacterial growth. Qualitative assay with 6-week biofilm showed growth when treated with Triphala, GTP and MTAD whereas NaOCl has shown complete inhibition. All groups except NaOCl showed eight log reduction when compared to control when analyzed quantitatively. 5% NaOCl showed maximum antibacterial activity against *E. faecalis* biofilm formed on tooth substrate.

19. **Retamozo B et al.,**(42) in 2010 determined the concentration of NaOCl and the irrigation time required to disinfect dentin cylinders infected with *E. faecalis*. Four hundred fifty dentin cylinders (5 mm in diameter and 4 mm in height) with a lumen (2–3 mm in width) were prepared from freshly extracted bovine incisors. The cementum and predentin were then removed. The tubules were opened by using a 4-minute application with 17% ethylenediaminetetraacetic acid and 5.25% NaOCl and then exposed to *E. faecalis* (ATCC 4082) for 3 weeks in brain-heart infusion broth. The cylinders were then divided into 3 groups, and a 1.3%, 2.5%, or 5.25% concentration of NaOCl was applied in 5-, 10-, 15-, 20-, 25-, 30-, 35-, and 40-minute intervals for a total of 30 subgroups including positive and negative controls. The most effective irrigation regimen was 5.25% at 40 minutes, whereas irrigation with 1.3% and 2.5% NaOCl for this same time interval was ineffective in removing *E. faecalis* from infected dentin cylinders. High concentration and long exposure to NaOCl are needed for elimination of E.faecalis contaminated dentin.

20. **Wang R et al**.,(43) in 2011 evaluated the effect of an atmospheric pressure, direct current nonthermal plasma microjet on reducing the infection and

prevention of reinfection in the root canal. Forty-eight samples were equally divided into two groups: Group A was treated with gas flow (without plasma) for 2, 4, 6, and 8 min, respectively, and used as negative control. Group B was treated by CAP plasma jet for the same time periods. Results showed that 98.8% *E. faecalis* was inactivated in 8 min by PMJ. It was found that a 30-min PMJ treatment could effectively prevent the reinfection.

21. **Stojicic S et al**.,(18) in 2012 evaluated the efficacy of a novel root canal irrigant, Qmix, against *E. faecalis* and mixed plaque bacteria in planktonic phase and biofilms. *E. faecalis* and mixed plaque bacteria were exposed to Qmix, 2% CHX, MTAD and 1% NaOCl for 5s, 30s and 3 min. Qmix and NaOCl were superior to CHX and MTAD under laboratory conditions in killing *E. faecalis* and plaque bacteria in planktonic and biofilm culture.

22. **Wang Z et al**.,(44) in 2012 compared the antibacterial effects of different disinfecting solutions on young and old *E. faecalis* biofilms in dentin canals. After one day and 3 weeks of incubation, 40 infected dentin specimens were subjected to 1 and 3 minutes of exposure to 2% NaOCl, 6% NaOCl, 2% CHX and Qmix. They concluded six percent NaOCl and Qmix were the most effective disinfecting solutions against the young *E. faecalis* biofilm whereas against the 3-week-old biofilm, 6% NaOCl was the most effective followed by Qmix.

23. **Jiang C et al**.,(45) in 2012 evaluated the effect of CAP plasma jet and 5.25% NaOCl for the treatment of *E. faecalis* biofilm. A total of 27 Hydroxyapatite discs incubated with *E. faecalis* for six days were divided into three groups, the negative control group, positive control group (5.25% NaOCl) and the plasma

treatment group. The room temperature CAP plasma jet showed comparable antimicrobial effect as 5.25% NaOCl against *E. faecalis* biofilms on Hydroxyapatite discs.

24. **Meire MA et al.,**(46) in 2012 compared the antimicrobial efficacy of two high power lasers (Nd:YAG and Er:YAG) and two commercial antimicrobial photodynamic therapy (APDT) systems with that of NaOCl action on *E. faecalis* biofilms grown on dentine discs. Significant reductions in viable counts were observed for APDT (2 log reduction), ErYAG irradiation using 100 mJ pulses (4.3 log reduction) and all NaOCl treatments (>6 log reduction). NaOCl (2.5%) for 5 min effectively eliminated all bacteria. Within the limitations of this particular laboratory set-up, NaOCl was the most effective in *E. faecalis* biofilm elimination, while Er:YAG laser treatment (100 mJ pulses) also resulted in high reductions in viable counts. The use of both commercial aPDT systems resulted in a weak reduction in the number of *E. faecalis* cells. Nd:YAG irradiation was the least effective.

25. **Soares JA et al.,**(47) in 2012 aimed to evaluate the antimicrobial effectiveness of an alternating irrigation regimen during chemomechanical preparation (CMP). Canals were irrigated with saline solution (control group), with 5.25% NaOCl followed by a final rinse with 17% EDTA (conventional irrigation group), or with the alternating use of NaOCl and EDTA (alternating irrigation [AI] group). The irrigation regimen based on the alternating use of NaOCl and EDTA seems to be a promising endodontic tool because it promoted the elimination of root canal *E. faecalis* biofilms throughout the experimental period.

26. **Morgental RD et al.,**(19) in 2013 conducted an in vitro study aimed to compare the antibacterial effect of a new irrigant, Qmix with 6% NaOCl, 1% NaOCl, 2% CHX gluconate, 17% EDTA and sterilized saline solution against *E. faecalis* (ATCC 29212). After10 seconds of contact with the bacterial suspension, 6% NaOCl showed the lowest bacterial count. After 30 seconds, 6% NaOCl displayed 0 colony-forming units per milliliter, whereas 1% NaOCl and Qmix showed reduced number of colonies in comparison with the negative control. After 1 minute, both concentrations of NaOCl presented no bacterial growth and Qmix reduced the number of colonies, but EDTA and CHX had bacterial counts similar to the negative control. According to the results of this study, six percent NaOCl was the most effective irrigant against *E. faecalis*.

27. **Pan J et al**.,(48) in 2013 evaluated the efficiency of cold plasma therapy over calcium hydroxide in treating the infected root canals with *E. faecalis*. The seventy test samples were divided into seven groups and tested for efficiency of different exposure times of cold plasma over calcium hydroxide placed in the root canals as intra canal medicament for seven days. A significant decrease in the number of CFU after prolonged cold plasma treatment was observed when compared with calcium hydroxide. It was concluded that the cold plasma had a high efficiency in disinfecting the *E. faecalis* biofilms of dental root canal treatment.

28. **Du T et al.,**(49) in 2013 conducted a study that evaluated the antibacterial activity by atmospheric pressure nonequilibrium plasmas (APNPs) against bacterial biofilms in root canal systems during endodontic therapy. Sterile cover slips were placed into the wells of tissue culture plates to permit the formation of *E. faecalis* biofilms. Biofilms were treated for 5 minutes with APNPs or 2%

CHX digluconate. In addition, infected single-rooted teeth were exposed to APNPs or 2% CHX for 5, 10, and 15 minutes. Treatment for 5 minutes with APNPs or 2% CHX killed the majority of bacteria in the *E. faecalis* biofilms. Bacterial survival after treatment with APNPs or 2% CHX remarkably reduced with increasing exposure times. APNPs can be an effective adjunct to standard endodontic antimicrobial treatment.

29. **Schauddin C et al.,**(50) in 2013 conducted an in vitro study to evaluate the efficacy of a nonthermal plasma (NTP) at atmospheric pressure on ex vivo biofilm in root canals of extracted teeth. Intracanal contents from three teeth with root canal infections were collected, pooled and grown in thirty-five micro-CT mapped root canals of extracted and instrumented human teeth. One group of teeth was treated with NTP, another with 6% NaOCl and one set was left untreated. The intracanal contents from twenty-seven teeth (nine teeth in each group) were plated on agar and colony forming units were determined. Treatment with nonthermal plasma decreased the number of viable bacteria in biofilms by one order of magnitude, whilst the NaOCl control achieved a reduction of more than four magnitudes. The nonthermal plasma displayed antimicrobial activity against endodontic biofilms in root canals, but was not as effective as the use of 6% NaOCl.

30. **Habib M et al.,**(51) in 2014 conducted a study to investigate the effects of non-thermal atmospheric plasma on an *E. faecalis* biofilm within the canals of extracted human teeth. A significant decrease in number of CFU were observed after 2 minutes cold plasma treatment with an average kill rate of 99.999%. MTT (tetrazolium salt) reduction assay showed a significant reduction in the viability of bacteria with a reduction rate of 98.939%. Both 2 min cold plasma

and 6% NaOCl greatly reduced the viability and metabolic activity of *E. faecalis* of bacteria.

31. **Ureyen Kaya B et al.,**(52) in 2014 conducted an invitro study that was aimed to compare the antimicrobial efficacy of low temperature atmospheric pressure plasma (LTAPP) design and gaseous ozone delivery system with 2.5% NaOCl on *E. faecalis* in root canal walls and dentine tubules. The samples were divided into LTAPP (n = 12), ozone (n = 12), NaOCl (positive control, n = 12) and saline (negative control, n = 6) groups. The microbial sampling with paper points showed antibacterial efficacy of NaOCl, LTAPP, ozone and saline in descending order, respectively. The microbial sampling with dentin chips demonstrated a superior efficacy of LTAPP compared with NaOCl in the middle third, while both had similar effects in coronal and apical third. NaOCl and LTAPP were better than ozone at the coronal and middle parts of the root canals.

32. **Ariaz Moliz MT et al.,**(53) in 2014 evaluated the antimicrobial activity of a 2.5% NaOCl /9% etidronic acid (HEBP) irrigant solution on *E. faecalis* growing in biofilms and a dentinal tubule infection model. The highest viability was found in the distilled water group and the lowest in the NaOCl-treated dentin. Both NaOCl solutions killed 100% of the *E. faecalis* biofilms and showed the highest antimicrobial activity inside dentinal tubules, without statistical differences between the two. The HEBP isolated solution killed bacteria inside dentinal tubules but did not present any significant effect against *E. faecalis* biofilms. The incorporation of HEBP to NaOCl did not cause any loss of available chlorine within 60 minutes. HEBP did not interfere with the ability of NaOCl to kill *E. faecalis* grown in biofilms and inside dentinal tubules.

33. **Baca P et al**.,(54) in 2014 evaluated the residual antimicrobial activity of four final irrigation regimens in root canals contaminated with *E. faecalis*. Biofilms of *E. faecalis* were grown in uniradicular roots for 4 weeks. After preparing the roots chemomechanically, four final irrigation regimens were applied: (1) group EDTA-NaOCl, 17% EDTA followed by 5.25% NaOCl; (2) group MA-NaOCl, 7% maleic acid (MA) followed by 5.25% NaOCl; (3) group EDTA-CHX + CTR,17% EDTA followed by 2% CHX + 0.2% cetrimide (CTR); and (4) group MA-CHX + CTR,7% MA followed by 2% CHX + 0.2% CTR. Samples were collected for 60 days to denote the presence of bacterial growth. All root canals in which the final irrigant was 5.25% NaOCl yielded positive cultures on the fifth day. Groups EDTA-CHX + CTR and MA-CHX + CTR with a final irrigation of 2% CHX + 0.2% CTR showed respective percentages of samples without regrowth of 72.1% and 66.8% at 60 days. There were no statistically significant differences between these groups. The combination of 2% CHX + 0.2% CTR would be an effective alternative final irrigation regimen given its antimicrobial action over time.

34. **Li Y et al**.,(55) in 2015 evaluated the cold plasma treatment and safety in disinfecting 3 week old root canal *E. faecalis* biofilm. Fifty root canals were randomly divided into 8 groups of 10 teeth in which group 1 was the negative control and (groups 2-5) are treated with AC argon/oxygen (Ar/O2) cold plasma for various treatment times. Then they were compared with the positive control (groups 6-8) treated with $Ca(OH)_2$, 2% CHX gel, and $Ca(OH)_2$/CHX for a week. There were no detectable live bacteria observed after 12 minutes of cold plasma treatment. They concluded that atmospheric pressure cold plasma is an effective

therapy in endodontics for its strong sterilization effect on fully matured biofilm within a few minutes.

35. **Liu Y et al**.,(56) in 2015 compared the antimicrobial effectiveness of Qmix as a final irrigating solution. The teeth were randomly assigned into six groups EDTA/NaOCl, 17% EDTA followed by 5.25% NaOCl; EDTA/CHX, 17% EDTA followed by 2% CHX; EDTA/cetrimide (CTR), 17% EDTA followed by 2% CTR; MTAD; Qmix; and control, 0.9% saline. The antimicrobial activity of Qmix was comparable to that of EDTA/CHX and EDTA/CTR and more effective than that of EDTA/NaOCl against intracanal *E. faecalis.*

36. **Zhang C et al**.,(7) in 2015 conducted a systematic review and compared the prevalence of *E. faecalis* in primary and persistent intraradicular infections. A total of 10 studies covering 972 teeth were included. Among them, 2 studies used the culture technique, 6 studies used polymerase chain reaction, and the other 2 used both the techniques. It was concluded that detection rate of *E. faecalis* by both the methods was higher in persistent infections compared with untreated chronic periapical periodontitis as primary infection.

37. **Simoncelli E et al.,**(57) in 2015 reported the efficiency of a handheld plasma gun in the decontamination of the tooth root canals. The antibacterial efficacy of the plasma gun was first assessed on *E. faecalis* contaminated agar plates to determine optimal operating conditions that were then quantitatively evaluated treating contaminated liquid suspensions. Moreover, two different procedures for the inactivation of bacteria in realistic tooth models, resembling procedures conventionally accepted in endodontic practice were investigated. Irrigation of the contaminated tooth models with plasma activated water produced using the

plasma gun and direct activation of the contaminated tooth models to the plasma plume produced by the plasma gun. The experiments were performed with the root canal model both in wet (root canal filled with bacteria suspension) and in dry (root canal contaminated and dried) conditions. From the obtained results, the direct treatment under dry conditions turned out to be the the most effective, leading to a bacterial load mean reduction of 4.1.

38. **Herbst SR et al.,**(58) in 2015 conducted an in vitro study to analyse the bactericidal efficacy of cold plasma in different depths of infected dentin. 32 standardized root canals of human mandibular premolars were infected with *E. faecalis* and incubated for one week. Specimens were randomly selected for one of four disinfection methods: control (5mL NaCl), 5mL CHX, cold plasma alone (CP), and a combination of 5mL CHX and cold plasma (CHX+CP). CHX was ultrasonically activated for 30s, while cold plasma was used for 60s in the root canals. The highest overall logarithmic reduction factors (RF) were obtained from CHX+CP, followed by CP and CHX alone related to the control. All disinfection methods showed significantly lower CFU counts compared to the control group. The adjuvant use of CP might be beneficial in highly infected root canals to improved disinfection. However, the disinfection effect against *E. faecalis* of CP is comparable to ultrasonically activated CHX.

