THESIS QUERIES

Query no.1:- English correction needed.

Ans:- Language correction has been done.

Query 2:- Method for ozonated water preparation in detail.

Ozonated water was produced from ozone unit (ADC Dentozone) by electric discharge bubbled into 1 L sterile distilled water. The generator produces ozone at a rate of 250mg/hr. The final concentration acquired was 4mg/L after bubbling for 15mins.

Sulaymon, Abbas. (2009). The Factors Affecting the Absorption of Ozone in Water. Iraqi Journal of Chemical and Petroleum Engineering. 10. 29-34.

Query 3:- Aims and objectives 4mg/ml discussion 24mg/l. correct it

Answer: correct concentration used is 4mg/L. It was a typing mistake in discussion. Kindly consider.

Query 4:- Final concentration of ozonated water assessed and confirmed?

The ozone generator produces ozone gas at a rate of 250mg/hr.(4.166mg/min)

So, to achieve the required concerntration only 1 min of exposure is required.

Because ozone gas is never absorbed 100% and it is affected by factors such as contact time, pH of distilled water.

Suloyman A.H.(2009)[72], the contact time should be 15 minutes to acquire a conc of 4mg/L

So, the pH of distilled water was kept in accordance with the above study to achieve the above concentration.

But, we regret to inform the final concentration of ozone in water was not confirmed.

Sulaymon, Abbas. (2009). The Factors Affecting the Absorption of Ozone in Water. Iraqi Journal of Chemical and Petroleum Engineering. 10. 29-34.

Query 5:- Transport used and how was it prepared?

Liquid transport media™,[HiMedias, Mumbai] was prepared as per manufacturer's instructions. As per the instructions, 18.5gm of the media requires to be suspended in 1L of distilled water. Since, 500ml was prepared, 0.940 grams of media was added and it was heated to completely dissolve the medium then it was autoclaved at 121^{o} C .

Photos added. (Page no. 45)

Query 6:- Colonial morphology & pigmentation is explained. However, pictures (photographs) of the culture plates with colony growth for microorganism are needed.

Answer: photos have been added (pg 61)

Query no.7:- Gram staining test to be explained on pictures..

Answer:- Gram staining(explained on page 47). Photos(page 65,64)

Procedure:

1. The slide was placed on the slide holder or a rack.
2. The primary stain (crystal violet) was applied for 60 seconds followed by rinsing with water for 5 seconds.
3. The iodine solution (mordant) was added to form a crystal violet-iodine (CV-I) complex. It was kept for a minute and rinsed with water for 5 seconds
4. The decolorization agent that is solvent such as acetone or ethanol was added which extracts the blue dye complex. The ethanol was added dropwise until the blue-violet color was no longer seen from specimen, followed by rinsing with the water for 5 seconds..
5. The red dye safranin was added by flooding the slide with the dye as in steps 1 and 2. It was kept for a minute to allow the bacteria to incorporate the saffranin and again, rinsed with water for 5 seconds to remove any excess of dye.

Query No.8:- What biochemical preformed in study? Explain methods alongwith pictures?

Answer: following biochemical tests were performed:

1. **Sugar fermentation test**
2. **Catalase test**
3. **Nitrate reduction test**

photos have been added. (pg 65-66)

Sugar fermentation tests, catalase test and nitrate reduction test were performed. (page page 47-49,)

For sugar fermentation test:-

Procedure:-

1. Basal medium(peptone water) containing a single carbohydrate source such as glucose, lactose, sucrose and mannitol was added in a concentration of 0.5-1%.
2. Bromothymol blue was present in the medium as an indicator.
3. The medium was inoculated with isolated colonies from the culture of the organism.
4. The inoculated media was incubated aerobically at 35-37ºC. for 3-5 days.
5. It was observed daily for development of a yellow color in the medium.

Catalase test:-

Procedure:-

1. A loop or sterile wooden stick was used to transfer a small amount of colony growth in the surface of a clean, dry glass slide.
2. A drop of 3% H_2O_2 was placed on the glass slide and observed for the evolution of oxygen bubbles.

Nitrate Reduction test:

Procedure:

1. The nitrate broths were inoculated with bacterial suspension.
2. The tubes were inoculated at the optimal temperature 30°C or 37°C for 24 hours.
3. They were checked for N2 gas first before adding reagents.
4. 6-8 drops of nitrite reagent A followed by nitrite reagent B were added.
5. The reaction was observed for color development for a minute or less.
6. Zinc powder was added to see the red color development if no color development was seen within a minute in step 5.

Query no.9:- Re-evaluate flow chart

Answer:- flow chart evaluated(page no-50)

Query no.10:- How cfu/ml was counted. Explain with pictures

Answer:- The colony counting was done manually. By using the plate count method or spread plate technique the colonies visible to the naked eye and the number of colonies on a plate were counted. Since only a part of medium of medium was inoculated the total number of colonies was determined by formula,

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate (page no. 48)

Query no.11:- Intergroup comparison p- value has discrepancy with value mentioned in table.

Answer:- Yes, the p-value mentioned is 0.004 and the correction has been done in the results section.(page no. 67)

Query no. 12:- Writing merely statiscally significant difference does not justify. Which group performed better????

Answer:-The comparison of three groups according to their performance have been explained in their respective groups. **(page no. 67-69)**

“COMPARATIVE EVALUATION OF THE ANTI-MICROBIAL EFFICACY OF SODIUM HYPOCHLORITE AND AQUEOUS OZONE AS ROOT CANAL IRRIGANT IN NON-VITAL PRIMARY TEETH.- A RANDOMIZED CONTROLLED STUDY.”

Dissertation submitted to

Maharashtra University of Health Sciences, Nashik

in the Partial Fulfillment of Regulations

for the award of the Degree of

MDS IN

PAEDODONTICS AND PREVENTIVE DENTISTRY

BRANCH VIII

2018-2021

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LIST OF COLOUR PLATES

ABBREVIATIONS

Short Form	Full Form
ppm	Parts per million
AAPD	American Academy of Pediatric Dentistry
P value	Probability value
S. mutans	Streptococcus mutans
P.gingivalis	Porphyromonas Gingivalis
Prevotella sp.	Prevotella species
P.micros	Peptostreptococcus micros
E. faecalis	Enterococcus faecalis
O_3	Ozone
Aq. Ozone	Aqueous ozone
NaOCl	Sodium hypochlorite
ml	millitlitres
µg	micrograms
BMP	Biomechanical preparation
CI	Confidence Interval
SD	Standard Deviation
WHO	World Health Organization
ANOVA	Analysis of variance
IOPA	Intra oral periapical
CFU	Colony forming Unit
S	Significant
N.S.	Not significant
D	Distal
M	Mesial
Ni-Ti	Nickel titanium
g/h	Grams/hour
L	Litre
S	Seconds
CO_2	Carbon dioxide
O_2	Oxygen

INTRODUCTION

The objective of preparation of root canals during endodontic therapy is thorough debridement of root canal tissue. Debridement is done by cleaning and shaping which involves two procedures: the mechanical cleansing by instruments and the use of irrigating solutions. One of the important aspect of chemomechanical preparation is effective irrigation.[(1)] The mechanical action of the instruments alone is not effective in cleaning a root canal satisfactorily owing to the complexity of the internal dental anatomy, for example, apical, deltas, lateral canals, and accessory canals.[(2)] Irrigation is the only way to clean these areas of the root canal wall that are not touched by mechanical instrumentation. Also, large areas in the oval and flat canals are often not cleaned properly despite careful instrumentation and contain tissue remnants and biofilms which can be cleaned by means of chemicals using irrigation. [(3)]

Hence, chemomechanical preparation not only includes mechanical action of the endodontic instrument on the canal walls, but also the chemical action of the irrigating solutions, and the physical action of the irrigation/aspiration process. [2]

The most important route of pulpal invasion by the bacteria is through the tubules of carious dentin and can occur even before the pulp is exposed directly to the oral environment through cavitation. Brook I[4], mentioned that the organismns that predominates in pulpitis and dentoalveolar abscess are *Prevotella, Porphyromonas, Fusobacterium* and *Peptostreptococcus.* Pazelli et al, found that in human deciduous root canals with necrotic pulp and periapical lesions, the infection is polymicrobial with a large number of micro-organisms and a predominance of streptococci, anaerobic micro-organisms and black pigmented bacilli. Rana V[5], in his review article also mentioned that the most common bacteria associated with necrotic pulp are Prevotella, Porphyromonas, Fusobacterium, *and* Peptostreptococcus spp. Fabris[6], 2014 during the Bacteriological analysis of necrotic pulp and fistulae in primary teeth had found that there was predominance of gram-positive cocci (81.8%)out of the 103 samples examined and found a high prevalence of *Enterococcus* spp. and Black pigmented Bacteria.

Emphasis has been laid upon irrigation as an important steps for disinfection of root canal before root filling because the bacteria and their by‑products play an important role in initiation and perpetuation of pulpal and periapical disease.[2] The irrigating solution facilitates the killing and removal of microorganisms, necrotic and inflamed tissue, and dentine debris but also prevents the lodging of the hard and soft tissue into the apical root canal and extrusion of bacteria and their by-products into the periapical tissues.[3]

The most common irrigant used for endodontic irrigation are sodium hypochlorite, chlorhexidine and EDTA. During 1915, Dakin introduced sodium hypochlorite solution (concentrations 0.45 to 0.50%) to carry out the disinfection of open or infected wounds in World War I. In 1917, Barret used the Dakin solution in dentistry for root canal irrigation and found that it was an efficient antiseptic. Many years later, Coolidge used sodium hypochlorite to refine root canal cleansing and disinfection procedures. Dr. Blass was one of the pioneers in the use of 5% sodium hypochlorite (chlorinated soda) as organic material solvent as well as potent germicide. Their concentration can vary from 0.5 to 5.25%.[7]

Siqueira JF[8],1998 proved it's antibacterial activity against black pigmented bacteria and streptococcus spp. in the concentration of 4%, 2.5%, followed by 0.5% and then again, in another study by Siqueira[9], 2000 he proved the Chemomechanical Reduction of the Bacterial Population in the Root Canal after Instrumentation and Irrigation with 1%, 2.5%, and 5.25% Sodium Hypochlorite when tested against e. faecalis. Thus, It has pronounced antimicrobial activity and the ability to dissolve organic matter but it has an adverse effect on vital tissues on extrusion into the periradicular tissues and can damage permanent tooth follicles, surrounding tissues and oral mucosa. Also, it is unable to remove the smear layer, and can damage the clothing and damage the retina of the patient or operator on contact. Chlorhexidine gluconate damages the bacterial cytoplasmic membranes, causing loss of osmotic balance, leading to leakage of intracellular material. It also binds to hydroxyapatite and soft tissues, changing their electrical field to compete with bacterial binding. [10,11] Irrigation with 2.5% sodium hypochlorite solution, can preserve sufficient stock of chlorine to eliminate significant amounts of bacterial cells, compensating the irritant effect caused by use of higher concentrations.[7,8,12] Hence, this concentration was chosen for this study.

German chemist Christian Friedrich Schonbein, of University of Basel in Switzerland was the first to discover ozone in 1840. Dr. Fish, a Swiss dentist used ozone either as gas or ozonated water in his practice but due to lack of ozone resistant materials such as Dacron, nylon, and Teflon there were restrictions on the use of ozone therapy, until 1950 when manufacturing of ozone resistant material began. At the end of 1980s, medical ozone became a subject of dental research and practice and was used increasingly for medical and dental purposes. Goals of ozone therapy include[13]

(i) inactivates and eliminates pathogens,

(ii) stimulation of immune system and improves circulation,

(iii) reduction of inflammation and pain,

(iv) stimulates humoral anti-oxidant system,

(v) restore proper oxygen metabolism.

Ozone has got a high oxidation potential which is 1.5 times greater than chloride when used as an antimicrobial agent. The antibacterial effect of Ozone occurs as a result of its oxidant action on cells by damaging its cytoplasmic membrane due to ozonolysis of dual bonds and also causes Ozone-induced modification of intracellular contents because of secondary oxidant effects.[13,14]

Ozone is generated with a Corona discharge system and is formed through an electrical discharge that is diffused over an area using a dielectric to create a corona discharge. The oxygen molecule which passes through this corona discharge is converted into ozone. The handling of this design is easy and the ozone production rate can be controlled, hence most commonly used in the medical and dental fields.[13]

There are three basic forms of ozone application.