39. **Zhou C et al.,**(25) in 2016 conducted a study that was aimed to assess the antimicrobial activity of CAP plasma jet with helium (He) flowing through 3% hydrogen peroxide in root canals infected with *E. faecalis*. A total of 42 single-rooted anterior teeth were randomly divided into six experimental groups (including groups treated by CAP plasma jet with or without He for different time durations) and one control group treated without plasma. The greatest

reductions in CFU/ml were observed for groups which were treated by CAP plasma jet sterilization with He flowing through 3% hydrogen peroxide for 4 min or for 2 min, respectively. In conclusion, CAP plasma jet with or without He flowing through 3% hydrogen peroxide can effectively sterilized root canals infected with *E. faecalis* and should be considered as an alternative method for root canal disinfection in endodontic treatments.

40. **Chen W et al.,**(59) in 2016 designed an atmospheric cold plasma brush suitable for large area and low-temperature plasma-based sterilization is designed and used to treat *E. faecalis* bacteria. The results show that the efficiency of the inactivation process by helium plasma is dependent on applied power and exposure time. After plasma treatments, the cell structure and morphology changes can be observed by scanning electron microscopy. Optical emission measurements indicate that reactive species play a significant role in the sterilization process.

41. **Bhukhary et al.,**(60) in 2017 evaluated the antibacterial effectiveness of Octenisept, 1% alexidine and 2% CHX against *E. faecalis* biofilm using confocal laser scanning microscopy. Root dentin discs were prepared from extracted human teeth, sterilized, and inoculated with *E. faecalis* strain (ATCC 29212) to establish 3-week-old biofilm model. Dentin discs (n = 15) exposed to 5.25% NaOCl were used as a positive control, whereas specimens exposed to saline (n = 15) were used as a negative control. NaOCl had significantly greater antimicrobial activity against *E. faecalis* biofilms compared with OCT, CHX, and ALX. OCT was more effective than CHX and ALX.

42. **Hong SW et al.,**(61) in 2017 treated the structurally intact Lipoteichoic acid purified from *E. faecalis*(EfLTA) with NaOCl at various concentrations and time periods. Murine macrophage cell line RAW 264.7 was treated with interferon gamma followed by treatment with intact or NaOCl treated EfLTA to determine the inducibility of inflammatory mediators such as nitric oxide, interferon gamma–inducible protein 10, and macrophage inflammatory protein-1a. Reporter gene assays assessed by flow cytometry were used to examine the ability of intact or NaOCl treated EfLTA to activate Toll-like receptor 2 (TLR2), which is known to recognize EfLTA on host cells. Structural damage of EfLTA by NaOCl was examined using silver staining and thin layer chromatography. NaOCl-treated EfLTA showed markedly less induction of nitric oxide, interferon gamma–inducible protein 10, and macrophage inflammatory protein-1a in RAW 264.7 cells compared with intact EfLTA. In contrast to intact EfLTA that potently stimulated TLR2 activation, NaOCl-treated EfLTA did not activate TLR2. Structural analysis showed that NaOCl damaged EfLTA structure by deacylation. NaOCl deacylates the glycolipid moiety of EfLTA, which fails to activate TLR2, leading to the reduced production of inflammatory mediators.

43. **Solana C et al.,**(62) in 2017 determined the antimicrobial activity of mixed alkaline tetrasodium EDTA (EDTANa4)/NaOCl solutions with and without the addition of cetrimide (CTR) against *E. faecalis* biofilms. The antimicrobial solutions were evaluated on a 3-week biofilm of *E. faecalis* grown on radicular dentin blocks. The irrigating solutions were 2.5% NaOCl group, 20% EDTANa4 group, 10% EDTANa4 group, 2.5% NaOCl/10% EDTANa4 group, 2.5% NaOCl/5% EDTANa4 group, 2.5% NaOCl/10% EDTANa4/0.2% CTR

group, 2.5% NaOCl/5% EDTANa4/0.2% CTR group, and 0.9% saline solution group. Cell viability was determined by adenosine triphosphate assay, and culture techniques were used to determine colony-forming unit (CFU) counts. In the NaOCl groups there was no bacterial growth. The lowest antimicrobial efficacy was found for the EDTANa4 groups. Mixed alkaline EDTANa4/NaOCl solutions with and without the addition of CTR do not interfere with the antimicrobial activity of NaOCl.

44. **Hufner A et al.,**(63) in 2017 conducted an invitro study that was aimed to compare the effect of atmospheric pressure plasma (APP) with NaOCl or the combination of APP and NaOCl on *E. faecalis* biofilm in root canals of extracted human teeth. The adjunctive treatment with Plasma O_2 has a small additive, effect in the CFU reduction of an *E. faecalis* biofilm compared to the 12 min monotherapy with NaOCl. The effectiveness of Plasma O_2 could be significantly increased by longer treatment time.

45. **Ballout H et al**.,(64) in 2017 compared the effects of CAP plasma jet, dielectric barrier discharge, photodynamic therapy and NaOCl on infected curved root canals. 50 standardized curved human root canals were infected with *E. faecalis* and assigned to five groups that are negative control, CAP plasma jet (CAP I), dielectric barrier discharge (CAP II), photodynamic therapy (PDT) and NaOCl ultrasonically activated for 30 s. NaOCl was significantly more effective at reducing the Colony Forming Units (CFU) than all test groups. CFUs in PDT were significantly lower than those in CAP II, and those in CAP-I were significantly lower than those in CAP II.

46. **Vaid D et al.,** (65) in 2017 Evaluated the additive effect of photodynamic therapy (PDT) on the antibacterial activity of 2.5% NaOCl and Qmix against 6-week *E. faecalis* biofilms contaminated root canals. A 6-week *E.faecalis* (ATCC 29212) biofilm was formed in 190 extracted teeth that were subsequently subjected to following irrigation protocols. Group A1: normal saline, Group A2: 2.5% NaOCl, Group A3: Qmix, Group B1: normal saline and photoactivated disinfection (PAD), Group B2: 2.5% NaOCl and PAD, Group B3: Qmix and PAD, Group C: no irrigation. Maximum percentage of disinfection (99%) was seen in Group B2 (NaOCl with PDT), which was similar to Groups A2 (97.6%) and B3 (98.8%).

47. **Ye W et al.,**(66) in 2018 conducted an ex vivo study to investigate the anti-biofilm efficacy of root canal irrigants in canal spaces, isthmi and dentinal tubules of root canals. Fifty-one single-rooted premolars, each containing an isthmus, were instrumented, autoclaved and inoculated with *E. faecalis* for 4 weeks. One specimen was sectioned for bacteria-specific staining to confirm the presence of biofilms using light microscopy. The remaining specimens were randomly divided to five groups: (1) 0.9% NaCl, (2) SilverSol/H2O2, (3) HYBENX, (4) Qmix 2 in1, (5) 6% NaOCl. 6% NaOCl was the most effective solution in disrupting biofilms.

48. **Armand A et al.,**(67) in 2019 conducted a study that evaluated and compared the antibacterial effect of plasma and photodynamic therapy (PDT) in root canals infected with *E. faecalis*. One hundred single-rooted extracted human teeth (no treatment) were prepared and contaminated with *E. faecalis*. 60 specimens were randomly selected for three disinfection groups: He and He/O2 plasma (n=20) in 5-s groups (control, 2, 4, 6 and 8 min treatments) and PDT

(n=20) in 2-s groups (control and PDT treatment). Next, for comparison, 40 remaining teeth were divided into four groups: control, 8 min He plasma, 8 min He/O2, and PDT. He/O2 plasma was more effective against *E. faecalis*, followed by PDT and He plasma respectively.

49. **Alghamdi F et al**.,(8) in 2020 conducted a systematic review which compiled all the current studies concerning *E. faecalis* as a dental root canal pathogen that causes endodontic failure. The study included 11 articles that studied different aspects of *E. faecalis* including its prevalence, characteristics, resistance mechanisms, express survival genes, and treatment. Most of the included studies conferred the high prevalence of *E. faecalis* within the root canal systems. It presented the ability of *E. faecalis* to affect the size of the periapical lesion and the microbial load within the root canal systems during the endodontic treatment.

50. **Jungbauer G et al.,**(68) in 2021 conducted a systematic review on the antimicrobial effect of CAP plasma on dental pathogens. A database search was performed (PubMed, Embase, Scopus). Data concerning the device parameters, experimental setups and microbial cultivation were extracted. 55 studies were included (quality score 31–92%). The reduction factors varied strongly among the publications although clusters could be identified between groups of set pathogen, working gases, and treatment time intervals. A time-dependent increase of the antimicrobial effect was observed throughout the studies. CAP may be a promising alternative for antimicrobial treatment in a clinically feasible application time. Further studies, including multi-species biofilm models, are needed to specify the application parameters of CAP before CAP should be tested in randomized clinical trials.

AIMS AND OBJECTIVES

Aim:

To evaluate the disinfection effectiveness of Cold Atmospheric Pressure (CAP) plasma jet in comparison to irrigation with 5.25% NaOCl and Qmix in *E. faecalis* infected root canals at different time intervals.

Objectives:

Primary objective:

To find if any significant difference exists between CAP plasma jet, 5.25% NaOCl and Qmix in their bacterial disinfection capacity when used as a root canal irrigant in *E. faecalis* infected root canals.

Secondary Objective:

To determine the disinfection effectiveness of experimental irrigating regimens (CAP plasma jet, 5.25% NaOCl and Qmix) at different exposure times (2, 5 and 10 minutes) when tested in *E. faecalis* infected root canals.

MATERIALS AND METHODS

Study Design:

The present in-vitro study was conducted in the Department of Dentistry (AIIMS, Jodhpur) in collaboration with Department of Microbiology (AIIMS, Jodhpur) and Department of Physics (IIT, Jodhpur).

Materials:

- Human natural teeth (Mandibular premolars)
- Micromotor handpiece (Confident, India)
- Diamond discs (DFS, India)
- Normal saline (0.9%) (B|Braun group company, India)
- Pro Taper universal hand files (Dentsply, Maillefer, India)
- NaOCl (3% and 5.25%) (SafeEndo Dental India Pvt. LTD)
- EDTA (17%) [Neelkanth Healthcare (P.) LTD, India]
- Qmix (Dentsply Tulsa Dental Specialities, US)
- Barbed broach (Dentsply, Maillefer, India)
- 5ml Irrigation syringe and needle 24 gauge (ONE TOUCH Medical Products (P.) LTD)
- Composite resin (3M, ESPE, India)
- BHI (Brain Heart Infusion) broth (HI MEDIA, Mumbai, India,)
- Blood agar plates (HI MEDIA, Mumbai, India, MV073 500G)
- F3 Paper points (META BIOMED, India)
- Falcon tubes 50ml and 5 ml (Eppendorf, Mumbai, India)
- Anaerobic gas pack and jar (HI MEDIA, Mumbai, India, LE002A)

Equipments:

- Autoclave
- Anaerobic incubator
- CAP plasma jet unit

Sample collection:

Two hundred and ten human caries-free single-rooted mandibular premolar teeth with mature apices extracted for orthodontic/periodontal reasons were collected from Oral and Maxillofacial Surgery section, Department of Dentistry, AIIMS Jodhpur. A digital radiograph was taken for each tooth so as to confirm inclusion or exclusion of the sample unit according to the criteria followed in the study given below.

Inclusion criteria:

- Single rooted mandibular premolar teeth with intact crown and root
- Mandibular premolars without any carious lesion or defects.
- Teeth with intact and mature root apex.
- Degree of root curvature ≤ 25 degree.
- Teeth having single root canal with single canal orifice and single apical foramen.

Exclusion criteria:

- Teeth with caries, cracks, endodontic treatments, or restorations.
- Teeth with any calcification, extra roots and canals, internal and external resorptions, and open apices.

Sample Preparation:

Teeth were stored in NaOCl (3%) for 1 hour and were further cleaned with an ultrasonic scaler to remove any calculus and/or debris. Teeth were autoclaved for 20

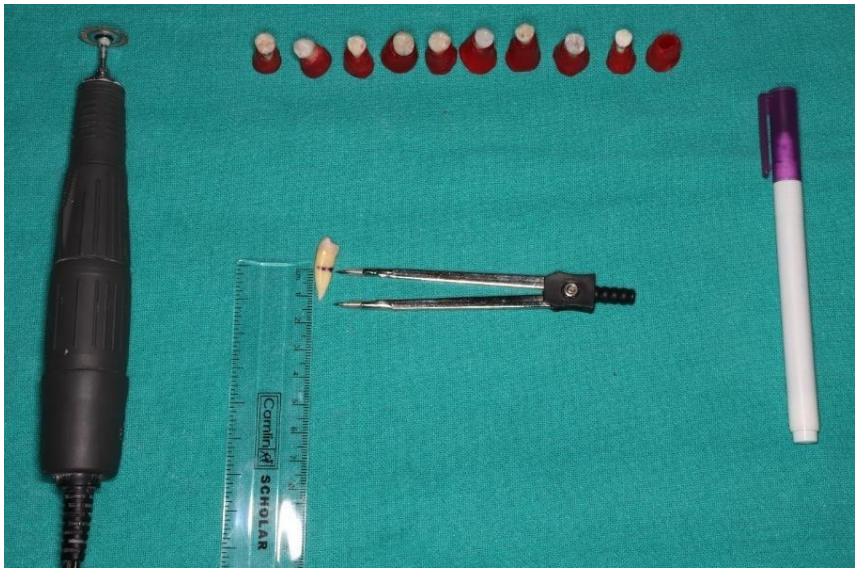


Figure 1: Armamentarium for decoronation of samples

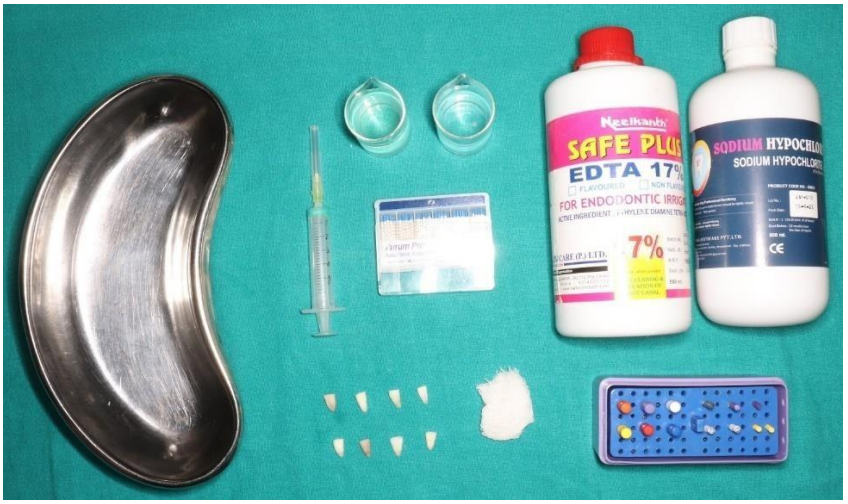


Figure 2: Armamentarium for preparation of samples

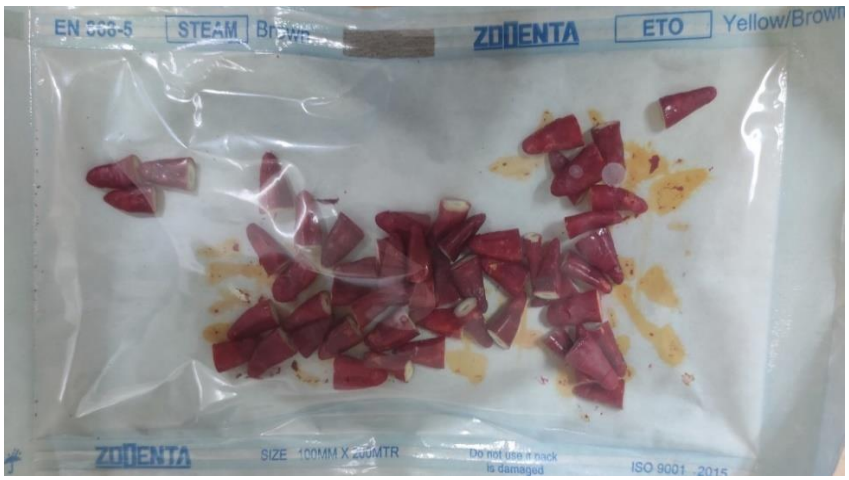


Figure 3: Autoclaved samples

minutes under 15 psi pressure at 121°C temperature, following which they were stored in 0.9% normal saline solution until used.