1) Ozone gas
2) Ozonated water
3) Ozonated oil

In a study Pace et al[15], determined the effect of ozone gas on cells in-vitro and found that even low concentrations of ozone gas can retard cell proliferation, damage the cell membrane, and increase the vacuolar content causing consequent "swelling" of the cells and concluded that this effect depends upon concentration of the gas and length of exposure. Huth KC [16] investigated whether gaseous ozone and aqueous ozone exerted any cytotoxic effect on oral cells and found that aqueous ozone had high level of biocompatibility on human oral epithelial (BHY) cells, gingival fibroblast (HGF-1) cells and periodontal cells. They concluded that the advantages of aqueous ozone are its potency, ease of handling, lack of mutagenicity, rapid microbicidal effects. Also, aqueous ozone fulfils optimal cell biological characteristics in terms of biocompatibility for oral application whereas the ozone gas was found to have toxic effects on both cell types. He did not observe any cytotoxic signs for aqueous ozone. Literature also states that, ozone does not damage healthy human body cells owing to the free radical scavengers present in cells like superoxide dismutase, catalase, hydrolase and antioxidant nutrients like vitamin C, E, beta-carotene, selenium, methionine, glutathione. Hence, the infectious cells of organisms such as bacteria, viruses, fungi, parasites which are devoid of these antioxidants and scavengers are destroyed. (13,14) Nagayoshi et al (17), 2004 had demonstrated the ability of ozone against p.ginivalis, porphyromonas, streptococcus and e. faecalis in- vitro. Pinheiro et al(18), 2018 had investigated the antimicrobial efficacy of 2.5% sodium hypochlorite and ozonated water in the mesial root of mandibular molar in- vitro.

The apical root canal poses a special challenge to irrigation as the balance between safety and effectiveness is particularly important in this area. There are studies which have demonstrated the efficacy of ozonated water in-vitro but no published studies have assessed microbial load reduction in the root canal system in comparison to 2.5% sodium hypochlorite in-vivo in children. Also, due to drawbacks of the above mentioned irrigants, the need for a substitute with either equal or better anti- microbial efficacy arises. Hence, the purpose of this study is to evaluate and compare the antimicrobial efficacy of sodium hypochlorite and aqueous ozone against the bacteria found in the non- vital tooth which are- anaerobes- black pigmented bacilli like- P.Gingivalis, Prevotella, P. Micros, E. Faecalis and aerobes- streptococcus species.

AIM AND OBJECTIVES

Aim:

To evaluate and compare the anti- microbial efficacy of sodium hypochlorite and aqueous ozone as root canal irrigant in non-vital primary teeth.

Primary objective:

To compare the anti- microbial efficacy of 4mg/L aqueous ozone with 2.5% NaOCl as root canal irrigant in non- vital primary teeth.

Other objectives:

- To evaluate the anti- microbial efficacy of 2.5% sodium hypochlorite as root canal irrigant in non- vital primary teeth.
- To evaluate the anti- microbial efficacy of 4mg/L of aqueous ozone as root canal irrigant in non- vital primary teeth.

REVIEW OF LITERATURE

For decades, one of the most popular ideas in relation to root canal irrigation is that sodium hypochlorite has anti-microbial action and tissue dissolving capacity. This field is maturing with a wealth of well-understood methods and algorithms about black pigmented bacteria, sodium hypochlorite and ozone's forms, safety and anti-microbial efficiency.

A systematic search was opted for published studies and confined to databases like PubMed, ResearchGate and Google Scholar and for grey literature the references of the articles were manually searched. The key terms were tooth, 'deciduous', Root Canal Irrigants, sodium hypochlorite and ozone. Scientific articles on importance of root canal irrigation, use of sodium hypochlorite and ozonated water in endodontics were found. However, the data regarding the use of ozone as root canal irrigants is scarce.

For simplicity the literature has been categorized and into in-vitro, in-vivo and review articles and has been presented in the review of literature under following headings:-

a) Black pigmented bacteria in root canals

b) sodium hypochlorite- use and adverse effects.

c) ozone- various forms, anti-microbial efficacy and safety.

1) Studies demonstrating the presence of black pigmented bacteria in teeth:-

In- vitro studies:-

Winkelhoff A.J. VAN, Carlee AW, Graaff JDE (1985)[19] assessed Bacteroides endodontalis and Other Black-Pigmented Bacteroides Species in Odontogenic Abscesses. In their study, 28 patients who suffered from oral pyogenic infections of odontogenic origin were selected. Pus was collected with a sterile cotton swab and were streaked directly on four 5% horse blood agar plates supplemented with 0.05% hemin and 0.01% menadione. All pure cultures were examined for the following characteristics: Gram staining, cell morphology, aerotolerance, production of catalase, and fermentation of 1% glucose in BM medium. They observed that out of 28 odontogenic abscesses, 26 proved to contain one or more species of black-pigmented Bacteroides. It was found that Bacteroides endodontalis, a newly described species of asaccharolytic black-pigmented Bacteroides, was isolated almost exclusively from periapical abscesses of endodontic origin. B. intermedius proved to be the most frequently isolated species in all of the samples. B. gingivalis was present in all of the periodontal abscesses studied, as well as in two endodontic abscesses. B. melaninogenicus was recovered once from a pericoronal abscess.

Baumgartner C, Falkler W (1991)[20] evaluated bacteria in apical 5mm of Infected root canals. They included 10 freshly extracted teeth which had carious pulpal exposures and periapical lesions contiguous with the root apex, which were placed inside an anaerobic chamber and then apical 5 mm of the root canals was cultured. In addition to anaerobic incubation, duplicate cultures were incubated aerobically. 50 strains of bacteria from the 10 root canals were isolated and identified. The most prominent bacteria cultured from the 10 root canals were Actinomyces, Lactobacillus, black-pigmented Bacteroides, Pepto streptococcus, nonpigmented Bacteroides, VeilIonella, *Enterococcus faecalis, Fusobacterium nucleatum,* and *Streptococcus mutans.* Of the 50 bacterial isolates, 34 (68%) were strict anaerobes. This study demonstrated the presence of predominately anaerobic bacteria in the apical 5 mm of infected root canals in teeth with carious pulpal exposures and periapical lesions.

Pazelli LC, Campos De Freitas A, Ito IY, Monteiro De Souza-Gugelmin MC, Medeiros AS, Nelson-Filho P, (2003)[21] conducted a study to evaluate the bacterial prevalence of microorganisms in root canals of human deciduous teeth with necrotic pulp and chronic periapical lesions**.** A total of 31 root canals were selected for the study and the samples were taken from maxillary incisors and canines, and maxillary and mandibular molars. Bacteriological samples were collected immediately after crown access by introducing 3-4 sequential sterile absorbent paper points, of a size visually compatible with the root canal diameter, 2-3 mm before the radiographic apex and after approximately 1 min, The paper points were removed from each root canal and placed in a test tube containing 2.0 ml of reduced transport fluid following which microbiological analysis was carried out. The results showed that Anaerobic microorganisms were found in 96.7% of the samples, black-pigmented bacilli in 35.5%, aerobic microorganisms in 93.5%, streptococci in 96.7%, and *S. mutans* in 48.4%. From the study they concluded that in human deciduous teeth root canals with necrotic pulp and periapical lesions the infection is polymicrobial, with a large number of microorganisms and a predominance of streptococci and anaerobic microorganisms.

In- vivo evidence:-

Brown LR. Rudolph CE.(1957)[22]. performed isolation and identification of microorganisms from unexposed canals of pulp-involved teeth. In this study, teeth having unexposed pulp canals, no deep carious lesions, no discernible gross anatomic defects, no large restorations, no extensive periodontal involvement, and no history of severe trauma were selected. From the lingual or occlusal surface, entrance to the pulp chamber was effected with sterile burs. When access to the pulp chamber was gained, a dry, sterile paper point was inserted into the pulp canal and samples were obtained for microbial culture and further stored in storage medium. They used phase contrast and darkfield microscopy and found that predominant bacteria in an infected root canal were often not cultivable using existing culturing methods. Very few strains of black-pigmented Bacteroides/Porphyromonas were isolated and described.

Sabiston, C. B., Grigsby, W. R., & Segerstrom, N. (1976)[23] performed a study to evaluate bacteria present of pyogenic infections of dental origin. In their study, abscesses were isolated with disposable syringes and dried sterile gauze. The exudate was obtained by aspiration with the use of a sterile disposable syringe with a No. 16 gauge needle while other abscesses were sampled by sterile paper point after the tooth had been opened for endodontic therapy. Each specimen was examined grossly, by a wet preparation for phase-contrast microscopy and a gram preparation. Representative colonies were picked in 3 to 5 days. The blood media were further incubated for 10 days in order to detect Bacteroide melaninogenicus. Methods of identification relied on morphology, Gram reaction, a battery of biochemical tests, and gas chromatographic analysis of fatty acids and alcohols produced in culture medium. They found that more than 65% of the species isolated were obligate anaerobes, which indicates the need for anaerobic methods in studying these infections. Anaerobic gram-negative rods and facultative streptococci were the groups isolated most frequently.

Da Silva LAB, Nelson-Filho P, Faria G, De Souza-Gugelmin MCM, Ito IY(2006)[24], evaluated the bacterial profile in root canals of human primary teeth with necrotic pulp and periapical lesions using bacterial culture**.** A total of 20 primary teeth with necrotic pulp and radiographically visible radiolucent areas in the region of the bone furcation and/or the periapical region were selected. After crown access, 4 sterile absorbent paper points were introduced sequentially into the root canal for collection of material. After 30 s, the paper points were removed and placed in a test tube containing reduced transport fluid (RTF) and were sent for microbiological evaluation. Anaerobic microorganisms were found in 100% of the samples, black-pigmented bacilli in 30%, aerobic microorganisms in 60%, streptococci in 85%, Gram-negative aerobic rods in 15% and staphylococci were not quantified. Mutans streptococci were found in 6 root canals (30%), 5 canals with *Streptococcus mutans* and 1 canal with *Streptococcus mutans* and *Streptococcus sobrinus*. It was concluded that in root canals of human primary teeth with necrotic pulp and periapical lesions, the infection is polymicrobial with predominance of anaerobic microorganisms.

Cogulu[(11)](2008), evaluated the presence of the selected pathogens in samples from deciduous and permanent tooth root canals by using PCR method and associated these organisms with clinical symptoms. One hundred forty-five endodontic samplings were obtained from deciduous (n =79) and permanent molars (n =66) during the first visit of the root canal therapy Access to the root canal was made using sterile burs without water spray. Aseptic techniques were used for instrumentation, during access to and removal of the contents from the pulp space, and sample collection. All samples were processed within 2 hours. After thoroughly shaking the endodontic sample in a mixer for60 seconds, all were frozen immediately at 20^{o}C and stored until assayed by PCR. *P. gingivalis* were associated with tenderness to percussion in both deciduous and permanent teeth.

REVIEW ARTICLES:-

G. Sundqvist, (1992)[(25)] wrote a review on Ecology of the Root Canal Flora**.** It mentions about the bacterial interrelationships and how the final bacteria grow and prevail over the other bacterial species. It mentions that there are three phases of bacterial growth inside a root canal. First, the carbohydrates in serum are consumed by rapidly growing saccharolytic bacteria, leading to lactic and formic acid production. In a second phase, proteins were hydrolysed, due to which amino acid fermentation takes place, and the remaining carbohydrates were used. Growth during this phase is dominated by B. intermedius, V. parva, Eubacterium sp., and F. nucleatum. In a final phase, progressive protein degradation and extensive amino acid fermentation takes place. The predominant species during this phase were P. micros, F. nucleatum, and Eubacteria sp. The black-pigmented anaerobic rods B. intermedius, P. endodontalis, and Porphyromonas (Bacteroides) gingivalis have a high ability to degrade serum proteins and make peptides and amino acids available for fermentation. Bacteriocin which is a protein, produced by a microorganism and has the capacity to inhibit the growth of a limited number of other species. The black-pigmented Bacteroides produce bacteriocins, which are able to suppress not only Gram-positive bacteria, but have an inhibitory influence on other Bacteroides strains as well. Streptococci also have the capacity to inhibit the growth in vitro of many anaerobic bacteria, through the production of hydrogen peroxide by the streptococci. The anaerobiosis is broken when the canal is opened and biomechanical treatment eliminates bacteria as well as deprives the canal of nutrients and interferes with bacterial interactions.

M. Haapasalo,[(26)] (1993) wrote a review article on black-pigmented Gram-negative anaerobe in endodontic infections which stated that the composition of the bacterial flora in endodontic infections is influenced by three major factors: the origin of the infection, the ecological conditions in the infected root canal, and the host defence mechanisms. He has opined that the bacterial flora in apical periodontitis constitute about 70- 95% anaerobes, the most frequent isolates belong to the genera Prevotella, Fusobacterium, Wolinella, Porphyromonas, Pepto streptococcus, Eubacterium and Streptococcus. The frequency of isolation of black-pigmented Gram-negative anaerobes in endodontic infections varies from 25% to > 50%. Pr.

intermedia is the most commonly found pigmented species, followed by Pr. Denticola and two Porphyromonas species, P. gingivalis and P. endodontalis.