The teeth were decoronated below the cemento enamel junction (CEJ) to obtain a standard root length of 12 mm using a diamond disc mounted on a mandrel in a micromotor straight handpiece (Figure 1). The patency of the apical foramina was established using a size 15 K-file and then pulp tissue of the test samples was extirpated using a barbed broach. All samples were prepared with ProTaper universal hand files up to size F3. The canals were irrigated with 5 ml of NaOCl (3%) after each instrumentation with 24-gauge irrigation needle during root canal preparation. After completing the root canal preparation, canals were irrigated with NaOCl (3%) for 2 minutes followed by EDTA (17%) for 1 minute (Figure 2). Subsequent to the completion of irrigation, the apical foramen of each test sample was sealed with composite resin and outer surface of root was coated with nail varnish. The samples were sterilized again in an autoclave for 20 minutes under 15 psi pressure at 121°C temperature (Figure 3).

Division of test samples:

The test samples in the present study were randomly divided into four groups. The test samples were divided into three experimental (CAP plasma jet, 5.25% NaOCl and Qmix) groups and one control (0.9% normal saline) group. The test samples in experimental group I were further divided into 3 subgroups (n=21 each) based upon the duration of CAP plasma jet exposure in the root canals of test samples (subgroup IA=2 min, subgroup IB=5 min, subgroup IC=10 min). The test samples in experimental group II were further divided into 3 subgroups (n=21 each) based upon the duration of irrigation with 5.25% NaOCl in the root canals of test samples (subgroup IIA=2min, subgroup

Flow chart depicting the division of test samples into experimental and control groups

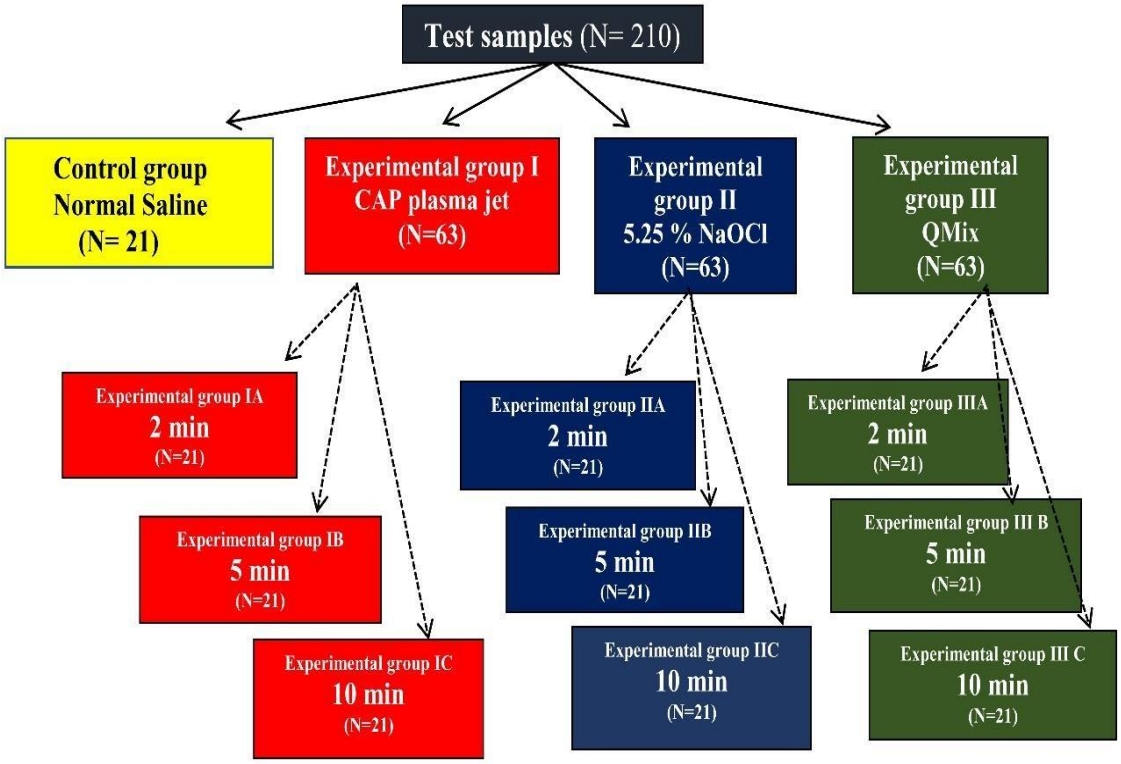


Figure 4: Division of test samples

Figure 5: Anaerobic gas pack Jar

Figure 6: Anaeraobic Incubator

IIB=5 min, subgroup IIC=10 min). The test samples in the pack jar (Figure 5) and experimental group III were also divided into 3 subgroups (n=21 each) based upon the duration of irrigation with Qmix in the root canals of test samples (subgroup IIIA=2min, subgroup IIIB=5 min, subgroup IIIC=10 min) (Figure 4).

Bacteria Culture:

The *E. faecalis* (American Type Culture Collection 29212) was cultured in a sterile 50 ml falcon tube with 30 ml brain-heart infusion (BHI) broth under aerobic condition at 37°C. The bacterial concentration used in the experiment was adjusted to 10^5 colony-forming units (CFUs)/ml.

Experimental root canal infection:

The test samples of each subgroup were immersed in 30 mL of BHI broth containing 10^5 CFUs/ml of *E. faecalis* in falcon tubes. Falcon tubes were placed in Anaerobic gas pack jar (Figure 5) and the samples were incubated anaerobically in incubator (Figure 6) for 7 days. 5 mL of sterile BHI broth was refreshed every 2 days to ensure the viability of *E.faecalis.*

Cold atmospheric pressure (CAP) plasma jet generation:

A dielectric barrier discharge CAP plasma jet consists of a SS/copper tube as the central electrode and an axially aligned SS/copper ring as the grounded electrode. A dielectric barrier (Teflon/Quartz) was used between two electrodes to reduce the flowing current and prevent electrical arching and control plasma discharge in the flow region. The produced plasma was cold at atmospheric pressure. The central electrode was connected through a low power bi-polar high-voltage source with a 5 KV peak-to-peak voltage using 0-6 KV, 1 amp, 2µm PW power source. Helium was used as a working gas at atmospheric pressure, at a flow rate of 2.5 standard litre per min. The generated CAP plasma plume was exposed via a 23 gauge needle (Figure 7) and the geometry developed was unique to extend the plasma jet length in a controlled way.

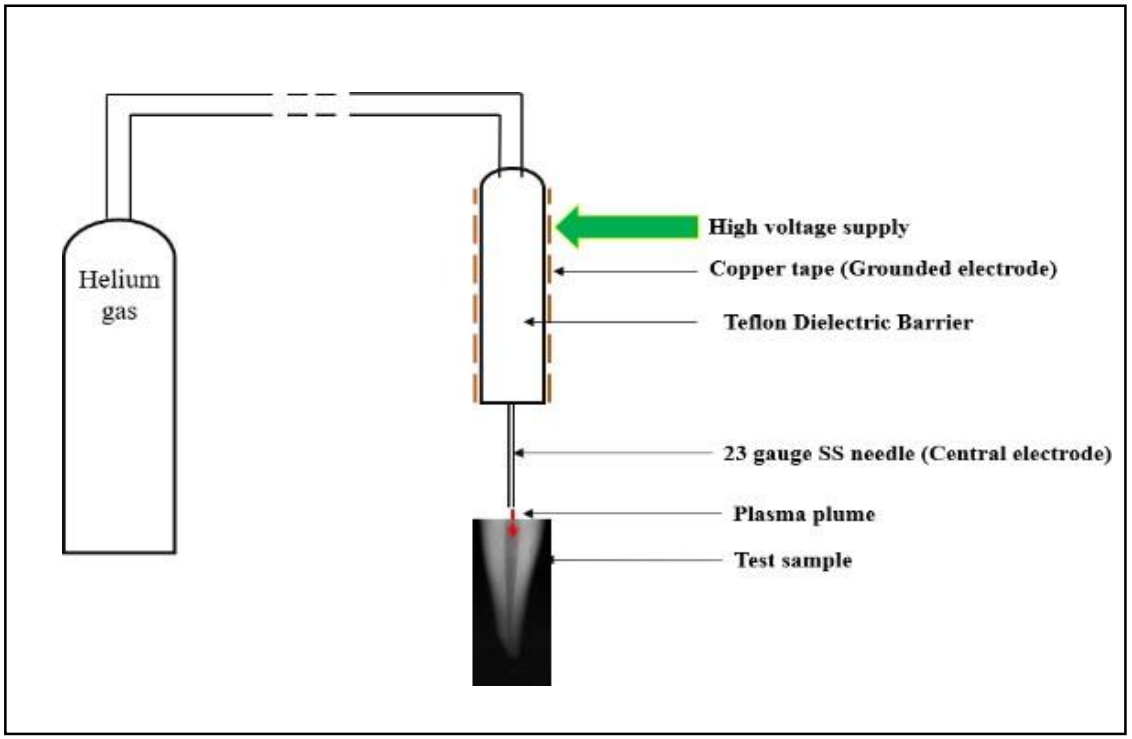


Figure 7: Generation of CAP plasma jet using Helium gas

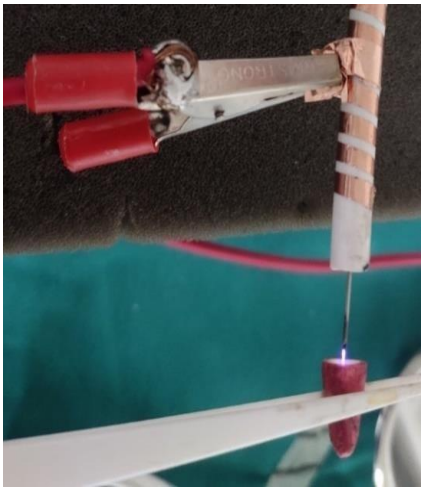


Figure 8: CAP plasma jet exposure to a sample

Figure 9: 5.25% NaOCl

Figure 10: Qmix

Figure 11: Normal saline

Assessment of Antibacterial Activity of Cold Atmospheric Pressure (CAP) plasma jet:

The test samples infected with *E.faecalis* in each subgroup of CAP plasma jet were held with a plastic tweezer to orient the coronal orifices of the root canals towards the plasma plume (Figure 8). Then the samples were adjusted to direct the plasma plume into the root canal space for respective time durations of 2, 5, and 10 minutes. A sterile barbed broach was inserted into the test samples and churned clockwise and anti-clockwise directions for 10 times to collect the dentinal mud in to the root canal space. Then F3 paper point which snugly fit inside the root canals was used to collect the dentinal mud with residual bacteria and the same process was repeated 3 times. The paper points with residual bacteria were transferred to the 5ml falcon tubes containing 3 ml of BHI broth. The falcon tubes were then shaken for 1 minute and 0.25 ml of infected BHI broth was transferred to blood agar plates for bacterial culture.

Assessment of Antibacterial Activity of 5.25% NaOCl:

A 23 gauge irrigation needle mounted on a syringe loaded with 5.25% NaOCl (Figure 9) was inserted 2 mm short of the working length and the solution was expelled slowly to fill the canals in the test samples. The experimental solution was retained in the canals for different time durations of 2, 5 and 10 minutes according to the division of subgroups. The residual bacteria were collected similarly as explained in the experimental group I.

Assessment of Antibacterial Activity of Qmix:

A 24 gauge irrigation needle mounted on a syringe loaded with Qmix (Figure 10) was inserted 2 mm short of the working length and the solution was expelled slowly to fill the canals in the test samples. The experimental solution was retained in the canals for different time durations of 2, 5 and 10 minutes according to the division of subgroups. The residual bacteria were collected similarly as explained in the experimental group I.