2) Studies demonstrating the effective of sodium hypochlorite against the black pigmented bacteria:-

In- vitro evidence:-

Baumgartner, J. C., and Cuenin, P. R. (1992) (27) studied the Efficacy of Several Concentrations of Sodium Hypochlorite for Root Canal Irrigation. In their study, single canal bicuspids which had been extracted for orthodontic purposes were used. The roots of the control teeth were fractured into longitudinal halves using wire cutters and were divided into 4 experimental groups. They were used to evaluate the debridement capabilities of four concentrations of sodium hypochlorite (NaOCl-5.25%, 2.5%, 1.0%, and 0.5%). The root canals were instrumented using sequential K-type files, #10 through #50. A total of 30 ml of NaOC1 irrigant was used with 27-gauge blunt endodontic needles. A photomicrograph was taken at x150, x500 and x4000 in the middle third of each root canal. Evaluation of the scanning electron micrographs focused on the amount of superficial debris, the nature of the smear layer on the instrumented side, and the characteristics of the surface on the non-instrumented surface of the root canal. They found that all of the concentrations of NaOCI were very effective in flushing out loose debris from the root canals. A smear layer with some exposed dentinal tubules was seen on all instrumented surfaces regardless of concentration of NaOCI or irrigation device. Hence, they inferred, NaOCI in concentrations of 5.25%, 2.5%, and 1% completely removed pulpal remnants and predentin from the non-instrumented surfaces. Although 0.5% NaOCI removed the majority of pulpal remnants and predentin from the un-instrumented surfaces, it left some fibrils on the surface.

Siqueira J.F., (1998)[8] studied the antibacterial effects of endodontic irrigants on black-pigmented gram negative anaerobes and facultative bacteria. In his experiment he used the following irrigants- 0.5% NaOC1; 2.5% NaOC1; 4.0% NaOC1; 0.2% chlorhexidine digluconate; 2.0% chlorhexidine digluconate; 10% citric acid; and 17% EDTA. He evaluated their antibacterial activities against four black-pigmented Gram-negative anaerobes and four facultative anaerobic bacteria commonly isolated from infected root canals. The black-pigmented strains used were *Porphyromonas endodontalis* (BN l la-f), *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedia* (ATCC 25611), and *Prevotella nigrescens* (DAL 5). The facultative strains used were *Enterococcus faecalis* (ATCC 29212), *Streptococcus mutans* (clinical isolate), *Streptococcus sanguis* (ATCC 10556), and *Streptococcus sobrinus* (ATCC 27609). The Facultative anaerobes were maintained in brain heart infusion (BHI) broth. Obligate anaerobic bacteria were maintained in preproduced anaerobically sterilized BHI, supplemented with hemin (5 rag/L) and menadione (0.5 mg/L). The blood agar plates were inoculated with the facultative anaerobes and incubated aerobically at 37°C for 2 days whereas the, bacterial agar plates inoculated with the black-pigmented were placed into anaerobic jars. Anaerobic conditions were produced by the evacuation replacement procedure, in which the air in the jar was removed using a vacuum pump and was replaced with a mixture containing 10% H 2 and 10% CO2 in nitrogen. Then the jars were incubated at 37°C for 7 days. Afterward, the diameters of the zones of bacterial inhibition were measured. Based on the averages of the diameters of the zones of bacterial growth inhibition, thereupon the antibacterial effects of the solutions were ranked from strongest to weakest as follows: 4% NaOCI; 2.5% NaOCI; 2% chlorhexidine; 0.2% chlorhexidine, EDTA, and citric acid; and 0.5% NaOCI**.**

Siqueira Jr, J., Rocas, I., Favieri, A., & Lima, K [12]. (2000) performed a study on Chemomechanical Reduction of the Bacterial Population in the Root Canal after Instrumentation and Irrigation with 1%, 2.5%, and 5.25% Sodium Hypochlorite. In their study, 40 extracted human lower bicuspids were selected and after access preparations the root canals were instrumented 1 mm beyond the apical foramen with K-type files up to size 20. After sealing the apical foramen by epoxy resin, the root canal was completely filled with the E. faecalis suspension. Sterile K-type #15 files were used to carry the bacterial suspension to the entire root canal length. Apical preparation was completed by enlargement through a #40 Niti-flex file. The root canals were divided into three experimental groups accordingly to the NaOCl solution used in irrigation. In group 1, 10 root canals were irrigated with 1% NaOCl during instrumentation. In group 2, 10 other canals were irrigated with 2.5% NaOC1. In group 3, 10 root canals were irrigated with 5.25% NaOCl. A total volume of 7 ml of the irrigants tested was used for each tooth. They found that the samples taken before and after root canal preparation showed that all test solutions significantly reduced the number of bacterial cells in the root canal. Instrumentation and irrigation with 2.5% NaOCl provided a decrease of 99.9% (in absolute numbers) and 59.5% (in log numbers) in the number of viable bacteria in the root canal. The mean reduction for 5.25% NaOCl group was 99.8% (in absolute numbers) and 65.9% (in log numbers). Comparisons between groups using the ANOVA and the Tukey's test failed to find a significant difference between the three NaOCl solutions tested. They deduced that anti-bacterial action was dependent on concentrations and could be ranked from the strongest to the weakest solutions as follows: 5.25% NaOCl, 2.5% NaOC1, and 1% NaOCl.

Siqueira JF, Rôças IN, Lopes HP [28](2002), conducted a study to investigate the patterns of microbial colonization in primary root canal infections in untreated teeth associated with chronic peri radicular lesions by use of scanning electron microscopy. In the study, 15 extracted single-root teeth with asymptomatic peri radicular lesions were collected. After fixation, the tooth crowns were cut off and the lesions were removed with scalpel blades. Longitudinal grooves following the root length axis were cut and roots were then split into 2 halves. Roots were dried in ethanol concentrations followed by which they were dehydrated and sputter-coated with gold under vacuum. Then dentinal walls and the dentinal tubules of both apical and middle thirds of the root canals were examined by using a scanning electron microscope. The results showed that the root canal microbiota consisted of cocci and/or rods in all specimens, often forming mixed communities. Different forms of rods were found, such as filaments, straight rods, curved rods, and coccobacilli. Bacterial penetration into dentinal tubules was usually close to the main root canal. In some specimens some tubules were infected up to 300 mm deep.

Vianna ME, Gomes BPFA, Berber VB, Zaia AA, Ferraz CCR, De Souza-Filho FJ.[29], (2004) conducted a to determine in vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite in the concentration of 0.2%, 1%, and 2% chlorhexidine gluconate against endodontic pathogens and compare the results with the ones achieved by 0.5%, 1%, 2.5%, 4%, and 5.25% sodium hypochlorite (NaOCl). The species of microorganisms used in this experiment were (1)*E faecalis* ATCC 29212, (2) *C albicans* NTCC 3736, (3) *S aureus* ATCC 25923 (all of them grown on 5% sheep blood–Brain Heart Infusion [BHI] agar plates for 48 hours at 37°C); and (4) *P gingivalis*, (5) *P endodontalis*, and (6) *P intermedia* (all of which were isolated from the root canal infections and identified by using conventional biochemical tests. Six wells were used for each time period, irrigant, and microorganism, respectively. Overall, 5616 wells were used: 4752 for all the test irrigants and 864 for the control group. They found that all irrigants eliminated Porphyromonas endodontalis, Porphyromonas gingivalis, and Prevotella intermedia in 15 seconds. The timing required for 1.0% and 2.0% CHX liquid to eliminate all microorganisms was the same required for 5.25% NaOCl. They gave the inference that the antimicrobial action is related to type, concentration, and presentation form of the irrigants as well as the microbial susceptibility

In-vivo studies:-

Vianna ME, Horz HP, Gomes BPFA, Conrads G.(2006) [(30)], conducted an in vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. In their experiment they used 32 single rooted teeth with necrotic pulp which were divided into two groups. One group was irrigated with 2.5% NaOCl, whilst the other group was irrigated with 2% CHX gel. In the CHX-group, use of each instrument was followed by irrigation with CHX gel and immediately after with 4 mL of physiological saline solution. In the NaOCl-group use of each instrument was followed by irrigation of the canal with 5 mL of 2.5%NaOCl solution. For culturing technique, the serial dilutions were inoculated on blood agar plates supplemented with5% horse blood, 5 mg L)1 haemin and 1 mg L) menadione. Plates were incubated anaerobically (80% N2, 10% H2, 10% CO2) at 37^{o}C for 7 days. After incubation, the total CFU were counted using a stereomicroscope at 16x magnification. The results showed that the bacterial reduction in the NaOCl-group was significantly greater than in the CHX-group. Also, 75% of cases were free of bacteria in the NaOCl-group. He concluded that NaOCl not only had a higher capacity to kill microorganisms but also good ability to remove cells from the root canal.

Siqueira JF, Magalhães KM, Rôças IN, (2007)[(9)] conducted a study to assess the bacterial Reduction in Infected Root Canals Treated With 2.5% NaOCl as an Irrigant and Calcium Hydroxide/Camphorated Paramonochlorophenol Paste as an Intracanal Dressing. 12 single-rooted teeth were selected for this study based on stringent inclusion/exclusion criteria. Only teeth with intact pulp chamber walls, necrotic pulps as confirmed by negative response to sensitivity pulp tests, and clinical and radiographic evidence of chronic apical periodontitis lesions were included in this study. Bacterial samples were taken before treatment (S1), after chemomechanical preparation using hand NiTi files and 2.5% NaOCl (S2), and following a 7-day medication with a Ca (OH)2 paste in CPMC (S3). Cultivable bacteria recovered from infected root canals at the three stages were counted and identified by means of 16S rRNA gene sequencing analysis. The results showed significantly high reduction in bacterial counts between S1 and S2, and S1 and S3. Significant differences were also observed for comparisons involving S2 and S3 samples with regard to both quantitative bacterial reduction and number of culture-negative cases. It was concluded that chemomechanical preparation with 2.5% NaOCl as an irrigant significantly reduced the number of bacteria in the canal but failed to render the canal free of cultivable bacteria in more than one-half of the cases.

Rocas I and Siqueira JF[31](2011), Comparison of the In Vivo Antimicrobial Effectiveness of Sodium Hypochlorite and Chlorhexidine Used as Root Canal Irrigants: A Molecular Microbiology Study. 50 patients with intact pulp chamber walls and necrotic pulps, contributed a single-rooted single-canal tooth. Under rubber dam isolation access to the pulp chamber was achieved followed by proper disinfection of the operative field. Bacterial samples were taken at the baseline (S1) and after (S2) chemomechanical preparation using 2.5% NaOCl or 0.12%CHX as the irrigant. For broad range PCR, DNA was extracted by using the QIAamp DNA Mini Kit followed by which Bacterial, archaeal, and fungal presence was evaluated whereas bacterial identifications were performed by a closed-ended reverse-capture checkerboard approach. The results showed that the Initial (S1) samples from all teeth yielded positive PCR results for bacteria. In the post- irrigation samples, for 2.5% NaOCl group, 12 of 30 (40%) S2 samples were PCR negative for bacterial presence whereas in the CHX group, 8 of 17 (47%) cases exhibited negative PCR results for bacteria in S2. The most prevalent taxa in S2 samples from the NaOCl group were Propionibacterium acnes, Streptococcus species, Porphyromonas endodontalis, and Selenomonas sputigena. From this study it is concluded that 2.5% is effective in significantly reducing the number of bacterial taxa and their levels in infected root canals.

REVIEW ARTICLE:-

Estrela C et al [32] (2002), in her review on mechanism of action of sodium hypochlorite mentions the antimicrobial effectiveness of sodium hypochlorite, based in its high ph. The high pH of sodium hypochlorite interferes in the cytoplasmic membrane integrity with an irreversible enzymatic inhibition, biosynthetic alterations in cellular metabolism and phospholipid degradation observed in lipidic peroxidation. The amino acid chlorination reaction forming chloramines interferes with cellular metabolism. Oxidation promotes irreversible bacterial enzymatic inhibition replacing hydrogen with chlorine. This enzyme inactivation can be observed in the reaction of chlorine with amino groups (NH2 -) and an irreversible oxidation of sulfhydryl groups (SH) of bacterial enzymes (cysteine). Thus, sodium hypochlorite acts on bacterial essential enzymatic sites promoting irreversible inactivation originated by hydroxyl ions and chlorination action. Dissolution of organic tissue takes place by saponification reaction when sodium hypochlorite degrades fatty acids and lipids resulting in soap and glycerol. Pulp tissue dissolution capacity of 5% sodium hypochlorite is about 20 min to 2 h.

Cárdenas-bahena Á, Sánchez-garcía S, Tinajero-morales IIC, González-rodríguez VM, Baires-várguez L.[7],(2012), In her study on Use of sodium hypochlorite in root canal irrigation wrote about the history of hypochlorite as follows:- During 1915, in World War I, Dakin introduced sodium hypochlorite solution (concentrations 0.45 to 0.50%) for disinfection of open or infected wounds. In 1917, Barret spread the use of Dakin solution in dentistry, especially for root canal irrigation. He informed that the solution was a very efficient antiseptic. Years later, Coolidge used sodium hypochlorite to improve root canal cleansing and disinfection procedures. Dr. Blass was one of the pioneers in the use of 5% sodium hypochlorite (chlorinated soda) as organic material solvent as well as potent germicide. His experiences were published in the 5th Edition of the National Formulary, in 1936, Walker described usage of 5% sodium hypochlorite for preparation of root canal in tooth with necrotic pulps. In 1954, Lewis informed of use of sodium hypochlorite, commercial brand- name Clorox, due to the fact that this product contained 5.25% available chlorine concentration13. In 1970 Shih studied in vitro antibacterial action of 5.25% sodium hypochlorite on *E. faecalis* and *S. aureus*. In an *in vitro* study, Trepagnier& et al, concluded that 5% sodium hypochlorite was a potent tissue solvent. Dilution of this solution with water, at equal parts, (2.55) did not appreciably alter its solvent action. Frequent and abundant irrigation with 2.5% sodium hypochlorite solution, preserves sufficient stock of chlorine to eliminate significant amounts of bacterial cells, compensating thus the irritant effect caused by use of higher concentrations.

Spencer HR, Ike V, Brennan PA. **[33](2014),** reviewed the use of sodium hypochlorite in endodontics — potential complications and their management. The author mentions Sodium hypochlorite (NaOCl) was first recognised as an antibacterial agent in 1843 when hand washing with hypochlorite solution between patients produced unusually low rates of infection transmission between patients. It was first recorded as an endodontic irrigant in 1920 and is now in routine worldwide use. Sodium hypochlorite is used as an endodontic irrigant as it is an effective antimicrobial and has tissue-dissolving capabilities. It has low viscosity allowing easy introduction into the canal architecture, an acceptable shelf life, is easily available and inexpensive.

Neelakantan P, Herrera DR, Pecorari VGA, Gomes BPFA. [34] (2019). Wrote a systematic review and metanalysis on topic Endotoxin levels after chemomechanical preparation of root canals with sodium hypochlorite or chlorhexidine: a systematic review of clinical trials and meta-analysis. Four electronic databases (MEDLINE via PubMeb, Scopus, Web of Science and Cochrane Library) were searched from their start dates to 1 March 2017. From 712 articles that resulted from the initial search, 37 studies were included for full-text appraisal; four studies met the inclusion criteria for quantitative synthesis. A single meta-analysis was performed to compare the endotoxin levels before and after chemomechanical preparation with NaOCl or CHX. The forest plot of lipopolysaccharide (LPS) levels indicated that the use of NaOCl and CHX during chemomechanical preparation significantly reduced the LPS levels compared to the initial ones. When NaOCl was used during chemomechanical preparation, endotoxins levels were lower than those obtained after chemomechanical preparation with chlorhexidine.

Literature quoting the adverse effects of sodium hypochlorite

In- vitro evidence:-

Sim TPC, Knowles JC, Ng YL, Shelton J, Gulabivala K.[35](2001), performed an in-vitro study to determine the effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. 50 intact, human teeth after extraction were sectioned longitudinally using a motorized saw to create bars. The bars were randomly allocated to be soaked in one of three solutions 37 in saline, 31 in

0.5% NaOCl, 32 in 5.25% NaOCl. The bars were soaked in 200 mL of the respective Solutions and the total immersion time was 2hours where the solutions were changed intermittently every 10 mins. The bars were then subjected to a three-point bend test using a custom-made jig mounted in a load testing machine. Changes in strain of each of 10 teeth on cyclical non-destructive occlusal loading were measured using electrical resistance strain gauges bonded to the cervical aspects. The results showed that there was significant decrease in elastic modulus and the flexural strength of the dentine bars immersed in 5.25% NaOCl compared with the saline group. He concluded that 5.25% NaOCl reduced the elastic modulus and flexural strength of dentine. Irrigation of root canals of single, mature rooted premolars with 5.25% NaOCl affected their properties sufficiently to alter their strain characteristics when no enamel was present.

Hariharan V, Nandlal B, Srilatha K.[(36)]**(2010),** evaluated the efficacy of various root canal irrigants on removal of smear layer in the primary root canals after hand instrumentation: A scanning electron microscopy study. In the study, 30primary incisors with at least two third of root intact were included in the study. Chemo mechanical preparation was done with K files by conventional step back preparation till 45 sizes. Intermittently, canal was irrigated with 3 ml saline following which the samples were divided randomly into five groups - Saline group, Sodium hypochlorite group, Sodium hypochlorite +EDTA group, Citric acid group, Chlorhexidine group and final irrigation was carried out using 10 ml of the solution in each group as final irrigant. The specimens were split along the longitudinal axis using a chisel and one half of the sample was subjected to scanning electron microscopy analysis. The results proved that SEM pictures of the groups like sodium hypochlorite, chlorhexidine irrigants does not have the capacity to remove the smear layer in primary teeth. The pictures from the 10%EDTA + 5.25% sodium hypochlorite group showed that even though it removed the smear layer, it adversely affected the dentine structure.

M. Hülsmann & W. Hahn[(10)](1999) wrote a review on complications during root canal irrigation – literature review and case reports. In their review, they have mentioned that hypochlorite complications may occur in case of inadvertent injection of sodium hypochlorite beyond the apical foramen with wide apical foramina or when the apical constriction has been destroyed during root canal preparation or by resorption. Also, extreme pressure during irrigation or binding of the irrigation needle tip in the root canal with no release for the irrigant to leave the root canal coronally may result in contact of large volumes of the irrigant to the apical tissues. If this occurs, sodium hypochlorite will lead to tissue necrosis. The Symptomatology of hypochlorite accidents are concentration dependent and leads to- severe pain, oedema of neighbouring soft tissues, profuse bleeding from the root canal, profuse interstitial bleeding with haemorrhage of the skin and mucosa (ecchymosis). Reversible anaesthesia or paraesthesia is also possible. Apart from these it causes damage to clothing as it is a common household bleaching agent. While using hand irrigation, the irrigation needle and syringe should be securely attached and should not be separated to prevent leakage over clothing. Damage to the eye on contact with the patient's or operator's eyes results in pain, profuse watering, intense burning, and erythema. Loss of epithelial cells in the outer layer of the cornea may occur.

Wong T.S. and cheung S.P.[37](2013) Extension of Bactericidal Effect of Sodium Hypochlorite into Dentinal Tubules. For this study, extracted, human, single-rooted teeth were chemomechanically prepared and then decoronated, their roots were split buccolingually into 2 halves. These dentin specimens were then autoclaved and half-root served as specimen. A species biofilm consisting of initially equal proportions of E. faecalis (ATCC 2922) and Porphyromonas gingivalis (ATCC 33277) was cultivated on each specimen the dentin specimen was then immersed in the bacterial suspension for 7 days at 37^{o}C in a shake-incubator. The canal wall, with a biofilm, was irrigated with either 0.5% or 3% sodium hypochlorite solution. A control Group was irrigated with sterile physiological saline which was delivered via a sterile 27-gauge notched-tip needle, A total of 3 mL solution was delivered, and total contact time was 3 minutes. The roots were then split horizontally at 5-, 7-, and 9-mm levels from root apex and examined under confocal laser scanning microscope and then scanning electron microscope. The proportions of viable cells situated on different depths into root canal dentin were compared, both concentrations of sodium hypochlorite significantly reduced the number of live bacteria in the most superficial layer (first 0.1 mm) of root canal dentin. For the next 2 layers (0.1–0.3 mm into dentinal tubules), irrigation with 3% hypochlorite resulted in significantly lower amounts of viable bacteria than 0.5% hypochlorite or saline. No significant difference between 0.5% or 3% sodium hypochlorite and the positive control was observed in deeper (>0.3 mm) regions into the root canal wall. He concluded that Increasing the concentration of sodium hypochlorite improves the penetration depth of its antibacterial action into dentinal tubules, but that seemed unable to completely eradicate bacteria residing there. Total elimination of bacteria from dentinal tubules cannot be achieved by irrigation with sodium hypochlorite alone.

Safety of ozone in humans:-

Huth KC, Jakob FM, Saugel B, Cappello C, Paschos E, Hollweck R, et al.[16] (2006) conducted an in- vitro study to determine the effect of ozone on oral cells compared with established antimicrobials. They investigated whether gaseous ozone ($4x10^6/gm^3$) and aqueous ozone (1.25–20 lg ml)1) exert any cytotoxic effects on human oral epithelial (BHY) cells and gingival fibroblast (HGF-1) cells compared with established antiseptics [chlorhexidine digluconate (CHX) 2%, 0.2%; sodium hypochlorite (NaOCl) 5.25%, 2.25%; hydrogen peroxide (H2O2) 3%], over a time of 1 min. Human oral epithelial cells and gingival fibroblasts were cultured under standard conditions in Dulbecco's modified Eagle's minimal essential medium. Ozone gas was used at with pure oxygen as the control. Aqueous ozone was applied to the cells in the form of ozonated phosphate-buffered saline in photometrically confirmed concentrations of 1.25–20 lg ml). Total cell count and the number of dead cells were determined. Cell viability was monitored by assessing their metabolic activity after agent exposure. Essentially no cytotoxic signs were observed for aqueous ozone. CHX (2%, c0.2%) was highly toxic to BHY cells, and slightly (2%) and non-toxic (0.2%) to HGF-1 cells. NaOCl and H2O2 resulted in markedly reduced cell viability (BHY, HGF-1), whereas metronidazole displayed mild toxicity only to BHY cells. Taken together, aqueous ozone revealed the highest level of biocompatibility of the tested antiseptics.

Bocci V[38](2006), wrote a review article titled- Is it true that ozone is always toxic? The end of a dogma. In his review article, he states that Ozone is not always toxic and can be used as a real drug following are the evidence- based applications of ozone. Chronic osteomyelitis, pleural empyema, peritonitis, abscesses with intractable fistulae, infected wounds, bed sores, chronic ulcers and initial gangrene, diabetic foot, skin, mouth, vaginal and rectal bacterial, viral, fungal infections and burns. In these pathologies, simultaneous parenteral and topical therapy with ozonated oil is used to relieve the conditions and occurs because of lack of its antibiotic resistance, ozone's potent disinfectant activity and capacity for accelerating the healing process. In case of Age-related macular degeneration (ARMD, atrophic form even a small increase of oxygen delivery at the foveola level, improves the visual acuity acting on the photoreceptors and retinal pigment epithelium. Lumbar and cervical hernial discs as well as localized osteoarthrosis can be treated with a direct or intramuscular small injection of gas.

Studies quoting the efficacy of ozone as root canal irrigant:-

In- vitro evidence:-

Nagayoshi M, Fukuizumi T, Kitamura C, Yano J, Terashita M, Nishihara T[(17)]. (2004) determined the efficacy of ozone on survival and permeability of oral microorganisms. The study aimed to examined the effect of ozonated water on oral microorganisms and dental plaque. In their study the following bacteria were cultured- S. salivarius, S. sanguis, S. mutans, S. sobrinus, P. gingivalis, P. endodontalis according to their culture characteristics. They were harvested by centrifugation at 10,000 · g for 5 min, suspended in saline and adjusted to $1*10^6$ cells/ ml. they were exposed to 0.5, 2, and 4 mg/l of ozonated water for 10, 30, 60, or 120 s. they found that cell viability of S. mutans decreased to 58% after exposure to 0.5 mg/l of ozonated water for 10 s, and S. mutans was killed instantaneously in ozonated water (2 and 4 mg/l). The cell viabilities of S. sobrinus, S. sanguis and S. salivarius were very similar to that of S. mutans when the cells were exposed to ozonated water. The deductions put forth by their study were that the number of viable cells was significantly decreased when P. gingivalis, P. endodontalis were treated with ozonated water (0.5, 2, and 4 mg/l) and second and when it was maintained at 22^{o}C for 180 min, the concentration and bactericidal activity decreased with time whereas when it was stored on ice, bactericidal activity was maintained for 180 min.

Nagayoshi M, Kitamura C, Fukuizumi T, Nishihara T, Terashita M [(39)] (2004) performed a study to determine the Antimicrobial Effect of Ozonated Water on Bacteria Invading Dentinal Tubules. Freshly extracted bovine incisors with apical 5-mm portion of the root were selected. *E. faecalis* ATCC 29212 and *S. mutans* were cultured in brain-heart infusion (BHI) broth at 37°C for 18 h in an atmosphere of 5% CO2 in air. The blocks were kept in BHI broth inoculated with *E. faecalis* or *S. mutans* and incubated for 6 days. Each specimen was irrigated by flushing for10 min with the following solutions: 4 mg/L of ozonated water, (O3aq), 4 mg/L of O3aq with ultrasonication (US), distilled water (DW), or DW with US. To assess the MTT Assay for Cytotoxicity with Disinfectants L-929 mouse fibroblasts were cultured in minimum essential medium. Fibroblasts were rinsed, trypsin zed, and seeded at density of 2 *10^{5} cells/100 MEM/ well in 96-well plates. Each of the following solutions was added to the wells: phosphate-buffered saline (PBS), DW, 6 mg/L of O3aq (final concentration 4 mg/L), or 3.75% NaOCl (final concentration 2.5%). They found that After irrigation with ozonated water, the viability of *E. faecalis* and *S. mutans* invading dentinal tubules significantly decreased. When the specimen was irrigated with sonication, ozonated water had nearly the same antimicrobial activity as

2.5% sodium hypochlorite (NaOCl). The metabolic activity of fibroblasts was high when the cells were treated with ozonated water, whereas that of fibroblasts significantly decreased when the cells were treated with 2.5% NaOCl. They inferred that ozonated water application may be useful for endodontic therapy.