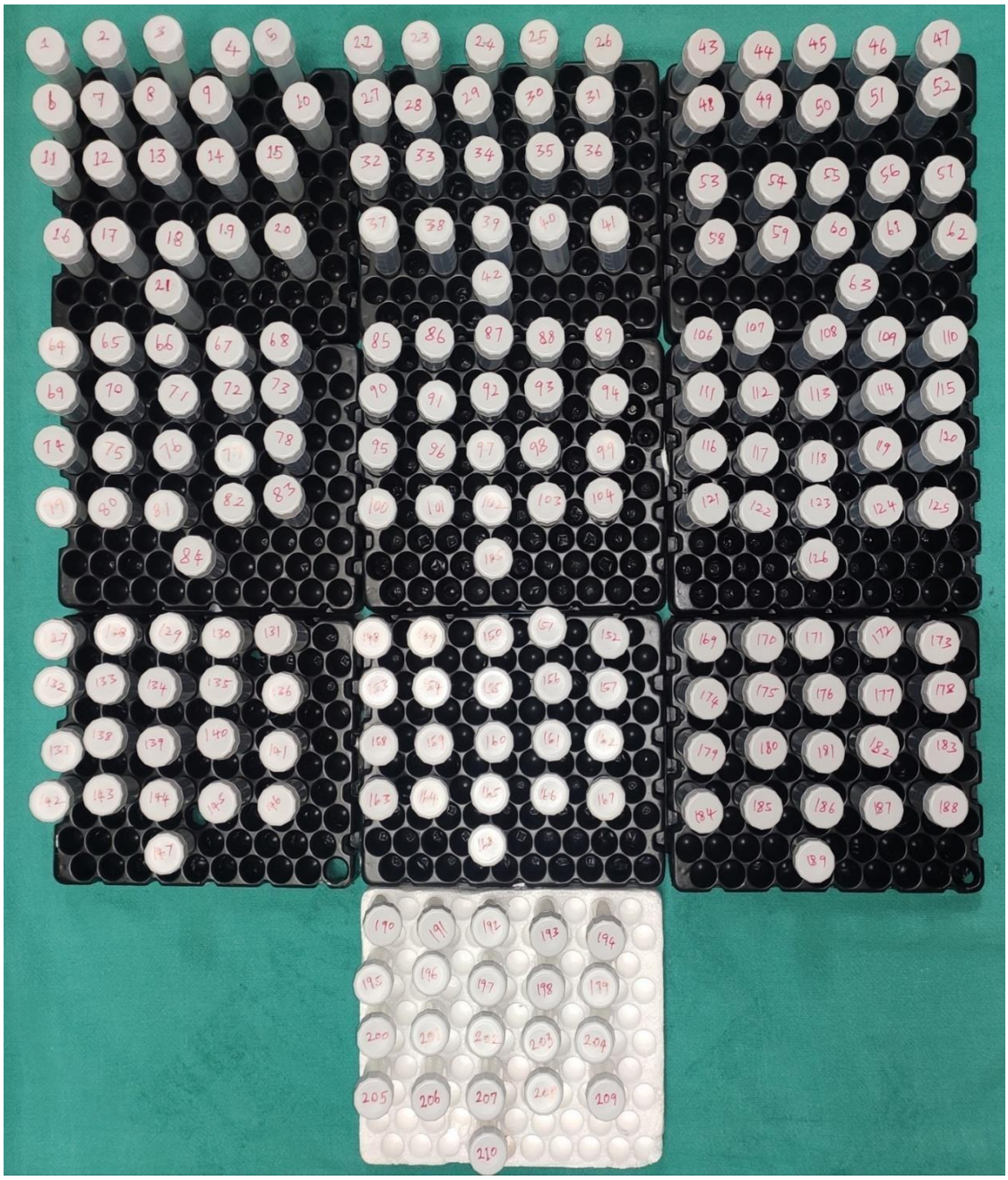


Figure 12: Falcon Tubes containing paper points with residual microorganisms submerged in BHI broth

Assessment of Antibacterial Activity of Normal saline (0.9%):

A 24 gauge irrigation needle mounted on a syringe loaded with normal saline (Figure 11) was inserted 2 mm short of the working length and the solution was expelled slowly to fill the canals in the test samples. The control solution was retained in the canals for 10 minutes. The residual bacteria were collected similarly as explained in the experimental group I.

A total of 210 falcon tubes containing the samples collected using the paper points were depicted in Figure 12.

The antimicrobial efficacy was ascertained by counting the colony forming units (CFU) so developed on the blood agar plates.

STATISTICAL ANALYSIS

The data was collected from four treatment groups referred to as Normal saline, CAP plasma jet, 5.25% NaOCl and Qmix at different time points of 2, 5 and 10 minutes.

All statistical analysis were performed using R software (version 4.0.5). At first, the count data measuring bacterial CFU was log transformed for further analysis. Next, analysis of variance (ANOVA) and Tukey's tests were performed in order to determine the significant difference between the various treatment groups. For three or more groups of data, an ANOVA is used to determine the relationship between the dependent and independent variables. A significant result in an ANOVA implies that at least two groups differ from one another, but it does not specify exactly which groups are different. Therefore, an ANOVA is usually followed by an analysis comparing all pairs of means to identify which groups exhibit a significant difference. The Tukey's test is the most common pairwise comparison test (69). The P-value of less than 0.05 was deemed to be statistically significant difference. In addition, Zero-Inflated Poisson (ZIP) model which is considered more suitable in dealing with excess zero count data was used (70). The overdispersion due to zero-inflated data can be handled by the ZIP model. The ZIP model was fitted using the *pscl* package in R software.

RESULTS

Total number of samples 210

Table 1: Number of CFU/ml observed after exposure with control and experimental irrigating regimens at different time periods.

Serial number	Normal saline N=21	CAP plasma jet N=63			5.25% NaOCl N=63			Qmix N=63		
		Exposure time			Exposure time			Exposure time		
	10 minutes N =21	2 minutes N =21	5 minutes N =21	10 minutes N =21	2 minutes N =21	5 minutes N =21	10 minutes N =21	2 minutes N =21	5 minutes N =21	10 minutes N =21
1	10^5	10^3	10	0	0	0	0	10	10	0
2	10^5	10	10^2	10^2	0	0	0	10	10	0
3	10^5	10	0	10	0	0	0	0	0	0
4	10^5	10	10	10	0	0	0	10	0	0
5	10^5	10	10	10	0	0	0	0	10^2	0
6	10^4	10^2	10	10^2	0	0	0	0	0	10
7	10^5	10^2	10	0	0	0	0	10^3	0	0
8	10^5	10	10	10	0	0	0	0	0	0
9	10^4	10^4	0	10	10	0	0	0	10	0
10	10^4	10	10^3	10	0	0	0	10	0	10
11	10^4	0	10^2	10^2	0	0	0	0	10	0
12	10^5	10^4	0	10	10	0	0	0	0	0
13	10^5	10^4	10^2	0	0	0	0	10	10	0
14	10^5	10^3	10	0	0	0	0	0	10	0
15	10^5	10^2	10^2	10	0	0	0	10	0	0
16	10^5	10^3	10	10	0	0	0	10	10	0
17	10^5	10^3	10	0	0	0	0	10^2	0	0
18	10^4	10^3	10	10	0	0	0	10	0	0
19	10^5	10^2	10^2	10^2	0	0	0	10	0	0
20	10^5	10^2	10	10	0	0	0	0	10^2	10
21	10^5	10^3	10	10	0	0	0	0	0	0

After 10 minutes of irrigation with normal saline, the number of test samples with no reduction in the CFU (10^5 CFU/ml) were 16 and 10^4 CFU count were 5 respectively. After 2 minutes of irrigation with CAP plasma jet, the number of test samples with the CFU count of 10^4 ,10^3, 10^2, 10 and 0 were 3, 6, 5, 6 and 1 respectively. After 5 minutes of irrigation with CAP plasma jet, the number of test samples with the CFU count of 10^3, 10^2, 10 and 0 were 1, 5, 12, and 3 respectively. After 10 minutes of irrigation with CAP plasma jet, the number of test samples with the CFU count of 10^2, 10 and 0 were 4, 12, and 5 respectively. After 2 minutes of irrigation with 5.25% NaOCl, the number of test samples with the CFU count of 10 were 2 and the number of test samples with the CFU count of zero were 19. After 5 and 10 minutes of irrigation with 5.25% NaOCl, all the test samples showed zero CFU count. After 2 minutes of irrigation with Qmix, the number of test samples with the CFU count of 10^3, 10^2, 10 and 0 were 1, 1 ,9 and 10 respectively. After 5 minutes of irrigation with Qmix, the number of test samples with the CFU count of 10^2, 10 and 0 were 2, 7, and 12 respectively. After 10 minutes of irrigation with Qmix, the number of test samples with the CFU count of 10 were 3 and the number of test samples with the CFU count of zero were 18 (Table 1).

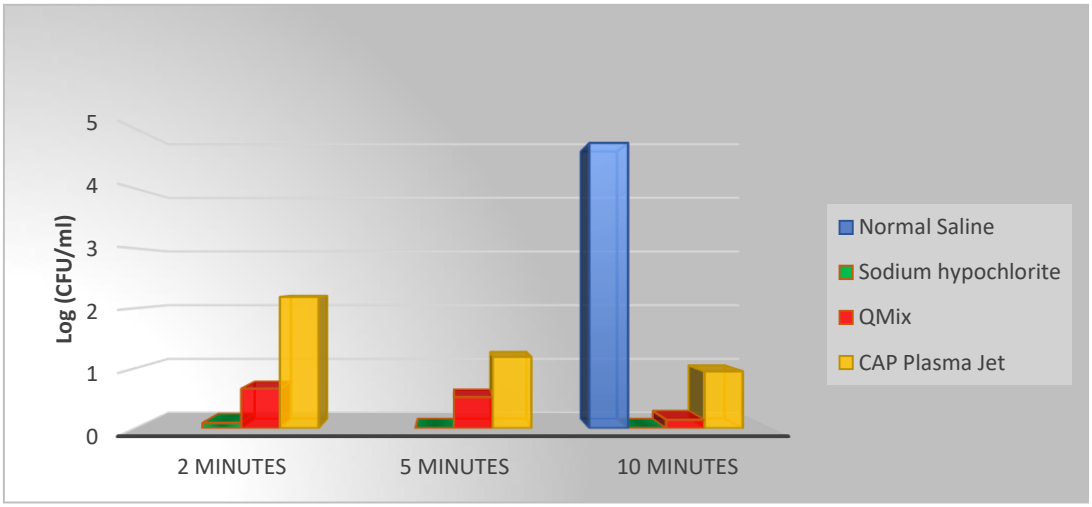


Figure 13: Graphical representation of mean CFU reduction by control and experimental groups at different time intervals.

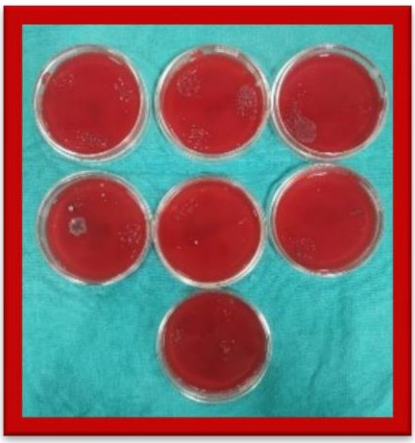


Figure 14: Culture growth in the experimental group IA

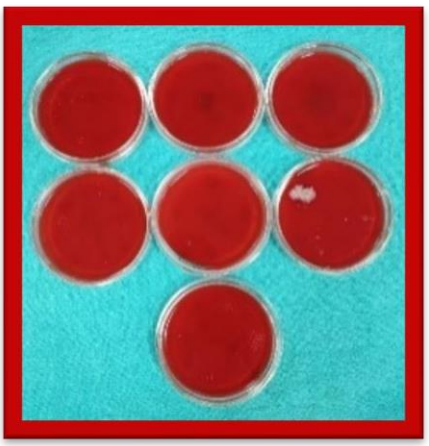


Figure 15: Culture growth in the experimental group IIA

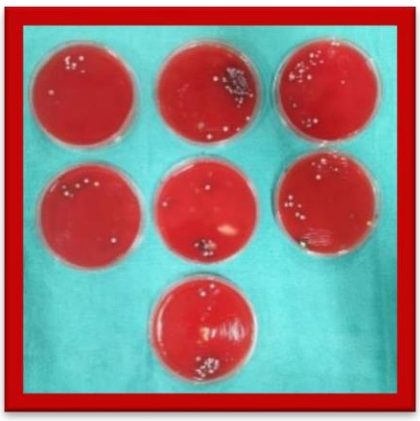


Figure 16: Culture growth in the experimental group IIIA

Table 2: Statistical analysis evaluating the effectiveness in CFU reduction by different experimental irrigating regimens exposed for 2 minutes.

S. no	Groups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	CAP plasma jet	2.19 ± 1.16	<0.001	<0.001	<0.001[a] 0.001[b]
2	5.25% NaOCl	0.09 ± 0.30			0.0753[c]
3	Qmix	0.66 ± 0.79			

a- comparison between CAP plasma jet and 5.25% NaOCl **b-** comparison between CAP plasma jet and Qmix **c-** comparison between 5.25% NaOCl and Qmix

The Mean ± SD values of Log (CFU/ml) observed after 2 minutes of irrigation with CAP plasma jet, 5.25% NaOCl and Qmix were 2.19 ± 1.16, 0.09 ± 0.30 and 0.66± 0.79 respectively. ANOVA and the zero-inflated models showed statistically significant differences ($p < 0.001$) between the treatment groups. At time period of 2 minutes, 5.25% NaOCl and Qmix showed a statistically significant difference ($p<0.001$) in CFU reduction than CAP plasma jet. There was no statistically significant difference ($p=0.07$) between 5.25% NaOCl and Qmix in CFU reduction after 2 minutes of irrigation (Table 2, Figure 13-16).

The results from the table 2 may be summarized as 5.25% NaOCl ≥ Qmix > CAP plasma jet.

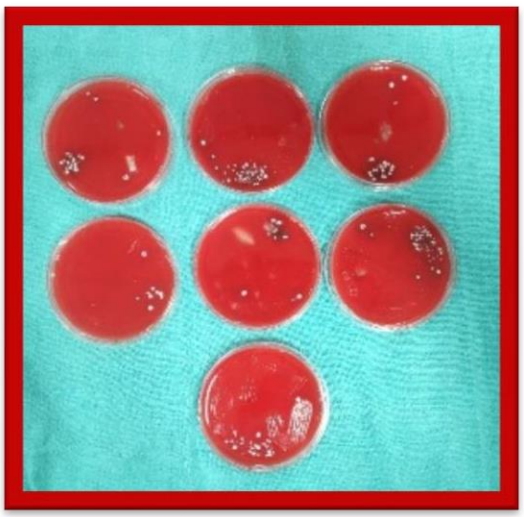


Figure 17: Culture growth in the experimental group IB

Figure 18: Culture growth in the experimental group IIB

Figure 19: Culture growth in the experimental group IIIB

Table 3: Statistical analysis evaluating the effectiveness in CFU reduction by different experimental irrigating regimens exposed for 5 minutes.

S. no	Groups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	CAP plasma jet	1.19 ± 0.74	<0.001	<0.001	<0.001[a] 0.001[b]
					0.01[c]
2	5.25% NaOCl	No CFU (0)			
3	Qmix	0.52 ± 0.67			

a- comparison between CAP plasma jet and 5.25% NaOCl **b-** comparison between CAP plasma jet and Qmix **c-** comparison between 5.25% NaOCl and Qmix

The Mean ± SD values of Log (CFU/ml) observed after 5 minutes of exposure to CAP plasma jet, 5.25% NaOCl and Qmix were 1.19 ± 0.74, 0 and 0.52 ± 0.67 respectively. ANOVA and the zero-inflated models showed statistically significant differences ($p < 0.001$) between the treatment groups. At time period of 5 minutes, 5.25% NaOCl showed a statistically significant difference ($p<0.001$) in CFU reduction than CAP plasma jet and Qmix. Qmix showed a statistically significant difference ($p<0.001$) in CFU reduction than CAP plasma jet after 5 minutes of irrigation (Table 3, Figure 17-19).

The results from the table 3 may be summarized as 5.25% NaOCl > Qmix > CAP plasma jet.