Estrela C, Estrela CRA, Decurcio DA, Hollanda ACB, Silva JA [40] (2007) performed a study to determine the antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in infected human root canals by e. faecalis. In the study, 30 human maxillary anterior teeth were prepared and inoculated with E. faecalis for 60 days. Eppendorf tubes were connected to the coronal portion of the teeth. Urethane hoses were attached to the tubes and to the entrance of a peristaltic pump. The exit of the apparatus was corresponding to the apical portion of the root canals. The test irrigating solutions were ozonated water, gaseous ozone, 2.5% sodium hypochlorite (NaOCl), 2% chlorhexidine that circulated at a constant flow of 50 mL/min for20 min. Samples from the root canals were collected and immersed in 7 mL Letheen Broth, followed by incubation at 37^{o}C for 48 h. Bacterial growth was analysed by turbidity of the culture medium and subculture on a specific nutrient broth. A 0.1 mL inoculum obtained from LB was transferred to 7 mL of brain heart infusion and incubated at 37^{o}C for 48h. Bacterial growth was checked by turbidity of the culture medium. They found that the irrigation of infected human root canals with ozonated water, 2.5% NaOCl, 2% chlorhexidine, or the application of gaseous ozone was not sufficient to inactivate *E. faecalis.* They concluded that irrigation of infected human root canals with ozonated water, 2.5% NaOCl, 2% chlorhexidine and the application of gaseous ozone for20 min was not sufficient to inactivate E. faecalis.

Cardoso MG, de Oliveira LD, Koga-Ito CY, Jorge AOC.[41] (2008), conducted an in- vitro study to test Effectiveness of ozonated water on *Candida albicans, Enterococcus faecalis,* and endotoxins in root canals. In his study, 24 single-rooted human teeth were inoculated with *C. albicans* and *E. faecalis*, and with *Escherichia coli* endotoxin. For the preparation of ozonated water, ozonation of the water was performed by bubbling ozone through sterile distilled water (O3 concentration 24 mg/L). Twenty-one days after the inoculation, contamination confirmation sampling was performed using #35sterile paper points that was placed inside the root canal for 1 min and then transferred to test tubes (Eppendorf) containing physiologic solution. Root canals were enlarged to a size 80 K-file. Instrumentation was followed by irrigation with 3 mL of the irrigating agent (ozonated water or sterile physiologic solution) for each file used. The irrigation was performed with pyrogenic syringe with needle and aspiration device. After the instrumentation, the second sampling of the root canals was performed. Antimicrobial effectiveness was evaluated by the reduction of microbial counts. They found that ozonated water significantly reduced the number of *C. albicans* and *E. faecalis* at the immediate sampling and concluded that ozonated water was effective against *C. albicans* and *E. faecalis*

Huth KC, Quirling M, Maier S, Kamereck K, Alkhayer M, Paschos E, et al. [42] (2009) performed a study to assess the effectiveness of ozone against endodontopathogenic microorganisms in a root canal biofilm model. They aimed to assess the antimicrobial efficacy of aqueous (1.25–20 lg mL)1) and gaseous ozone (1–53 g m)3) as an alternative antiseptic against endodontic pathogens in suspension and a biofilm model. In their study, Enterococcus faecalis, Candida albicans, Pepto streptococcus micros and Pseudomonas aeruginosa were grown in planktonic culture or in mono-species biofilms in root canals for 3 weeks. Cultures were exposed to ozone, sodium hypochlorite (NaOCl; 5.25%, 2.25%), chlorhexidine digluconate (CHX; 2%), hydrogen peroxide (H2O2; 3%) and phosphate buffered saline (control) for 1 min and the remaining colony forming units counted. They found that aqueous ozone completely eliminated E. faecalis and C. albicans when used in concentrations down to 5 lg mL)1, whereas lower concentrations (2.5 and 1.25 g/mL) reduced substantially but did not eliminate them totally. In the case of P. micros, aqueous ozone down to 2.5 g/mL) led to complete eradication whilst 1.25 g/mL) was less effective. In comparison, NaOCl and CHX led to a total elimination of the tested microorganisms. Aqueous ozone in the highest concentration (20 g/mL), 1 min) nearly eliminated E. faecalis, C. albicans and P. aeruginosa biofilms. They concluded that High-concentrated gaseous and aqueous ozone was dose-, strain- and time-dependently effective against the tested microorganisms in suspension and the biofilm test model.

Gozitans Z, Onate H, To sun G,[43] et al (2014) performed a study on to determine antimicrobial effect of ozonated water, sodium hypochlorite and chlorhexidine gluconate in primary molar root canals. The aim was to determine the antimicrobial effect of ozonated water, ozonated water with ultrasonication, sodium hypochlorite and chlorhexidine (CHX) in human primary root canals contaminated by Enterococcus faecalis (E. faecalis). In the study, 45 extracted primary molars were selected. Crowns of the teeth were removed telementor‑enamel junction by a diamond saw to obtain80 roots. An access cavity orifice was made, pulpal tissue extirpated by using a barbed broach and the root canals were enlarged to a size 30. 80 root canals were inoculated by e. faecalis (ATCC 29212) for 24 h. Bacterial cells were adjusted to final concentration of about 1.5×10^8 colony forming units per ml. The samples were randomly separated into four experimental groups (n = 20). The canal of each specimen was irrigated for 4 min with the 5 ml of following solutions: 25 mg/L of ozonated water (O3aq),2.5% NaOCl and 2% CHX. Microbial analysis was performed with spectrometer. They found that NaOCI, CHX and two types of O3aq were found statistically different than positive control group. NaOCI irrigation was found significantly most effective. They concluded that NaOCl, CHX and O3aq applications provide antibacterial effect in vitro conditions in primary root canals.

Savitri D, Shetty S, Sm SC, Kb J, Gowda M, Rai N, et al. [(44)] (2018) evaluated the efficacy of Ozonated Water, 2% Chlorhexidine and 5.25% Sodium Hypochlorite on Five Microorganisms of Endodontic Infection by an In vitro Study. In their study, E. faecalis, S. mutans, S. aureus, K. rhizophila strains cultured on blood agar and C. albicans cultured on Sabouraud dextrose agar. Antibacterial efficacy was evaluated using both agars well diffusion test and direct contact test. In Agar well-diffusion test, the turbidity was adjusted with normal isotonic saline which would result in a suspension approximately containing 1.5×10^8 CFU/ml. Four wells were cut in the agar medium using sterile templates of the diameter of 5 mm. 100 µl of antimicrobials were inoculated into the 4 wells. plates were preserved for 1 h at room temperature, and then incubation was done at 37°C for 24 h. E. faecalis and S. mutans were incubated in a candle extinction jar and other microbes incubated under aerobic conditions. For direct contact, In Eppendorf tube, 50 µl of antimicrobial agent was added and 50 µl of E. faecalis culture was adjusted to 0.5 McFarland (1.5 × 108

CFU/ml). After intervals of 2 min, 10 min, 20 min, 30 min and 1 h, 250 µl of sterile BHI broth was added and mixed well. The results showed that 2% chlorhexidine showed highest zone size and minimum colony forming units indicating its highest potency and ozonated water was showed the least efficacy with a significant difference between both groups.

Pinheiro SL, Cesar C, Augusto L, Cicotti MP, Eduardo C, Fontana CE, et al.[18](2018) performed a study to determine antimicrobial efficacy of 2.5% sodium hypochlorite, 2% chlorhexidine, and ozonated water as irrigants in mesiobuccal root canals with severe curvature of mandibular molars. In their study, 60 permanent mandibular molars were selected and the degree of curvature of the mesiobuccal root canal was determined. The crowns were removed and root length was standardized to 15 mm. The root canals were contaminated with standard strains of *E. faecalis*, *S. mutans*, and *C. albicans*. Instrumentation was performed using the WaveOne Gold. The specimens were randomly divided into four groups (n = 15) according to irrigating solution: 2.5% sodium hypochlorite, 2% chlorhexidine, ozonated water (40 µg/mL); and control: double-distilled water. canals were irrigated with the solutions for 3 min, using 5 mL of the irrigating solution during the chemomechanical preparation, for a total of 20 mL irrigant. In all groups, before and after instrumentation, samples were collected for viable bacterial counts. They found that after instrumentation, sodium hypochlorite, chlorhexidine, and ozonated water produced a significantly reduction in bacterial counts compared with double-distilled water and concluded that ozonated water may be an option for microbial reduction in the root canal system.

In-vivo studies:-

Ajay SS, Bhede RR, Chandak MG, Manwar NU, Nikhade PP[45](2011) performed a study to evaluate antimicrobial property of ozone in comparison with 3% sodium hypochlorite in eradication of enterococcus faecalis. In their study, 30 healthy individuals under the age group of 25-30 years, who required retreatment of previously performed root canal therapy, were included in the study. After rubber dam application in complete aseptic conditions Gutta-percha was removed by hand instrumentation and apical access was obtained. The patients were divided into three groups based on the solution used for irrigating the canal. In group 1 gaseous ozone at the rate of 5.8 cm3 atmospheric pressure and 21°C for 60 sec through a nozzle connected to the device into the orifice of root canal. In group 2, freshly prepared ozonated water was used as an irrigants. In group 3, 3% sodium hypochlorite (NaOCl) was used. Enterococcus faecalis was isolated and counted on colony forming unit. They found that reduction in bacterial colony count was seen in patients treated with aqueous ozone and 3% sodium hypochlorite, while minimal reduction was observed on samples of gaseous ozone. They concluded that gaseous ozone does reduce number of E. faecalis in planktonic form and had a minimal effect on E. faecalis biofilm. The aqueous ozone and 3% sodium hypochlorite was more effective in reducing E. faecalis Strain in planktonic phenotype

Review article: -

Mohammadi Z, Shalavi S, Soltani MK, Asgary S [46] (2013) wrote a review titled- A Review of the Properties and Applications of Ozone in Endodontics: An Update which enlightens on a brief review on the chemistry of ozone as well as its medical and dental applications focusing on its use in endodontics. Ozone's antimicrobial activity and its effect on dentin bonding. The review mentions the Ozone gas in a ~4 g/m3 concentration is used clinically for endodontic treatments.

K. Srinivasan and S. Chitra[14](2015) reviewed the Application of Ozone in Dentistry: A Systematic Review of Literature. They enlightened about the various forms of ozone used in dentistry and its antimicrobial effect. These are- Ozone gas, Ozonated water and Ozonated oil. These forms of application are used singly or in combination to treat dental disease. The anti- microbial effect is due to the high oxidation potential which is1.5 times greater than chloride when used as an antimicrobial agent. There is oxidant action on cells by damaging its cytoplasmic membrane due to ozonolysis of dual bonds and also Ozone-induced modification of intracellular contents because of secondary oxidant effects. It does not damage healthy human body cells because they have free radical scavengers like superoxide dismutase, catalase, etc. which inhibit the uncontrolled activity of free radicals. It is very efficient against the antibiotic resistant strains. In viral infections, Ozone relies on the susceptibility of infected cells to peroxides and mutates the reverse transcriptase, which takes part in synthesis of viral proteins.

Shiva Gupta, D. Deepa[13], (2016) Applications of ozone therapy in dentistry. German chemist Christian Friedrich Schonbein, of University of Basel in Switzerland was the first to discover ozone in 1840. The first ozone generator was developed by Werner Von Siemens (1857) in Germany and was first applied in medical field by Dr. C. Lender in 1870 to purify blood in test tubes. The first ozone generator for medical use was developed by Joachim Hänsler, a German physicist and Hans Wolff, a

German physician in 1957. However, it was only at the end of 1980s, medical ozone became a subject of dental research and practice. Subsequent to this, it was used increasingly for medical and dental purposes.

Silva EJNL, Prado MC, Soares DN, Hecksher F, Martins JNR, Fidalgo TKS. [47] **(2019)** performed a systemtic review- The effect of ozone therapy in root canal disinfection: a systematic review. The electronic databases PubMed, Science Direct, Scopus, Web of Science and Open Grey were searched for articles published until 2 November 2018. The search resulted in 180 published studies. After removal of duplicate studies and full-text analysis, eight studies were selected and seven were considered low risk of bias- seven ex vivo studies and one random clinical trial. He opined that ozone therapy provides significantly less microbial load reduction than NaOCl. Ozone performance was strongly associated with the application protocol used: it is dose, time and bacterial strain dependent, besides the correlation with the use of complementary disinfection sources. And he also mentioned that due to restricted number of randomized clinical trial and the difference amongst the methodology hindered a performance of meta-analysis.

MATERIALS AND METHOD

The present randomized, double- blinded study was carried out in the department of pediatric and preventive dentistry of the concerned dental college to evaluate and compare anti- microbial efficacy of sodium hypochlorite and aqueous ozone as root canal irrigant in non-vital primary teeth. The study was carried out after obtaining ethical clearance from the institutional ethics committee. Parent were explained the purpose and methodology of the study in local vernacular language and a signed informed consent with child's assent was obtained.

SAMPLE SIZE

Statistician was consulted to calculate the sample size for the study. The total sample size i.e. number of subjects to be included in the study was based on results of previous studies.