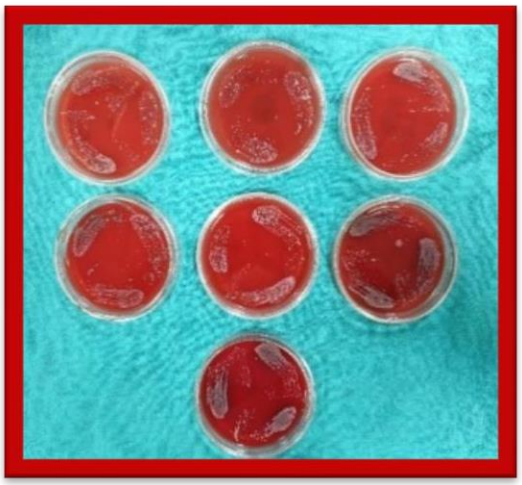


Figure 20: Culture growth in the control group

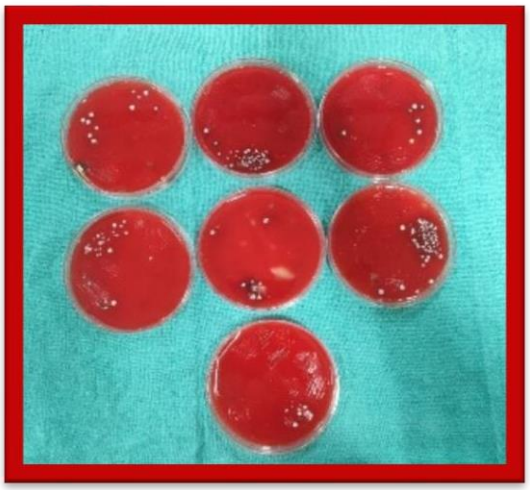


Figure 21: Culture growth in the experimental group IC

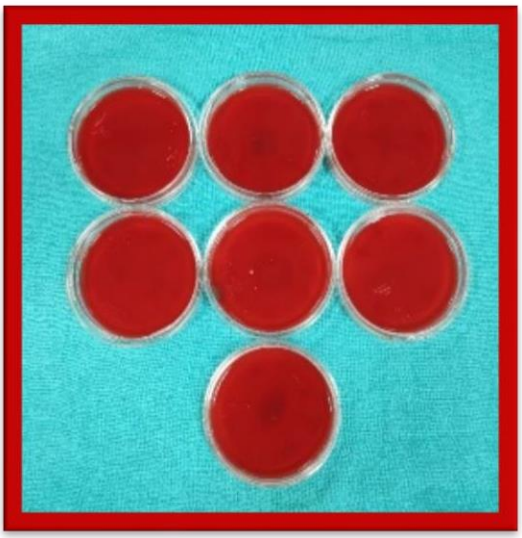


Figure 22: Culture growth in the experimental group IIC

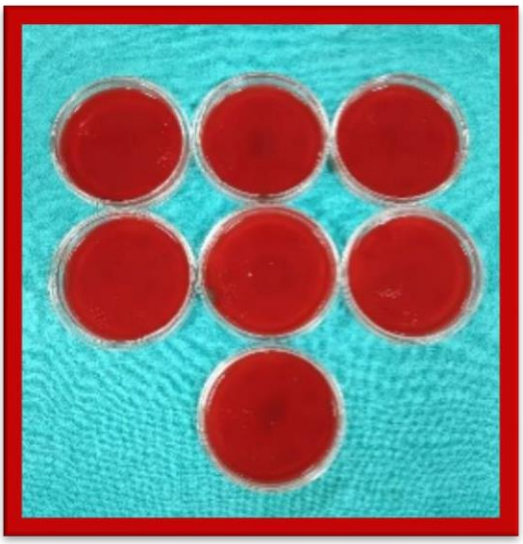


Figure 23: Culture growth in the experimental group IIIC

Table 4: Statistical analysis evaluating the effectiveness in CFU reduction by control and experimental irrigating regimens exposed for 10 minutes.

S. no	Groups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	Normal Saline	4.76 ± 0.43	<0.001	<0.001	<0.001[a] 0.001[b]
					<0.001[c]
2	CAP plasma jet	0.95 ± 0.66			<0.001[d] <0.001[e]
					0.545[f]
3	5.25% NaOCl	No CFU (0)			
4	Qmix	0.14 ± 0.35			

a- comparison between normal saline and CAP plasma jet **b-** comparison between normal saline and 5.25% NaOCl **c-** comparison between normal saline and Qmix **d-** comparison between CAP plasma jet and 5.25% NaOCl **e-** comparison between CAP plasma jet and Qmix **f-** comparison between between 5.25% NaOCl and Qmix

The Mean ± SD values of Log (CFU/ml) observed after 10 minutes of irrigation with normal saline, CAP plasma jet, 5.25% NaOCl and Qmix were 4.76 ± 0.43, 0.95 ± 0.66, 0 and 0.14 ± 0.35 respectively. ANOVA and the zero-inflated models showed statistically significant differences ($p < 0.001$) between the treatment groups. At time period of 10 minutes, all three experimental groups (CAP plasma jet, 5.25% NaOCl, and Qmix) showed a statistically significant difference($p<0.001$) in CFU reduction when compared with the control group. 5.25% NaOCl and Qmix showed a statistically significant difference ($p<0.001$) in CFU reduction than the CAP plasma jet. There was no statistically significant difference ($p=0.545$) between 5.25% NaOCl and Qmix in CFU reduction after 10 minutes of irrigation (Table 4, Figure 19-23).

The results from the table 4 may be summarized as 5.25% NaOCl ≥ Qmix > CAP plasma jet > Normal saline.

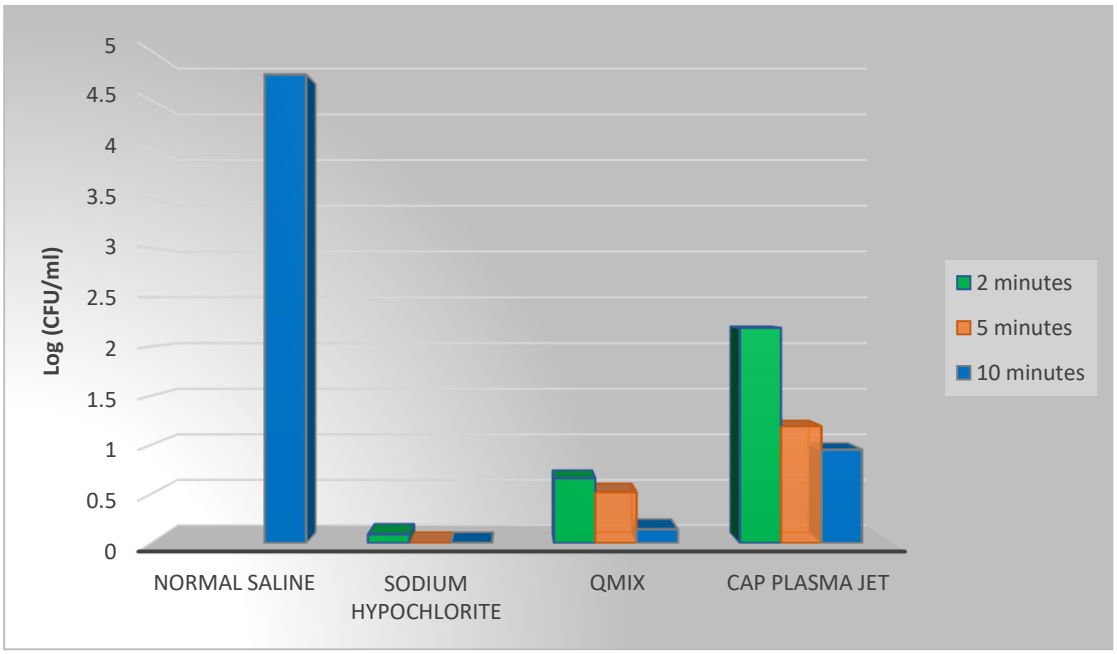


Figure 24: Graphical representation of mean CFU reduction at 2,5 and 10 minutes by control and experimental groups.

Table 5: Statistical analysis evaluating the effectiveness in CFU reduction with CAP plasma jet for different time intervals.

S. no	Subgroups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	A (2 minutes)	2.19 ± 1.16	<0.001	<0.001	0.001[a] <0.001 [b]
					0.66[c]
2	B (5 minutes)	1.19 ± 0.74			
3	C (10 minutes)	0.95 ± 0.66			

a- comparison between 2 and 5 minutes **b**- comparison between 2 and 10 minutes

c- comparison between between 5 and 10 minutes

The Mean ± SD values of Log (CFU/ml) observed after 2,5 and 10 minutes of irrigation with CAP plasma jet were 2.19 ± 1.16, 1.19 ± 0.74 and 0.95 ± 0.66 respectively. ANOVA and the zero-inflated models showed statistically significant differences ($p < 0.001$) between 2,5 and 10 minutes of irrigation with CAP plasma jet. 5 minutes of irrigation with CAP plasma jet showed a significant difference ($p=0.001$) in CFU reduction than 2 minutes of irrigation. 10 minutes of irrigation with CAP plasma jet showed a statistically significant difference ($p<0.001$) in CFU reduction than 2 minutes of irrigation. There was no statistically significant difference ($p=0.66$) in CFU reduction between 5 and 10 minutes of irrigation with CAP plasma jet (Table 5, Figure 24).

The results from the table 5 may be summarized as 10 minutes ≥ 5 minutes > 2 minutes.

Table 6: Statistical analysis evaluating the effectiveness in CFU reduction with 5.25% NaOCl for different time intervals.

S. no	Subgroups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	A (2 minutes)	0.09 ± 0.30	0.131	NA	0.186[a] 0.186[b]
					1[c]
2	B (5 minutes)	No CFU (0)			
3	C (10 minutes)	No CFU (0)			

a- comparison between 2 and 5 minutes **b**- comparison between 2 and 10 minutes

c- comparison between 5 and 10 minutes

The Mean ± SD values of Log (CFU/ml) observed after 2,5 and 10 minutes of irrigation with 5.25% NaOCl were 0.09 ± 0.30, 0 and 0 respectively. ANOVA test showed no statistically significant difference (p=0.131) between 2,5 and 10 minutes, however no CFU were seen at 5 and 10 minutes of exposure to 5.25% NaOCl (Table 6, Figure 24).

The results from the table 6 may be summarized as 10 minutes = 5 minutes ≥ 2 minutes

Table 7: Statistical analysis evaluating the effectiveness in CFU reduction with Qmix for different time intervals.

S. no	Subgroups	Mean ± SD values of Log (CFU/ml)	P value (ANOVA)	P value (Zero Inflated Poisson (ZIP) Model)	P value (Tukey's test)
1	A (2 minutes)	0.66 ± 0.79	0.0286	0.312	0.74[a] 0.02 [b]
2	B (5 minutes)	0.52 ± 0.67			0.13[c]
3	C (10 minutes)	0.14 ± 0.35			

a- comparison between 2 and 5 minutes **b**- comparison between 2 and 10 minutes

c- comparison between 5 and 10 minutes

The Mean ± SD values of Log (CFU/ml) observed after 2,5 and 10 minutes of irrigation with Qmix were 0.66 ± 0.79, 0.52 ± 0.67 and 0.14 ± 0.35 respectively. ANOVA test showed statistically significant difference (p =0.02) between 2,5 and 10 minutes of irrigation with Qmix. 5 minutes of irrigation with Qmix showed no statistically significant difference (p=0.74) in CFU reduction than 2 minutes of irrigation. 10 minutes of irrigation with Qmix showed a statistically significant difference (p=0.02) in CFU reduction than 2 minutes of irrigation. There was no statistically significant difference (p=0.13) in CFU reduction between 5 and 10 minutes of irrigation with Qmix (Table 7, Figure 24).

The results from the table 7 may be summarized as 10 minutes ≥ 5 minutes ≥ 2 minutes

DISCUSSION

The present invitro study was planned to evaluate the disinfection effectiveness of CAP plasma jet in comparison to irrigation with 5.25% NaOCl and Qmix in *E. faecalis* infected root canals at three different time intervals of 2, 5 and 10 minutes each. Normal saline was used as the control solution for 10 minutes as maximum time exposure.

E. faecalis was chosen as the test organism for this study as it is highly resistant to antimicrobial agents. *E. faecalis,* a gram-positive facultative bacterium is usually found in a biofilm in teeth with persistent periapical infection and post treatment diseases (8). The cell wall of the organism comprises of three main components including peptidoglycan, teichoic acid and polysaccharide. 40% of the cell wall is made up of peptidoglycan, while the rest of the cell wall is made up of a rhamnose containing polysaccharide and a ribitol-containing teichoic acid (71). The secretions of proteases such as serine protease, gelatinase, and collagen-binding protein aid in easy binding of *E. faecalis* to the dentin firmly. Because of these biological properties, *E. faecalis* can invade and live within dentinal tubules up to 1000 μm from the canal lumen, endure prolonged periods of starvation and recover when an adequate nutritional source is present (28). Its occurrence in root-filled teeth with peri radicular lesions was reported in a prevalence range from 24% to 77% (31). The ability of *E. faecalis* to grow as a biofilm on root canal walls and as a monoinfection in treated canals without synergistic support from other bacteria makes its elimination highly challenging (7). This microorganism seems to be the best organism to evaluate the disinfection efficiency of irrigating solutions. Numerous previous studies have also used *E. faecalis* as a test organism to evaluate the efficacy of root canal irrigating solutions and intracanal

medications (26,27,36,41,72,73). Therefore in this investigation, three active irrigation regimens were tested against *E. faecalis*.

E.Faecalis strain ATCC 29212 is an isolate from human urine (74) and was used as a control strain in this study to assess the disinfection efficiency of the irrigating regimens. This strain contains 20 out of 36 genetic loci associated with *E.faecalis* virulence including collagen adhesion protein (ace) (75). This strain was used in many studies that evaluated the antibacterial efficiency of endodontic irrigating solutions (19,76,77).

Single-rooted Mandibular preomolar teeth with curvatures less than 25 degrees were used in the present study to standardize and exclude the interference of the anatomical complexities of teeth. The degree of curvatures of the roots were calculated using schneider method. According to the schneider method, a line was drawn from the orifice (point A) parallel to the long axis of the canal, in the coronal third. The point where the first line left the long axis of the canal was considered as point B. A second line was drawn from the apical foramen (point C) to intersect the point B. The acute angle created was the Schneiders angle of root canal curvature (78). A canal that is having a curvature angle of greater than 20 degrees was considered a severely curved canal (79).

Mandibular premolars were prepared using ProTaper universal file system in this study. The ProTaper universal is a nickel titanium (NiTi) instrument manufactured with progressive tapering over the length of the cutting blades, convex triangular cross-sections, and noncutting tips. The taper angle of the file increases parabolically starting from the end (80). The File sequence to instrument the entire canal is S1, S2, SX, F1, F2 F3. Shaping files (S1, S2, SX) pre-enlarge and optimally shape the coronal 2/3 of

the canal. The multiple tapers ensure flexibility and cut dentin in specific canal zones. A brushing action creates lateral space and allows the files stronger and more efficient blades to passively move deeper into the canal. S1 is used in a brushing manner, expands the glide path, and its Eiffel Tower shape primarily pre enlarges the coronal portion of the canal. The S2 dominantly shapes the middle 1/3 and begins to expand the apical 1/3 in preparation for the first finishing file. The SX file can be used for additional coronal shaping, if needed. Finishing files (F1-F3) finish the apical 1/3. They produce the final deep shape in the canal. Each finishing file features a decreasing rate of taper that enhances flexibility, reduces the possibility of over-preparing the coronal 2/3 of a canal and reduces the potential for taper lock. More rounded tip on all ProTaper Universal finishing files and improved flexibility on the larger finishing files enable them to precisely follow the canal anatomy.

Advantages of ProTaper files includes a patented and progressive taper design that improves both flexibility and cutting efficiency, while reducing torsional loading and file fatigue. Greater cutting efficiency is achieved by the reduced contact area between the dentin and the cutting blades due to its convex triangular cross sectional design (81). A progressively changing helical angle and pitch balances each instrument, effectively reducing threading and aiding in debris removal. Studies have shown that ProTaper system maintains the canal anatomy and canal centering ability, provides good preparation shape, reduced risk of apical transportation and instrument fracture and reduced working time when compared with the other stainless steel hand files (82–85). F3 was considered as a last finishing file so as to ensure maintenance of curvature of the canal anatomy and at the same time to preserve greater radicular dentin.

NaOCl is the most common irrigating solution used in endodontics (86). NaOCl acts as an organic tissue and fat solvent, degrading fatty acids and transforming them into fatty acid salts (soap) and glycerol (alcohol), which reduces the surface tension of the solution (87). NaOCl neutralizes amino acids forming water and salt. With the exit of hydroxyl ions, there is a reduction of pH. When hypochlorous acid, a substance present in NaOCl solution, comes in contact with organic tissue it acts as a solvent and releases chlorine, which combines with the protein amino group to form chloramines. Hypochlorous acid ($HOCl^-$) and hypochlorite ions (OCl^-) lead to amino acid degradation and hydrolysis (88). The chloramination reaction between chlorine and the amino group (NH) forms chloramines that interfere in cell metabolism. Chlorine (a strong oxidant) has an antimicrobial action, inhibiting bacterial enzymes and leading to an irreversible oxidation of SH groups (sulphydryl group) of essential bacterial enzymes (89). Thus, the saponification, amino acid neutralization, and chloramination reactions that occur in the presence of microorganisms and organic tissue lead to the antimicrobial effect and tissue dissolution process. NaOCl is a very reactive oxidant that presents a well-documented dissolution and disorganization effect against biofilms (90). Hypochlorite's inability to effectively remove the smear layer from dentinal walls remains its main limitation. It was reported that smear layer harbour bacterial colonies which could also be the reason for post treatment disease in endodontics. Thus, an additional solution (EDTA) needs to be used for smear layer removal even after the use of any irrigating solution.

Qmix a relatively new irrigant has become more clinically acceptable as a single irrigating solution with both smear layer removal and anti-bacterial properties which consists of EDTA and CHX. CHX is a positively charged hydrophobic and lipophilic molecule that interacts with the negatively charged phosphate groups on microbial cell

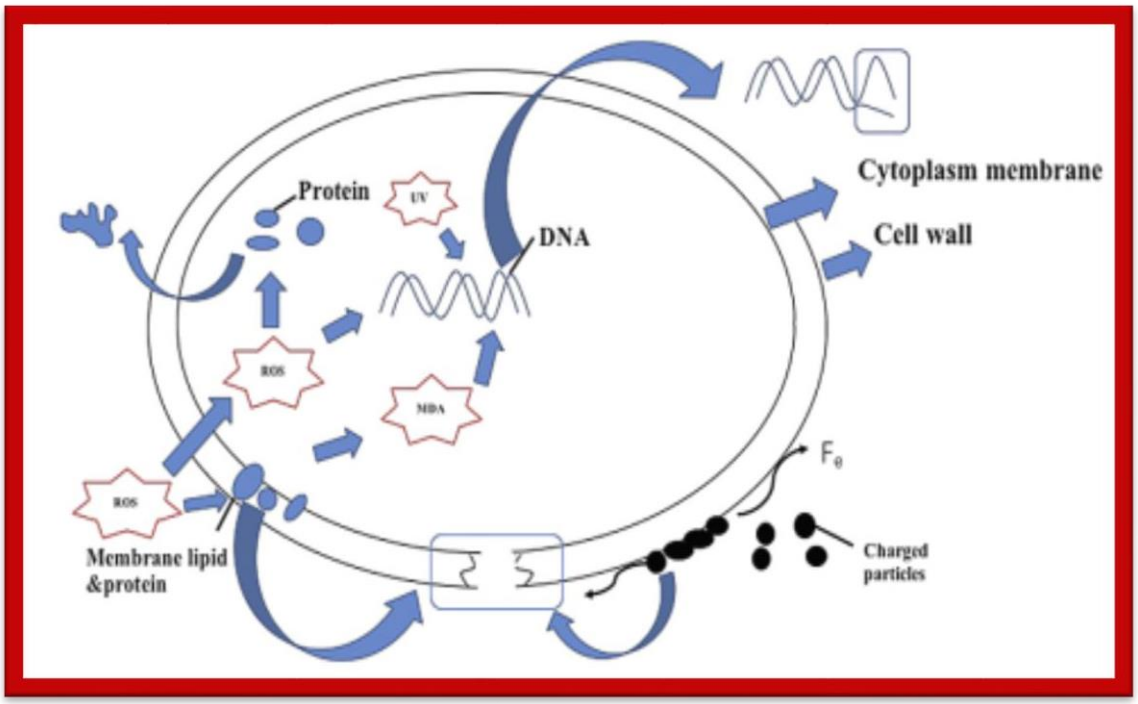


Figure 25: Microbial inactivation mechanisms of CAP plasma jet. (From Mizuno A. Destruction of biological particles using non-thermal plasma. J Clin Biochem Nutr.2017 Jan;60(1):12-24.)

walls, which alters the cells osmotic equilibrium (91). This increases the permeability of the cell wall, allowing the CHX molecule to penetrate into the bacteria. Damage to this delicate membrane causes leakage of intracellular constituents, particularly phosphate entities such as adenosine triphosphate and nucleic acids which ultimately results in cell death (92).

Cold atmospheric pressure plasma is a gas-phased alternative to conventional irrigants consisting of charged particles and chemically reactive species. The overall gas temperature is at or around the room temperature (20). Plasma-generated reactive species in the gas stream, can effectively reach the places that are hard or even impossible to reach by traditional medicaments (48). CAP plasma destroys micro-organisms by disrupting the cell wall using highly reactive free radicals, without the use of heat, chemicals or pressure (Figure 25).

CAP plasma was shown to be as effective as 6% NaOCl with a reduction of bacterial metabolic activity and viability similar to NaOCl (51). Plasma being in the gas phase can contact more areas of the root canals and reach in to the deeper parts of the canal than a solution. It was proved that the plasma diffused toward irregular surfaces, such as cracks and fissures, in order to kill living microorganisms (51). From the 3-dimensional reconstructed images of single rooted teeth treated with CAP plasma, root canal systems were found to be so complicated that more than 30% of the main root canals had several apical ramifications and lateral branches. There was no significant difference between the reduction of bacterial populations in the pure straight root canals and in the complex collateral pulp canals. This finding confirmed that the excited species generated by plasma could reach isthmuses, lateral canals, dentinal tubules (49). CAP plasma does not produce any side effects sometimes associated with NaOCl irrigation (51). The available evidence from the previous literatures suggests

that CAP plasma jet shows significant bacterial reductions at a feasible treatment timeline (68). Hence it shows potential to be tested as an alternative to conventional irrigants. Underlying mechanism of disinfection effectiveness and specific plasma microbe interactions are still not yet fully understood and discussed controversially in the literature.

Conventionally quantification of bacteria is usually performed by three methods including culture-based counting for colony forming units (CFU), spectrophotometer method of optical density (OD) measurement, and flow cytometry (FCM). CFU determination is the conventional method that has been used for decades to quantify bacteria (93). It detects those microorganisms that are able to grow on specific solid media. The low cost and ease of application makes the culture based plate counting method, the most commonly used one to determine the bacterial growth (94). It is considered as gold standard in determining the viable bacterial cells (95). But this method needs a period of incubation under suitable conditions before counting.

According to the results of this study NaOCl is the most effective irrigating solution at all the three time intervals of irrigation. The bactericidal effect of NaOCl results from the formation of hypochlorous acid (HOCl) on contact with organic matter. HOCl develops its bactericidal effect by oxidizing sulfhydryl groups in bacterial enzymes and thereby disrupting the metabolism of microorganisms, further leading to the destruction of bacterial cells. The effectiveness of 5.25% NaOCl against *E. faecalis* observed in the present study was in accordance with the previous literatures that compared CAP plasma jet and NaOCl (35,50,63,64). Few studies however have concluded that there is no significant difference between the antimicrobial action of CAP plasma jet and NaOCl (45,49).

The results of our present study was also in accordance with the previous studies that compared the antimicrobial effectiveness of NaOCl and Qmix (19,65,66). Qmix showed efficient reduction in the growth of CFU other than NaOCl at all time intervals in the present study. The antibacterial action of Qmix can be attributed to its composition which contains CHX and EDTA. The positively charged CHX molecules bind to the negatively charged phospholipids that causes rupture of the bacterial cell wall, which further results in cytoplasmic leakage and cell death. Though Qmix has shown clinically manageable levels of CFU reduction in the present study, it didn't achieve the complete bacterial elimination in the some of the tested samples at the 10 minutes of exposure time that would be counted as statistically significant. Some previous studies have reported that NaOCl is more effective than Qmix (19,65,66) in elimination of *E. faecalis* which corroborates the results of the present study. Whereas, studies by Wang et al., (44) and Stojicic et al., (18) have contradicted the results of the present study by stating that the antimicrobial effectiveness of Qmix is comparable with NaOCl. There is no comparison in the literature between Qmix and CAP plasma jet in their antimicrobial effectiveness.

In this present study 5 and 10 minutes of exposure to CAP plasma jet was significantly more effective than 2 minutes of exposure and there was no significant difference between 5 and 10 minutes of exposure with CAP plasma jet. According to the results of this study, increasing the exposure time has achieved more CFU reductions of *E. faecalis* but the significant reductions were observed only between 2 and 5 minutes of exposure time. This finding is in accordance with the study by ballout et al., (64) in 2007 who reported that there was no significant changes with prolonged application times. However, It has been found that increasing the exposure time increases the antimicrobial action of CAP plasma jet and prolonged plasma treatment of 10 minutes destroys *E. faecalis* biofilms completely (48). In our present study, at 10

minutes of exposure time CAP plasma jet showed maximum efficiency when compared with other time intervals, but did not result in complete CFU elimination. This could be due to the changes in geometry and difference in the gases used than the earlier studies. Previous studies have used Helium/Argon with oxygen as working gases however in the current study Helium was used as the only working gas. Helium/Oxygen plasma was more effective than Helium plasma, due to the presence of more reactive oxygen species in Helium/ Oxygen plasma. The lesser reduction in CFU counts by CAP plasma jet used in the present study may be attributed to this lack of oxygen utilisation as part of its geometry. In order to improve the antibacterial effectiveness of the CAP plasma jet modification in the following factors such as jet length, jet volume, flow rate of the gases can be considered.

According to the results of the present study there was no statistical difference in CFU reduction between 2, 5 and 10 minutes of irrigation with 5.25% NaOCl. NaOCl rapidly detoxifies lipoteichoic acid, a major virulence factor of *E. faecalis* in less than 1 minute of contact time (61). At 5 and 10 minutes of irrigation time NaOCl achieved complete elimination of *E. faecalis*. This finding is in accordance with a previous study which states that almost complete biofilm removal was achieved after 5 minutes of exposure to 5.25% NaOCl (35). Studies by Rematozo et al., (42) and Chau et al., (96) have also reported that the antibacterial effect of NaOCl increases with increasing exposure times.

In this present study 10 minutes of irrigation with Qmix was significantly more effective than 2 and 5 minutes of exposure and there was no statistically significant difference in CFU reductions between 2 and 5 minutes and 5 and 10 minutes of irrigation with Qmix. In some previous studies, irrigation with Qmix was done for 60-90 seconds and there were no comparisons or discussions on increasing the irrigation time with Qmix (18,19,44,56,66,97,98).

CONCLUSION

Hence it may be safely concluded from the results of the present study that

1. At all time intervals of 2,5 and 10 minutes 5.25% NaOCl is most effective in CFU reduction followed by Qmix and CAP plasma jet.
2. A minimum exposure time of 5 minutes is required for complete elimination of CFU with 5.25% NaOCl.
3. A minimum time interval of 10 minutes is required to achieve optimal CFU reduction with Qmix.
4. A minimum exposure time of 5 minutes is required to achieve substantial CFU reduction with CAP plasma jet. At 10 minutes of time interval better results were observed.

SUMMARY

Cold Atmospheric Pressure (CAP) plasma has shown successful antibacterial efficacy in different medical applications which has prompted researchers to explore its possible use in Endodontics in recent years. The present in-vitro study was aimed to evaluate the disinfection effectiveness of CAP plasma jet in comparison to irrigation with 5.25% NaOCl, Qmix and normal saline in *E. faecalis* infected root canals. 210 extracted human mandibular premolar teeth were randomly divided into 3 experimental groups (CAP plasma jet, 5.25% NaOCl, and Qmix) and 1 control group (Normal saline). The samples in the experimental groups were further divided into 3 subgroups depending on the exposure time (2, 5, and 10 minutes) of the irrigation regimens. Samples in the control group were irrigated with normal saline for 10 minutes. The test samples in each group were incubated in the BHI broth with *E. faecalis* (ATCC 29212) for one week. After root canal disinfection with the irrigation regimens, sterile paper points were used to collect the residual bacteria from the root canals. The paper points were then transferred to falcon tubes containing 3 ml of BHI broth and were further cultured on blood agar plates. The disinfection of the *E. faecalis* was evaluated by CFU counting method. Statistical analysis was performed using analysis of variance (ANOVA) and Tukey's tests to determine the significant difference between the treatment groups. At time period of 2 minutes, 5.25% NaOCl and Qmix showed a statistically significant difference ($p<0.001$) in CFU reduction than CAP plasma jet. At 5 minutes, 5.25% NaOCl showed a statistically significant difference ($p<0.001$) in CFU reduction than CAP plasma jet and Qmix. At time period of 10 minutes, all three experimental groups (CAP plasma jet, 5.25% NaOCl, and Qmix) showed a statistically significant difference($p<0.001$)

in CFU reduction when compared with the control group. 5.25% NaOCl and Qmix showed a statistically significant difference ($p<0.001$) in CFU reduction than the CAP plasma jet. The results obtained are encouraging and the newly developed CAP plasma jet has shown significant CFU reduction when compared with control group, as in line with the other experimental groups. At all time intervals of 2,5 and 10 minutes, 5.25% NaOCl is most effective in CFU reduction followed by Qmix and CAP plasma jet.