The formula used for estimation of sample size was as follows:-

$$n = \frac{(Z_{1-\alpha} + Z_{1-\beta})^2 (s1 + s2)^2}{(\mu1 - \mu2)^2}$$

Where,

($\mu1 - \mu2$) = mean difference

S1 and s2= are the standard deviation of the first and second group respectively.

$Z_{1-\alpha}$ and $Z_{1-\beta}$ are values for Standardized normal variate (Z) for specified alpha and beta errors respectively.

α error=5%

power of study($1-\beta$) = 80%

A sample size of 72 was obtained i.e. samples were collected from 72 teeth one canal each tooth. This study has three groups and accordingly 24 samples per group were required.

SAMPLING METHOD:- convenience sampling

Screening of children visiting the Department of Pediatric and preventive dentistry of the concerned college, for routine dental care was carried out and selected for the study as per the following selection criteria.

Inclusion criteria:

1. Children 3-7 years of age of both gender.
2. Healthy children without any systemic illness.
3. Teeth indicated for pulpectomy showing one or more clinical signs and symptoms such as tenderness to percussion, an abscess, sinus tract, fistula were selected [52,53,54,55]

4. Radiographically, deep carious lesions involving more than half of dentin and approaching pulp chamber, or involving pulp chamber with or without periradicular radiolucency in the furcation area
5. Teeth with intact roots or less than 2/3rd of physiological root resorption.

The samples were collected from patients complying to either of the clinical signs or symptoms, with either of the radiographic findings of the inclusion criteria from any of the primary teeth i.e. molars [maxillary of mandibular] or incisors were included in the study.

Exclusion criteria:

1. Any tooth found to be vital, upon opening the pulp chamber.
2. Patient with the history of antibiotic consumption within the past three months from the time of diagnosis.
3. Teeth excluded from the study were showing radiographic evidence of internal root resorption, perforated pulpal floor, excessive bone loss in furcation area involving underlying tooth germ.

STUDY DESIGN:- A randomized, double- blind study.

Patient and the microbiologist assessing the treatment outcome were blinded to the treatment allocation.

TREATMENT ALLOCATION:

The participants were allocated treatment according to simple block randomization list generated by the SEALED ENVELOPE software[56]. The three treatment groups i.e. Group A, Group B, Group C and the list length i.e. 72 were fed into the software and the block size of 6 was used. The software generated a block randomized list for 72 patients and respectively the root canal irrigant was used for each patient. The software generated a seed no 229564721059628- which is unique for every list generated and thereby helps in reviewing the list again in future.[56]

CLINICAL METHODOLOGY:

Preparation of access cavity:-

After allocation to treatment group, local anaesthesia with lignocaine containing 1:2,00,000 adrenaline was administered. An aseptic technique was used throughout the endodontic treatment. Before rubber dam isolation, each tooth which had supragingival biofilms were removed by scaling. Following the single tooth isolation using rubber dam (Hygienic, Coltene Whaledent) antisepsis of the was performed of the crown, the surrounding rubber dam with 10% povidone-iodine solution (Betadine). A high-speed handpiece and sterile round bur BR 46 (Mani, Inc., Dental products, Japan) were used to remove the carious tooth structure or restoration and access to the root canal was achieved. The walls were modified using safe end cutting diamond point (Mani, Inc., Tochigi, Japan).[49]

Method for biomechanical preparation

The sample were collected from teeth containing little or no pulp tissue upon access opening or contained at least one necrotic root canal were selected. In case of multirooted teeth with no pulp tissue, a single root canal was sampled, in order to confine the microbiological evaluation to single ecologic environment. The criteria used to select a canal to be microbiologically investigated for multirooted teeth, was the presence of exudation or in it's absence the largest canal, of canal related to periradicular radiolucency.[57]

After initial entry into the pulp space, patency of the root canal was established using size 10 K-file with minimal instrumentation and without the use of any chemical active irrigant. Under aseptic conditions, initial pretreatment root canal culture sample was taken with sterile paper points.[50] A working length was determined using pre-operative radiograph and measured 1-2 mm short of the apex. Biomechanical preparation was performed using crown-down technique with PRO-AF-Baby-Gold Ni-Ti rotary instruments as recommended by Marwah N.[48] It is a five file system which has 4-6% constant taper. It also has a unique orifice opener[B_0] and is available in sizes like B1, B2, B3, B4, B5. To prepare and shape the mesiobuccal, distobuccal and mesiolingual canals B2[#25/04] followed by B3[#25/06] was used. To prepare and shape the distal and palatal canals B2[#25/04] followed by B4[#30/04] were used.

Depending on the disinfection protocol to be tested and the group allocation, the canals were treated according to the groups.

Group I (24 teeth) – after each instrumentation saline was used by sterile side-vented needle and syringe.

Group II (24 teeth) – after each instrumentation, irrigation was done with the help of of 2.5% sodium hypochlorite using 27gauge needle with side vented bore. 5% sodium thiosulfate was used as final irrigant to neutralize the effect of sodium hypochlorite.[51]

Group III (24 teeth) –. Ozonated water was produced from ozone unit (ADC Dentozone) by electric discharge bubbled into 1 L sterile distilled water. The generator produces ozone at a concentration of 250mg/hr. The canals were irrigated using 27 gauge needle with side vented bore.

A total of 10 mL of solution of the respective test group was used during instrumentation. After the root canal preparation was complete 5 mL of the respective irrigant was used as a final rinse.[58]

Method of ozonated water preparation:-

The ozone generator produces ozone gas at a rate of 250mg/hr.(4.166mg/min) So, to achieve the required concerntration only 1 min of exposure is required.

Because ozone gas is never absorbed 100% and it is affected by factors such as contact time, pH of distilled water.

Suloyman A.H.(2009)[72], the contact time should be 15 minutes to acquire a conc of 4mg/L

So, the pH of distilled water was kept in accordance with the above study to achieve the above concentration.

After the second sample collection, the teeth were treated using standard operating protocol with a week of calcium hydroxide intracanal medicament followed by obturation and final restoration.

Microbiological Sampling:-

1) Sample collection:

The preoperative samples were taken with the help of sterile paper points placed in the canal for 30s and then transferred into presterilized tube containing 2 ml of transport liquid media. After completion of biomechanical preparation, sterile paper point was again placed in the root canal for 30s to collect postoperative samples. Paper point was immediately put in test tube containing transport liquid media and sent for microbiology laboratory. Microbial processing of samples was done and bacterial counts were measured by CFU in all the three study groups.[50] All the vials were labelled and contained patient identification number, tooth no., age/ sex, date, study group.

2)Preparation of Liquid Transport Medium:-

Liquid transport mediaTM,[HiMedias, Mumbai] was prepared as per manufacturer's instructions. As per the instructions, 18.5gm of the media requires to be suspended in 1L of distilled water. Since, 500ml was prepared, 0.940 grams of media was added and it was heated to completely dissolve the medium then it was autoclaved at 121^{o} C.

3)Microbiological culturing and identification of micro-organsims:-

Immediately on receipt in the laboratory, each sample was Vortex (Biorad BR-200 vortexer) for 30 s and plated on to Columbia blood agar and anaerobic blood agar by spread plate technique. 50 μl each of pre and the post samples collected from each patient was added on the media plates. The Columbia agar plates were kept for incubation at 37°C for 24 hours in the incubator. The Anaerobic blood agar base plates were kept with Anaero Gas Pack. (LE002A- Himedia**)** in the sealed 1.5 L jar and were kept for the incubation. The number of colonies was recorded. Identification of microorganisms was based on growth in the anaerobic chamber and growth in the aerobic incubator with 5% CO_2, colonial pigmentation, colonial morphology, Gram stain, and biochemical tests as done by Baumgartner C.[20] The streptococcus and enterococcus species was identified on Columbia agar. The colonies which were non-hemolytic, circular, convex colonies were identified as e. faecalis. Some strains of e.faecalis can grow with alpha-hemolysis whereas beta-hemolytic strains are rare. Cultivation 24 hours,37°C in an aerobic atmosphere.[59] The colonies of streptococcus are smooth, glossy, and mucoid.[60,61]

On anaerobic media, the Prevotella species have a colony morphology which is pinpointed black/brown pigmented and non-pigmented moist colonies of Prevotella species.[63] The porphyromonas species showed small, shiny, circular, black-pigmented, and mucoid colonies with or without hemolysis.[62] The P.micros had small, dome-shaped, bright white, nonhemolytic colonies on agar plates.[64]

Identification of microorganism using biochemical test and gram staining:

Gram staining:-

Procedure:-

1. The slide was placed on the slide holder or a rack.
2. **The primary stain (crystal violet) was** applied for 60 seconds followed by rinsing with water for 5 seconds.
3. The iodine solution (mordant) was added to form a crystal violet-iodine (CV-I) complex. It was kept for a minute and rinsed with water for 5 seconds
4. The decolorization agent that is solvent such as acetone or ethanol was added which extracts the blue dye complex. The ethanol was added dropwise until the blue-violet color was no longer seen from specimen, followed by rinsing with the water for 5 seconds..
5. The red dye safranin was added by flooding the slide with the dye as in steps 1 and 2. It was kept for a minute to allow the bacteria to incorporate the

saffranin and again, rinsed with water for 5 seconds to remove any excess of dye.

Biochemical test:

Sugar fermentation test:-

Procedure:-

1. Basal medium(peptone water) containing a single carbohydrate source such as glucose, lactose, sucrose and mannitol was added in a concentration of 0.5-1%.
2. Bromothymol blue was present in the medium as an indicator.
3. The medium was inoculated with isolated colonies from the culture of the organism.
4. The inoculated media was incubated aerobically at 35-37ºC. for 3-5 days.
5. It was observed daily for development of a yellow color in the medium.

Catalase test:-

Procedure:-

1. A loop or sterile wooden stick was used to transfer a small amount of colony growth in the surface of a clean, dry glass slide.

2. A drop of 3% H_2O_2 was placed on the glass slide and observed for the evolution of oxygen bubbles.

Nitrate Reduction test:

Procedure:

1. The nitrate broths were inoculated with bacterial suspension.

2. The tubes were inoculated at the optimal temperature 30°C or 37°C for 24 hours.

3. They were checked for N2 gas first before adding reagents.
4. 6-8 drops of nitrite reagent A followed by nitrite reagent B were added.
5. The reaction was observed for color development for a minute or less.
6. Zinc powder was added to see the red color development if no color development was seen within a minute in step 5.

Method of Colony Counting:

The colony counting was done manually. Using the plate count method or spread plate technique, the colonies visible to the naked eye were counted. Since only a part of medium of medium was inoculated the total number of colonies (CFU/ml)was determined by formula,

cfu/ml = (no. of colonies x dilution factor) / volume of culture plate.

Materials and equipment:-

A. Sterile disposable gloves, mouth mask and head cap **(Colour plate 1, fig. 1)**

B. Oral examination diagnostic instruments - mouth mirror, probe, and tweezer. **(Colour plate 1, fig. 2)**

C. Rubber dam kit **(Colour plate 2, fig. 3)**

D. biomechanical preparation instruments and materials.

- Airotor hand piece, Spoon excavator, **(Colour plate 2, fig.4)**
- Burs used: small Round bur (no.4). burs- for access opening, **(Colour plate 2, fig. 4)**
- Hand K- files and rotary files for cleaning and shaping of the root canal**(Colour plate 2, fig. 4)**
- Endomotor **(Colour plate 3, fig. 5)**
    - Rotary handpiece**(Colour plate 3, fig. 6)**

- Syringe with side-vented needle used for irrigation. **(Colour plate 4, fig. 7)**

E. transport media:

- Transport liquid media **(Colour plate 4 and 5, fig.8,9,10)**

F. for microbial analysis:

- Paperpoints**(Colour plate 5, fig. 11)**
- Blood agar [HiMedia Labs, Mumbai] **(Colour plate 5, fig.12)**
- Anaerobic blood agar[HiMedia Labs, Mumbai] **(Colour plate 6, fig. 13)**
- Anaero gas pack**(Colour plate 6, fig.14)**

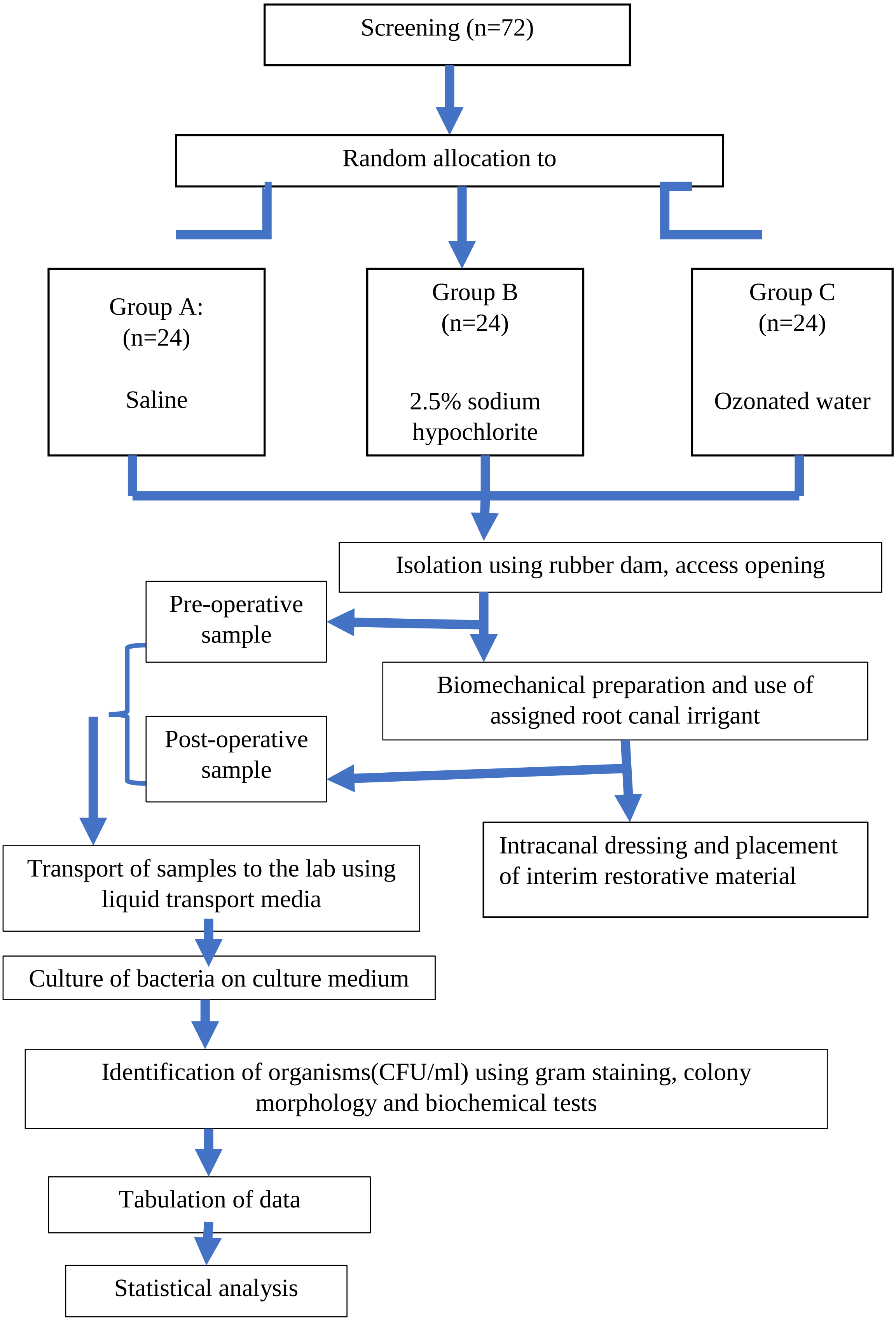
G. Ozonated water irrigation– steps in preparation of ozonated water **(Colour plate 7, fig.15,16)**

The application of rubber dam followed by povidone iodine scrub and sample collection has been represented in**(Colour plate 8and 9, fig. 17,18,19,20)**.

H. Growth of bacteria and identification:

- Culture demonstrating growth of organsims **(Color plate 11, fig. 21,22,)**
- Colony counting- **(Color Plate 12 fig 23)**
- Gram staining- **(Color Plate 12,13 fig 24,25)**
- Catalase test **(Color Plate 13 fig 26)**
- Biochemical tests- sugar fermentation test, nitrate reduction test, catalase test **(Color Plate 14, fig 27)**
- Nitrate reduction test **(color plate 14, fig 28)**

Study flow chart



COLOUR PLATE 1

Fig.1- Disposable pair of gloves, head-cap and mouth-mask.

Fig.2- diagnostic instruments- mouth- mirror, probe and tweezer

COLOUR PLATE 2

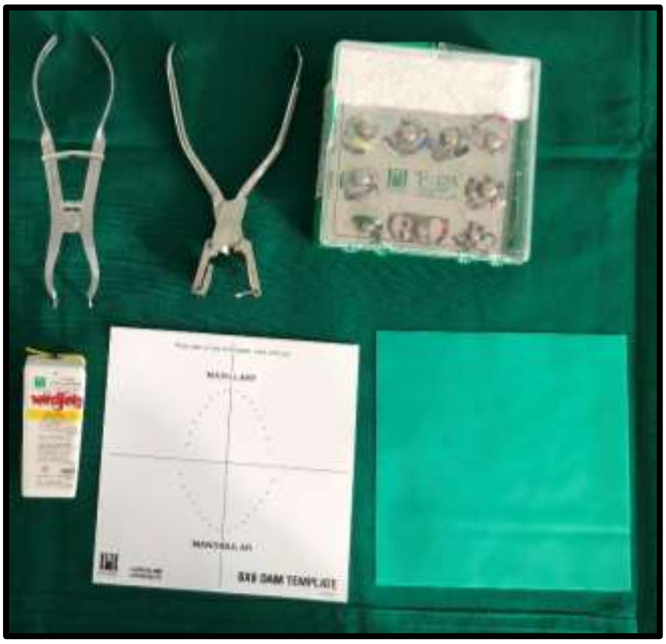


Fig. 3- rubber dam kit- clamp forcep, rubber dam punch, clamps, template, rubber dam sheets and wedjets.

Fig.4- instruments used for access opening and biomechanical preparation

COLOUR PLATE 3

Fig.5- Endomotor alongwith rotary handpiece used for biomechanical preparation

Fig.6- rotary files used for biomechanical preparation [Baby PRO AF system]

COLOUR PLATE 4

Fig.7- side-vented used for endodontic irrigation

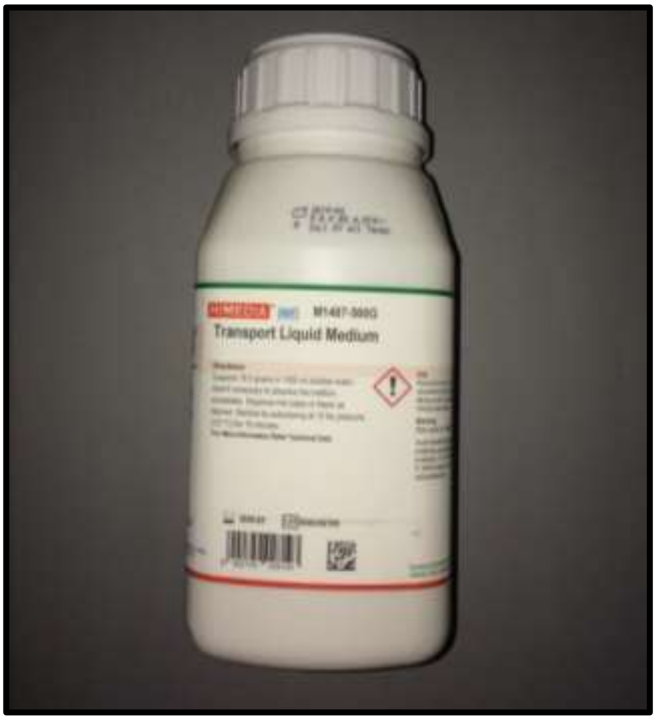


Fig.8- transport medium

COLOUR PLATE 5

Fig.9- transport media for preparation

Fig.10- transport media for collection of sample

COLOUR PLATE 6

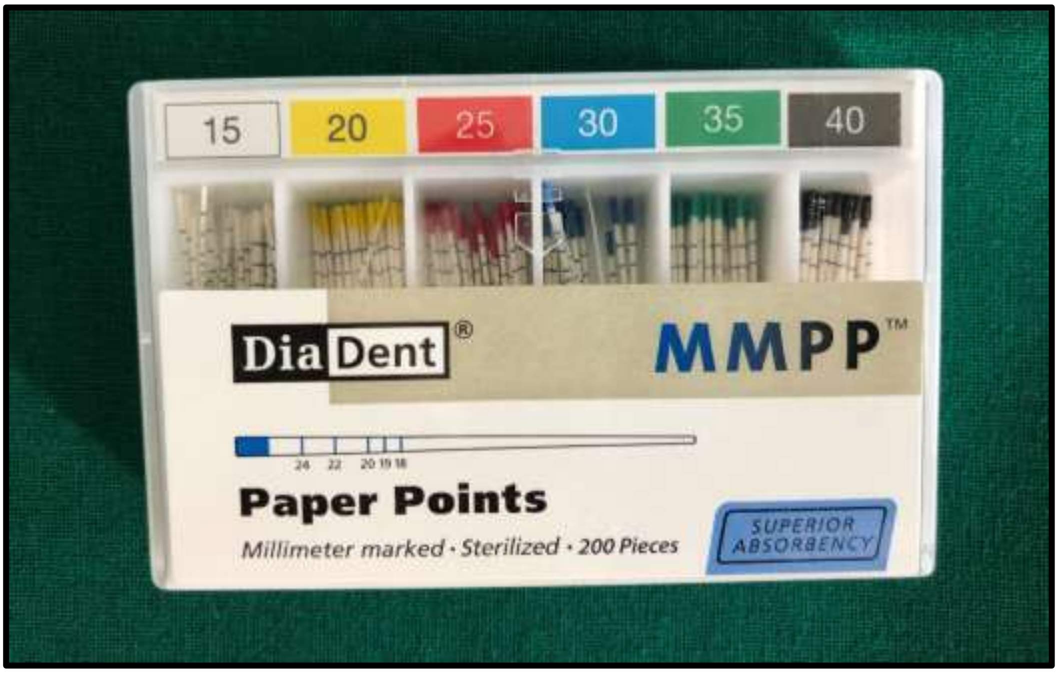


Fig. 11- paperpoints used for sample collection

Fig.12- Anaerobic media used for growth of anaerobic organisms

COLOUR PLATE 7

Fig.13- Colombia blood agar for growth of aerobes

Fig.14- anaerobic pouch for incubation of anaerobic organisms

COLOUR PLATE 8

Fig.15- ozone generator machine

Fig.16- Preparation of ozonated water through bubbling of ozone gas in distilled water