REFERENCES

1. Pereira RS, Rodrigues VAA, Furtado WT, Gueiros S, Pereira GS, Avila-Campos MJ. Microbial analysis of root canal and periradicular lesion associated to teeth with endodontic failure. Anaerobe. 2017 Dec;48:12–8.
2. Sjogren U, Figdor D, Spangberg L, Sundqvist G. The antimicrobial effect of calcium hydroxide as a short-term intracanal dressing. Int Endod J. 1991 May;24(3):119–25.
3. Kakoli P, Nandakumar R, Romberg E, Arola D, Fouad AF. The Effect of Age on Bacterial Penetration of Radicular Dentin. J Endod. 2009 Jan 1;35(1):78–81.
4. Kaur N. Hermetic Seal in Obturation: An Achievable Goal with Recently Introduced Cpoint. Int J Clin Pediatr Dent. 2019 Oct;12(5):410–3.
5. Kumar A, Alam A, Rani M, Ehtesham NZ, Hasnain SE. Biofilms: Survival and defense strategy for pathogens. Int J Med Microbiol. 2017 Dec;307(8):481–9.
6. Sakamoto M, Siqueira JF, Rôças IN, Benno Y. Bacterial reduction and persistence after endodontic treatment procedures. Oral Microbiol Immunol. 2007 Feb;22(1):19–23.
7. Zhang C, Du J, Peng Z. Correlation between Enterococcus faecalis and Persistent Intraradicular Infection Compared with Primary Intraradicular Infection: A Systematic Review. J Endod. 2015 Aug;41(8):1207–13.
8. Alghamdi F, Shakir M. The Influence of Enterococcus faecalis as a Dental Root Canal Pathogen on Endodontic Treatment: A Systematic Review. Cureus. 2020 Mar 13;
9. Hubble TS, Hatton JF, Nallapareddy SR, Murray BE, Gillespie MJ. Influence of Enterococcus faecalis proteases and the collagen-binding protein, Ace, on

adhesion to dentin. Oral Microbiol Immunol. 2003 Apr;18(2):121–6.

10. Metzger Z, Solomonov M, Kfir A. The role of mechanical instrumentation in the cleaning of root canals. Endod Top. 2013 Sep;29(1):87–109.
11. Arruda JAA de, Schuch LF, Pereira A, Monteiro JLGC, Melo-Junior PMR, Mesquita RA, et al. Investigation of different sodium hypochlorite volumes, concentrations and times of irrigation in endodontic therapy: a systematic review. Arch Heal Investig. 2019 Jul 8;8(4).
12. Mohammadi Z SS. Antimicrobial activity of sodium hypochlorite in endodontics. J Mass Dent Soc Spring. 2013;62((1)):28–31.
13. Siqueira JF, Machado AG, Silveira RM, Lopes HP, Uzeda M. Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of Enterococcus faecalis from the root canal,in vitro. Int Endod J. 2003 Oct 30;30(4):279–82.
14. Kandaswamy D, Venkateshbabu N. Root canal irrigants. J Conserv Dent. 2010;13(4):256.
15. Arslan D, Guneser MB, Kustarci A, Er K, Siso SH. Pulp tissue dissolution capacity of QMix 2 in1 irrigation solution. Eur J Dent. 2015 Jul 4;09(03):423–7.
16. Rasimick BJ, Nekich M, Hladek MM, Musikant BL, Deutsch AS. Interaction between Chlorhexidine Digluconate and EDTA. J Endod. 2008 Dec;34(12):1521–3.
17. Dai L, Khechen K, Khan S, Gillen B, Loushine BA, Wimmer CE, et al. The Effect of QMix, an Experimental Antibacterial Root Canal Irrigant, on Removal of Canal Wall Smear Layer and Debris. J Endod. 2011 Jan;37(1):80–4.
18. Stojicic S, Shen Y, Qian W, Johnson B, Haapasalo M. Antibacterial and smear layer removal ability of a novel irrigant, QMiX. Int Endod J. 2012

Apr;45(4):363–71.

19. Morgental RD, Singh A, Sappal H, Kopper PMP, Vier-Pelisser FV, Peters OA. Dentin Inhibits the Antibacterial Effect of New and Conventional Endodontic Irrigants. J Endod. 2013 Mar;39(3):406–10.
20. Xinpei Lu, Yinguang Cao, Ping Yang, Qing Xiong, Zilan Xiong, Yubin Xian, et al. An RC Plasma Device for Sterilization of Root Canal of Teeth. IEEE Trans Plasma Sci. 2009 May;37(5):668–73.
21. Ermolaeva SA, Sysolyatina E V., Gintsburg AL. Atmospheric pressure nonthermal plasmas for bacterial biofilm prevention and eradication. Biointerphases. 2015 Jun;10(2):029404.
22. Hoffmann C, Berganza C, Zhang J. Cold Atmospheric Plasma: methods of production and application in dentistry and oncology. Med Gas Res. 2013;3(1):21.
23. Brany D, Dvorska D, Halasova E, Skovierova H. Cold Atmospheric Plasma: A Powerful Tool for Modern Medicine. Int J Mol Sci. 2020 Apr 22;21(8):2932.
24. Stoffels E, Flikweert AJ, Stoffels WW, Kroesen GMW. Plasma needle: a non-destructive atmospheric plasma source for fine surface treatment of biomaterials. Plasma Sources Sci Technol. 2002 Nov 1;11(4):383–8.
25. Zhou XC, Li YL, Liu DX, Cao YG, Lu XP. Bactericidal effect of plasma jet with helium flowing through 3% hydrogen peroxide against Enterococcus faecalis. Exp Ther Med. 2016 Nov;12(5):3073–7.
26. Siqueira J, Rocas I, Favieri A, Lima K. Chemomechanical Reduction of the Bacterial Population in the Root Canal after Instrumentation and Irrigation with 1%, 2.5%, and 5.25% Sodium Hypochlorite. J Endod. 2000 Jun;26(6):331–4.
27. Gomes BPFA, Ferraz CCR, M. E. V, Berber VB, Teixeira FB, Souza-Filho FJ.

In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. Int Endod J. 2001 Sep;34(6):424–8.

28. Kayaoglu G, Orstavik D. Virulence Factors of *Enterococcus faecalis:* Relationship to Endodontic Disease. Crit Rev Oral Biol Med. 2004 Sep 11;15(5):308–20.

29. Radcliffe CE, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis. Int Endod J. 2004 Jul;37(7):438–46.

30. Sena NT, Gomes BPFA, Vianna ME, Berber VB, Zaia AA, Ferraz CCR, et al. In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms. Int Endod J. 2006 Nov;39(11):878–85.

31. Stuart C, Schwartz S, Beeson T, Owatz C. Enterococcus faecalis: Its Role in Root Canal Treatment Failure and Current Concepts in Retreatment. J Endod. 2006 Feb;32(2):93–8.

32. Berber VB, Gomes BPFA, Sena NT, Vianna ME, Ferraz CCR, Zaia AA, et al. Efficacy of various concentrations of NaOCl and instrumentation techniques in reducing Enterococcus faecalis within root canals and dentinal tubules. Int Endod J. 2006 Jan 3;39(1):10–7.

33. Kho P, Baumgartner JC. A Comparison of the Antimicrobial Efficacy of NaOCl/Biopure MTAD versus NaOCl/EDTA against Enterococcus faecalis. J Endod. 2006 Jul;32(7):652–5.

34. Krause TA, Liewehr FR, Hahn C-L. The Antimicrobial Effect of MTAD,

Sodium Hypochlorite, Doxycycline, and Citric Acid on Enterococcus faecalis. J Endod. 2007 Jan;33(1):28–30.

35. Giardino L, Ambu E, Savoldi E, Rimondini R, Cassanelli C, Debbia E. Comparative Evaluation of Antimicrobial Efficacy of Sodium Hypochlorite, MTAD, and Tetraclean Against Enterococcus faecalis Biofilm. J Endod. 2007 Jul;33(7):852–5.

36. Baumgartner JC, Johal S, Marshall JG. Comparison of the Antimicrobial Efficacy of 1.3% NaOCl/BioPure MTAD to 5.25% NaOCl/15% EDTA for Root Canal Irrigation. J Endod. 2007 Jan;33(1):48–51.

37. Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P. Enterococcus faecalis Biofilms Eradication by Root Canal Irrigants. J Endod. 2009 May;35(5):711–4.

38. Zhou X, Xiong Z, Cao Y, Lu X, Liu D. The Antimicrobial Activity of an Atmospheric-Pressure Room-Temperature Plasma in a Simulated Root-Canal Model Infected With Enterococcus Faecalis. IEEE Trans Plasma Sci [Internet]. 2010 Dec;38(12):3370–4.

39. Ozdemir HO, Buzoglu HD, Calt S, Stabholz A, Steinberg D. Effect of Ethylenediaminetetraacetic Acid and Sodium Hypochlorite Irrigation on Enterococcus faecalis Biofilm Colonization in Young and Old Human Root Canal Dentin: In Vitro Study. J Endod. 2010 May;36(5):842–6.

40. Pasqualini D, Cuffini AM, Scotti N, Mandras N, Scalas D, Pera F, et al. Comparative Evaluation of the Antimicrobial Efficacy of a 5% Sodium Hypochlorite Subsonic-activated Solution. J Endod. 2010 Aug;36(8):1358–60.

41. Prabhakar J, Senthilkumar M, Priya MS, Mahalakshmi K, Sehgal PK, Sukumaran VG. Evaluation of Antimicrobial Efficacy of Herbal Alternatives

(Triphala and Green Tea Polyphenols), MTAD, and 5% Sodium Hypochlorite against Enterococcus faecalis Biofilm Formed on Tooth Substrate: An In Vitro Study. J Endod. 2010 Jan;36(1):83–6.

42. Retamozo B, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. Minimum Contact Time and Concentration of Sodium Hypochlorite Required to Eliminate Enterococcus faecalis. J Endod. 2010 Mar;36(3):520–3.

43. Wang R, Zhou H, Sun P, Wu H, Pan J, Zhu W, et al. The Effect of an Atmospheric Pressure, DC Nonthermal Plasma Microjet on Tooth Root Canal, Dentinal Tubules Infection and Reinfection Prevention. Plasma Med [Internet]. 2011;1(2):143–55.

44. Wang Z, Shen Y, Haapasalo M. Effectiveness of Endodontic Disinfecting Solutions against Young and Old Enterococcus faecalis Biofilms in Dentin Canals. J Endod. 2012 Oct;38(10):1376–9.

45. Jiang C, Schaudinn C, Jaramillo DE, Webster P, Costerton JW. In Vitro Antimicrobial Effect of a Cold Plasma Jet against Enterococcus faecalis Biofilms. ISRN Dent. 2012 Jan 24;2012:1–6.

46. Meire MA, Coenye T, Nelis HJ, De Moor RJG. Evaluation of Nd:YAG and Er:YAG irradiation, antibacterial photodynamic therapy and sodium hypochlorite treatment on Enterococcus faecalis biofilms. Int Endod J. 2012 May;45(5):482–91.

47. Soares JA, Roque de Carvalho MA, Cunha Santos SM, Mendonça RMC, Ribeiro-Sobrinho AP, Brito-Júnior M, et al. Effectiveness of Chemomechanical Preparation with Alternating Use of Sodium Hypochlorite and EDTA in Eliminating Intracanal Enterococcus faecalis Biofilm. J Endod. 2010 May;36(5):894–8.

48. Pan J, Sun K, Liang Y, Sun P, Yang X, Wang J, et al. Cold Plasma Therapy of a Tooth Root Canal Infected with Enterococcus faecalis Biofilms In Vitro. J Endod. 2013 Jan;39(1):105–10.

49. Du T, Ma J, Yang P, Xiong Z, Lu X, Cao Y. Evaluation of Antibacterial Effects by Atmospheric Pressure Nonequilibrium Plasmas against Enterococcus faecalis Biofilms In Vitro. J Endod. 2012 Apr;38(4):545–9.

50. Schaudinn C, Jaramillo D, Freire MO, Sedghizadeh PP, Nguyen A, Webster P, et al. Evaluation of a nonthermal plasma needle to eliminate ex vivo biofilms in root canals of extracted human teeth. Int Endod J. 2013 Oct;46(10):930–7.

51. Habib M, Hottel TL, Hong L. Antimicrobial effects of non-thermal atmospheric plasma as a novel root canal disinfectant. Clin Plasma Med. 2014 Jul;2(1):17–21.

52. Ureyen Kaya B, Kececi AD, Guldaş HE, Cetin ES, Ozturk T, Oksuz L, et al. Efficacy of endodontic applications of ozone and low-temperature atmospheric pressure plasma on root canals infected with Enterococcus faecalis. Lett Appl Microbiol. 2014 Jan;58(1):8–15.

53. Arias-Moliz MT, Ordinola-Zapata R, Baca P, Ruiz-Linares M, Ferrer-Luque CM. Antimicrobial Activity of a Sodium Hypochlorite/Etidronic Acid Irrigant Solution. J Endod. 2014 Dec;40(12):1999–2002.

54. Baca P, Mendoza-Llamas ML, Arias-Moliz MT, González-Rodríguez MP, Ferrer-Luque CM. Residual Effectiveness of Final Irrigation Regimens on Enteroccus faecalis–infected Root Canals. J Endod. 2011 Aug;37(8):1121–3.

55. Li Y, Sun K, Ye G, Liang Y, Pan H, Wang G, et al. Evaluation of Cold Plasma Treatment and Safety in Disinfecting 3-week Root Canal Enterococcus faecalis Biofilm In Vitro. J Endod. 2015 Aug;41(8):1325–30.

56. Liu Y, Guo L, Li Y, Guo X, Wang B, Wu L. In vitro comparison of antimicrobial effectiveness of QMix and other final irrigants in human root canals. Sci Rep. 2016 Nov 3;5(1):17823.

57. Simoncelli E, Barbieri D, Laurita R, Liguori A, Stancampiano A, Viola L, et al. Preliminary investigation of the antibacterial efficacy of a handheld Plasma Gun source for endodontic procedures. Clin Plasma Med. 2015 Dec;3(2):77–86.

58. Herbst SR, Hertel M, Ballout H, Pierdzioch P, Weltmann KD, Wirtz HC, et al. Bactericidal Efficacy of Cold Plasma at Different Depths of Infected Root Canals In Vitro. Open Dent J. 2015 Dec 31;9(1):486–91.

59. Chen W, Huang J, Du N, Liu XD, Wang XQ, Lv GH, et al. Treatment of enterococcus faecalis bacteria by a helium atmospheric cold plasma brush with oxygen addition. J Appl Phys. 2012 Jul;112(1):013304.

60. Bukhary S, Balto H. Antibacterial Efficacy of Octenisept, Alexidine, Chlorhexidine, and Sodium Hypochlorite against Enterococcus faecalis Biofilms. J Endod. 2017 Apr;43(4):643–7.

61. Hong SW, Baik JE, Kang S-S, Kum K-Y, Yun C-H, Han SH. Sodium Hypochlorite Inactivates Lipoteichoic Acid of Enterococcus faecalis by Deacylation. J Endod. 2016 Oct;42(10):1503–8.

62. Solana C, Ruiz-Linares M, Baca P, Valderrama MJ, Arias-Moliz MT, Ferrer-Luque CM. Antibiofilm Activity of Sodium Hypochlorite and Alkaline Tetrasodium EDTA Solutions. J Endod. 2017 Dec;43(12):2093–6.

63. Hufner A, Steffen H, Holtfreter B, Schluter R, Duske K, Matthes R, et al. Effects of Non-Thermal Atmospheric Pressure Plasma and Sodium Hypochlorite Solution on Enterococcus faecalis Biofilm: An Investigation in Extracted Teeth. Plasma Process Polym. 2017 Mar;14(3):1600064.

64. Ballout H, Hertel M, Doehring J, Kostka E, Hartwig S, Paris S, et al. Effects of plasma jet, dielectric barrier discharge, photodynamic therapy and sodium hypochlorite on infected curved root canals. J Biophotonics. 2018 Mar;11(3):e201700186.

65. Vaid D, Shah N, Kothari D, Bilgi P. Additive effect of photoactivated disinfection on the antibacterial activity of QMix 2 in1 against 6-week Enterococcus faecalis biofilms: An in vitro study. J Conserv Dent. 2017;20(1):41.

66. Ye W, Fan B, Purcell W, Meghil MM, Cutler CW, Bergeron BE, et al. Anti-biofilm efficacy of root canal irrigants against in-situ Enterococcus faecalis biofilms in root canals, isthmuses and dentinal tubules. J Dent. 2018 Dec;79:68–76.

67. Armand A, Khani M, Asnaashari M, AliAhmadi A, Shokri B. Comparison study of root canal disinfection by cold plasma jet and photodynamic therapy. Photodiagnosis Photodyn Ther. 2019 Jun;26:327–33.

68. Jungbauer G, Moser D, Müller S, Pfister W, Sculean A, Eick S. The Antimicrobial Effect of Cold Atmospheric Plasma against Dental Pathogens—A Systematic Review of In-Vitro Studies. Antibiotics. 2021 Feb 20;10(2):211.

69. 4. Pairwise Comparisons. In: Pitman's Measure of Closeness. Society for Industrial and Applied Mathematics; 1993. p. 101–34.

70. Lambert D. Zero-Inflated Poisson Regression, with an Application to Defects in Manufacturing. Technometrics. 1992 Feb;34(1):1.

71. Ali L, Goraya M, Arafat Y, Ajmal M, Chen J-L, Yu D. Molecular Mechanism of Quorum-Sensing in Enterococcus faecalis: Its Role in Virulence and Therapeutic Approaches. Int J Mol Sci. 2017 May 3;18(5):960.

72. Shabahang S, Torabinejad M. Effect of MTAD on Enterococcus faecalis–Contaminated Root Canals of Extracted Human Teeth. J Endod. 2003 Sep;29(9):576–9.

73. Schafer E, Bossmann K. Antimicrobial Efficacy of Chlorhexidine and Two Calcium Hydroxide Formulations Against Enterococcus faecalis. J Endod. 2005 Jan;31(1):53–6.

74. Andrewes FW, Horder TJ. a Study of the Streptococci Pathogenic for Man. Lancet. 1906;168(4333):708–13.

75. Kim EB, Kopit LM, Harris LJ, Marco ML. Draft Genome Sequence of the Quality Control Strain Enterococcus faecalis ATCC 29212. J Bacteriol. 2012 Nov;194(21):6006–7.

76. Mallya L, Shenoy R, Mala K, Shenoy S. Evaluation of the antimicrobial efficacy of 20% Punica granatum, 0.2% chlorhexidine gluconate, and 2.5% sodium hypochlorite used alone or in combinations against Enterococcus faecalis: An in-vitro study. J Conserv Dent. 2019;22(4):367.

77. Kajwadkar R, Shin JM, Lin G-H, Fenno JC, Rickard AH, Kapila YL. High-purity Nisin Alone or in Combination with Sodium Hypochlorite Is Effective against Planktonic and Biofilm Populations of Enterococcus faecalis. J Endod. 2017 Jun;43(6):989–94.

78. Dhingra A, Kakar S. Shaping Potential of Manual NiTi K-File and Rotary ProTaper and Analyzing the Final Outcome of Shaped Canals using CT. J Contemp Dent Pract. 2013 Jun;14(3):451–5.

79. Qiao X, Xu T, Chen L, Yang D. Analysis of Root Canal Curvature and Root Canal Morphology of Maxillary Posterior Teeth in Guizhou, China. Med Sci Monit. 2021 Jan 13;27.

80. Celik G, Ozdemir Kısacık F, Yılmaz EF, Mersinlioglu A, Ertugrul IF, Orhan H. A comparative study of root canal shaping using ProTaper universal and ProTaper next rotary files in preclinical dental education. PeerJ. 2019 Aug 19;7:e7419.

81. Tavanafar S, Gilani PV, Saleh AM, Schafer E. Shaping Ability of ProTaper Universal, ProTaper NEXT and WaveOne Primary in Severely Curved Resin Blocks. J Contemp Dent Pract. 2019 Mar;20(3):363–9.

82. Vaudt J, Bitter K, Neumann K, Kielbassa AM. Ex vivo study on root canal instrumentation of two rotary nickel-titanium systems in comparison to stainless steel hand instruments. Int Endod J. 2009 Jan;42(1):22–33.

83. Peters OA, Peters CI, Schonenberger K, Barbakow F. ProTaper rotary root canal preparation: effects of canal anatomy on final shape analysed by micro CT. Int Endod J. 2003 Feb;36(2):86–92.

84. Javaheri H, Javaheri G. A Comparison of Three Ni-Ti Rotary Instruments in Apical Transportation. J Endod. 2007 Mar;33(3):284–6.

85. Azar NG, Ebrahimi G. Apically-Extruded Debris Using The ProTaper System. Aust Endod J. 2005 Apr;31(1):21–3.

86. Clarkson RM, Moule AJ. Sodium hypochlorite and its use as an endodontic irrigant. Aust Dent J. 1998 Aug;43(3):250–6.

87. Haapasalo M, Shen Y, Wang Z, Gao Y. Irrigation in endodontics. Br Dent J. 2014 Mar 21;216(6):299–303.

88. Mohammadi Z. Sodium hypochlorite in endodontics: an update review. Int Dent J. 2008 Dec;58(6):329–41.

89. Zehnder M. Root Canal Irrigants. J Endod. 2006 May;32(5):389–98.

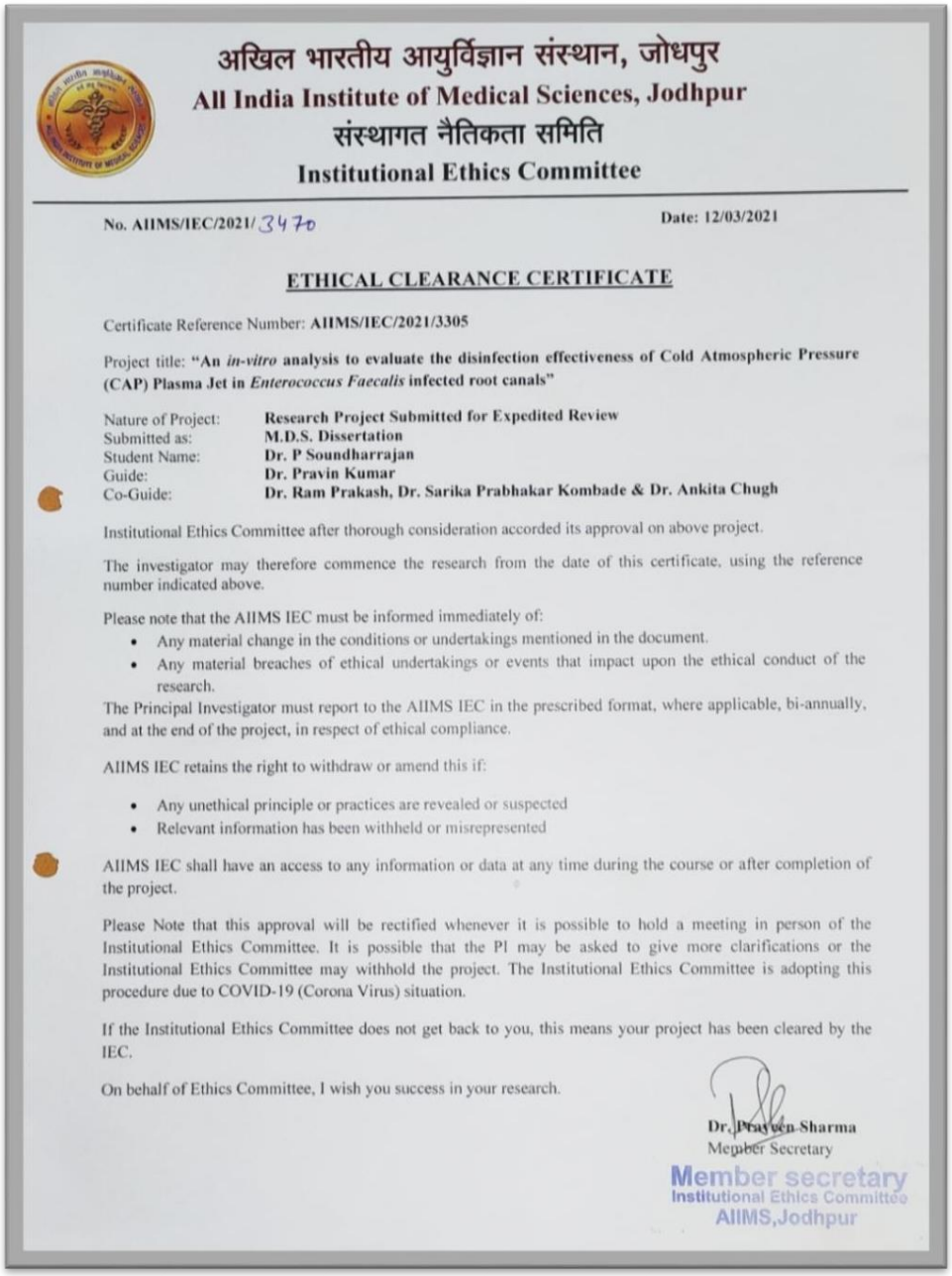
90. Alves FRF, Almeida BM, Neves MAS, Roças IN, Siqueira JF. Time-dependent

Antibacterial Effects of the Self-Adjusting File Used with Two Sodium Hypochlorite Concentrations. J Endod. 2011 Oct;37(10):1451–5.

91. Gomes BPFA, Vianna ME, Zaia AA, Almeida JFA, Souza-Filho FJ, Ferraz CCR. Chlorhexidine in Endodontics. Braz Dent J. 2013 Apr;24(2):89–102.
92. Kanisavaran ZM. Chlorhexidine gluconate in endodontics: an update review. Int Dent J. 2008 Oct;58(5):247–57.
93. Shuping G, Orstavik D, Sigurdsson A, Trope M. Reduction of Intracanal Bacteria Using Nickel-Titanium Rotary Instrumentation and Various Medications. J Endod. 2000 Dec;26(12):751–5.
94. Sieuwerts S, De Bok FAM, Mols E, De Vos WM, Van Hylckama Vlieg JET. A simple and fast method for determining colony forming units. Lett Appl Microbiol. 2008 Oct;47(4):275–8.
95. Hazan R, Que YA, Maura D, Rahme LG. A method for high throughput determination of viable bacteria cell counts in 96-well plates. BMC Microbiol. 2012 Dec 13;12(1):259.
96. Chau NPT, Chung NH, Jeon JG. Relationships between the antibacterial activity of sodium hypochlorite and treatment time and biofilm age in early Enterococcus faecalis biofilms. Int Endod J. 2015 Aug;48(8):782–9.
97. Grundling GL, Melo Taf De, Montagner F, Scarparo RK, Vier-Pelisser FV. QMix irrigant reduces lipopolysaccharide (LPS) levels in an in vitro model. J Appl Oral Sci. 2015 Aug;23(4):431–5.
98. Shetty S, Tejaswi S, U.K. A, Mittal A. Comparative Evaluation of the Antimicrobial Efficacy of Different Irrigating Solutions on Endodontic Pathogens - An In Vivo Study. J Evol Med Dent Sci. 2021 Mar 15;10(11):789–92.

ANNEXURES

Annexure I: Institutional Ethical Clearance certificate



अखिल भारतीय आयुर्विज्ञान संस्थान, जोधपुर
All India Institute of Medical Sciences, Jodhpur
संस्थागत नैतिकता समिति
Institutional Ethics Committee

No. AIIMS/IEC/2021/3470 **Date: 12/03/2021**

ETHICAL CLEARANCE CERTIFICATE

Certificate Reference Number: **AIIMS/IEC/2021/3305**

Project title: **"An *in-vitro* analysis to evaluate the disinfection effectiveness of Cold Atmospheric Pressure (CAP) Plasma Jet in *Enterococcus Faecalis* infected root canals"**

Nature of Project:	**Research Project Submitted for Expedited Review**
Submitted as:	**M.D.S. Dissertation**
Student Name:	**Dr. P Soundharrajan**
Guide:	**Dr. Pravin Kumar**
Co-Guide:	**Dr. Ram Prakash, Dr. Sarika Prabhakar Kombade & Dr. Ankita Chugh**

Institutional Ethics Committee after thorough consideration accorded its approval on above project.

The investigator may therefore commence the research from the date of this certificate, using the reference number indicated above.

Please note that the AIIMS IEC must be informed immediately of:

- Any material change in the conditions or undertakings mentioned in the document.
- Any material breaches of ethical undertakings or events that impact upon the ethical conduct of the research.

The Principal Investigator must report to the AIIMS IEC in the prescribed format, where applicable, bi-annually, and at the end of the project, in respect of ethical compliance.

AIIMS IEC retains the right to withdraw or amend this if:

- Any unethical principle or practices are revealed or suspected
- Relevant information has been withheld or misrepresented

AIIMS IEC shall have an access to any information or data at any time during the course or after completion of the project.

Please Note that this approval will be rectified whenever it is possible to hold a meeting in person of the Institutional Ethics Committee. It is possible that the PI may be asked to give more clarifications or the Institutional Ethics Committee may withhold the project. The Institutional Ethics Committee is adopting this procedure due to COVID-19 (Corona Virus) situation.

If the Institutional Ethics Committee does not get back to you, this means your project has been cleared by the IEC.

On behalf of Ethics Committee, I wish you success in your research.

Dr. Praveen Sharma
Member Secretary

Member secretary
Institutional Ethics Committee
AIIMS,Jodhpur