COLOUR PLATE 9

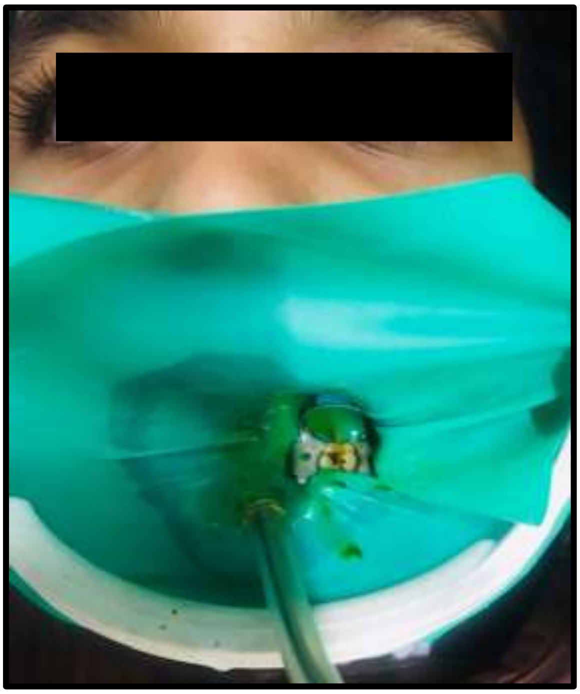


Fig.17- rubber dam application and povidone iodine scrub before access opening

Fig.18- insertion of paper point for collection of sample

COLOUR PLATE 10

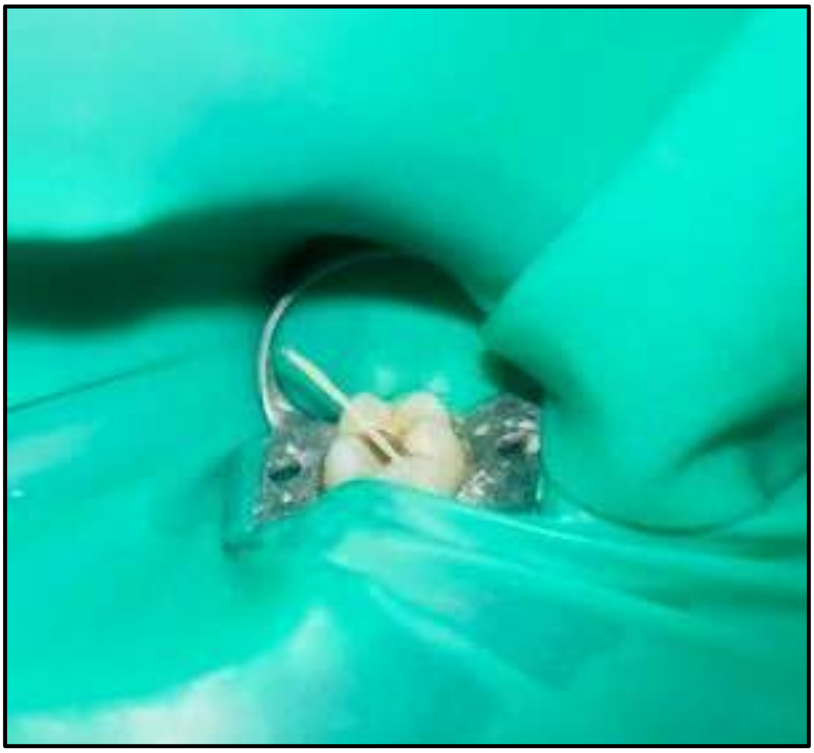


Fig.19- magnified picture showing paper point insertion for collection of sample

Fig.20- after removal the paper points were inserted in transport medium

COLOUR PLATE 11

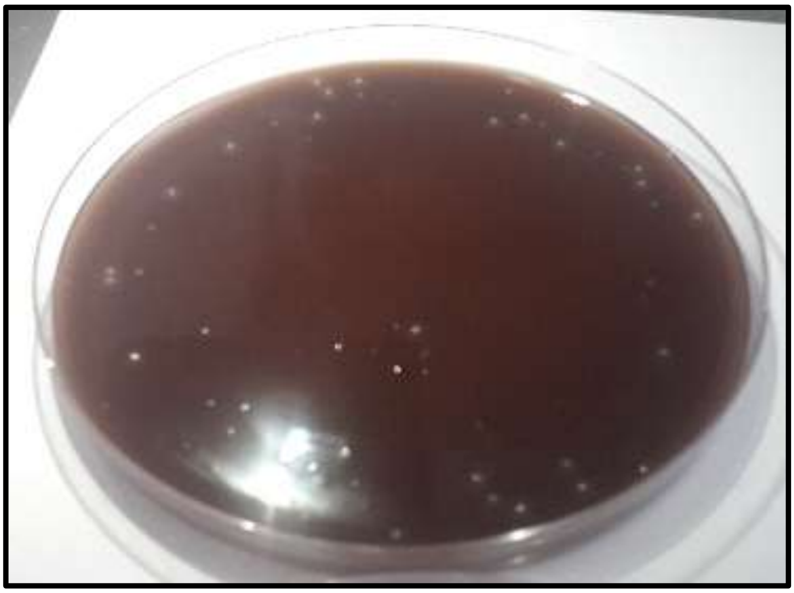


Figure 21- growth of micro-organisms on aerobic medium

Figure 22- growth of micro-organisms on anaerobic media

COLOUR PLATE- 12

Figure 23: colony counting

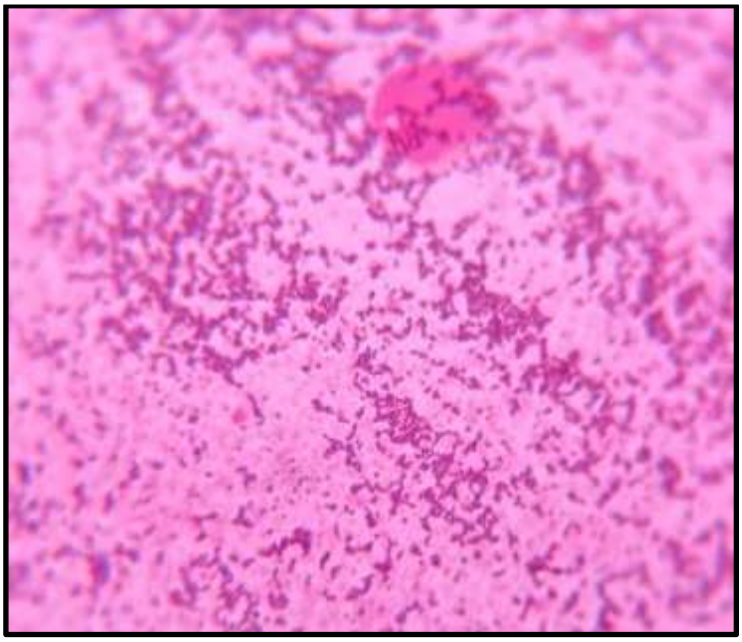


Figure 24: Gram stain- gram positive bacteria

COLOR PLATE- 13

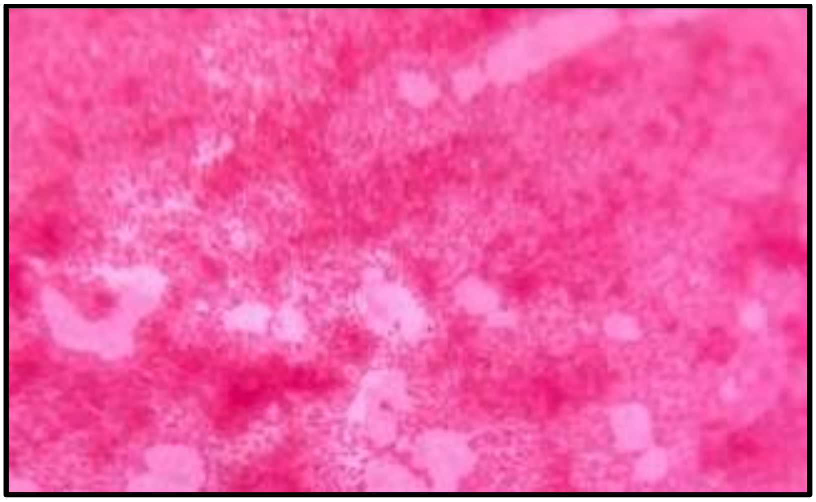


Figure 25: gram stain:- gram negative rods

Figure 26:- Catalase test (Positive)

COLOR PLATE 14

Fig 27: sugar fermentation test. Control(left) and test(right)

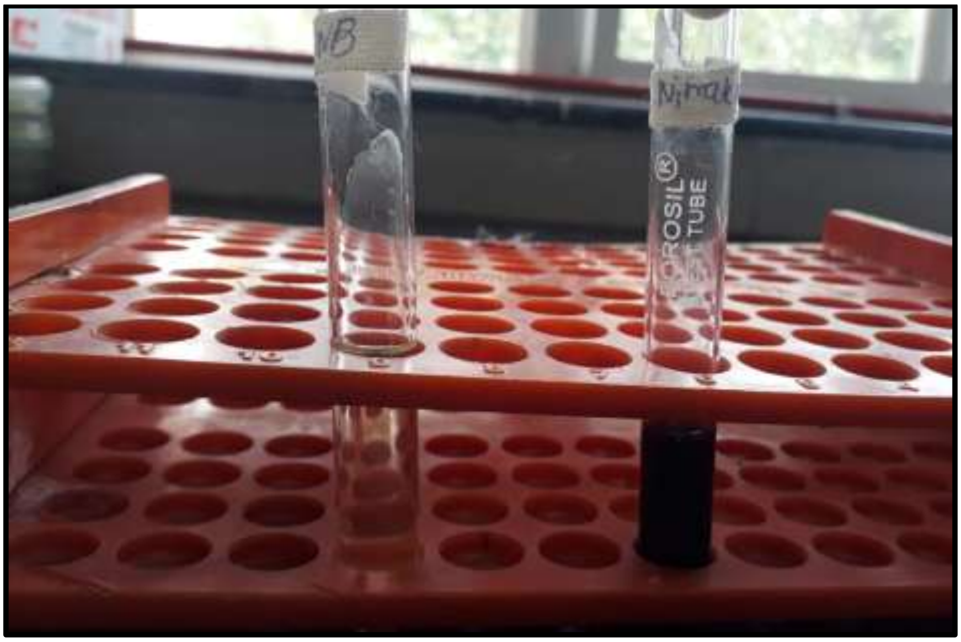


Fig 28: Nitrate Reduction test (Positive)

RESULTS

The present study was double blind, randomized controlled clinical study. 72 children aged 3-7 years, comprising of 32 boys and 40 girls were enrolled based on the inclusion criteria and were randomly divided into 3 groups of 24 each. The antimicrobial efficacy of aqueous ozone, 2.5% sodium hypochlorite, and normal saline as root canal irrigants was compared.

Group A: Normal saline

Group B: 2.5% sodium hypochlorite

Group C: Aqueous Ozone

The samples were collected from 30 maxillary teeth and 42 mandibular teeth. The distribution of sample collection across the three groups is as given in table 1. Each group consisted of 24 samples. In Group A i.e 0.9% normal saline 11 samples were collected from maxillary teeth and 13 samples were taken from mandibular teeth. In Group B i.e. 2.5% sodium hypochlorite which 8 samples were collected from maxillary teeth and 16 from mandibular teeth. In group C i.e. Aqueous ozone 24 samples were collected, out of which 11 samples were collected from maxillary teeth and 13 were collected from mandibular teeth.

The bacterial count was measured twice in the single visit. The initial bacterial count [pre-irrigation] was determined after access opening and final bacterial count[post-irrigation] was collected after biomechanical preparation. This reduction in bacteria across the same visit was converted to percentage bacterial reduction. Thereupon, an appropriate statistical analysis was carried out.

Statistical methods:

The greater the log reduction the more effective the product is at killing bacteria and other pathogens. 'Log' is short for logarithm, a mathematical term for a power to which a number can be raised. A log reduction takes the power in the opposite direction. For example, if the number of CFUs in the control was found to be 1,000,000 (or 10^6) and the end result using the product was only 1,000 (10^3), that would be a Log reduction of 3 or a reduction of 99.9%. The comparison of bacterial reduction in pre-irrigation and post-irrigation count was carried out using Paired t-test. To determine the difference between the inter-irrigation group one-way Anova was applied followed by post-hoc Tukey test was applied. The software used in the analysis were SPSS 21.0, Graph Pad Prism 7.0 version and $p<0.05$ is considered as level of significance.

The details of statistical methods are as used below:-

If x1, x2,xn are the observations on random variable X, then

Sample mean for a set of observations is given by

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$$

Standard deviation for a set of observations in given by

$$s = \sqrt{\frac{1}{(n-1)}\sum_{i=1}^{n}(x_i - \bar{x})^2}$$

Where,

x_i = observation on each object

n = number of objects

Paired t-test

The method is typically used for assessing the effectiveness of an experimental procedure that makes use of related observations resulting from dependent samples. The hypothesis test based on this type of data is known as paired comparison test. For n sample differences computed from n pairs of measurements, which are distributed normally, the test statistic for testing hypothesis about population mean difference μ_d is given by:

$$t = \frac{\bar{d} - \mu_d}{s_d / \sqrt{n}}$$

Where d is the sample mean, μ_d is the hypothesized population mean difference, SD

is the standard deviation of sample difference. Under the assumption that H0 is true, the test statistic is distributed as Student‟s t with n-1 degrees of freedom

Analysis of Variance test (ANOVA)

Analysis of variance (ANOVA) is a collection of statistical models and their associated estimation procedures (such as the "variation" among and between groups) used to analyze the differences among means. ANOVA was developed by the statistician Ronald Fisher. The ANOVA is based on the law of total variance, where the observed variance in a particular variable is partitioned into components attributable to different sources of variation. In its simplest form, ANOVA provides a statistical test of whether two or more population means are equal, and therefore generalizes the t-test beyond two means. At this point, it is important to realize that the one-way ANOVA is an **omnibus** test statistic and cannot tell you which specific groups were statistically significantly different from each other, only that at least two groups were. To determine which specific groups differed from each other, you need to use a **post hoc test.**

$$F = \frac{\text{Variance within the treatments}}{\text{Variance between the treatments}}$$

Post Hoc Tukey’s Test

Tukey’s test is known by several different names. Tukey's test compares the means of all treatments to the mean of every other treatment and is considered the best available method in cases when confidence intervals are desired or if sample sizes are unequal.

The test statistic used in Tukey's test is denoted qq and is essentially a modified t-statistic that corrects for multiple comparisons. q_q can be found similarly to the t-statistic:

$$q_{\alpha,k,N-k}$$

The studentized range distribution of qq is defined as:

$$q_s = \frac{Y_{max} - Y_{min}}{SE}$$

Where Y_{max} and Y_{min} are the larger and smaller means of the two groups being compared. SE is defined as the standard error of the entire design

Antimicrobial efficacy against Streptococcus mutans (S. mutans) across the irrigant groups:

The mean pre-irrigation count for s.mutans in group A, group B and group C was 1.89×10^6 , 1.49×10^7, 1.25×10^7 cfu/ml respectively whereas the post-irrigation count was 2.52×10^4, 9.20×10^2, 4.10×10^2cfu/ml respectively. On intragroup comparison between the pre and post-irrigation counts in all the three group A, group B, group C, using the paired t-test it was found that, there was statictically significant difference (table 2). The mean log reduction of s. mutans bacteria in group A was 90.3383%, in Group B was 99.3771% and in group C it was 99.4054%(table 3). On intergroup comparison by applying the Anova test significant difference was found between the irrigants for log reduction of s.mutans. On applying post-hoc Tukey test, a significant difference was observed between Group A & B, Group A & C (p- value 0.004). But the difference between group B and group C was insignificant (p-value- 1) for reduction of S. mutans. (table.4) All the three irrigants were effective in reducing the s.mutans count and on intergroup comparison 2.5% sodium hypochlorite and aqueous ozone were effective.

Antimicrobial efficacy of root canal irrigants against Porphyromonas gingivalis (P. Gingivalis):

The mean pre-irrigation count for P.gingivalis in group A, group B and group C was $1.46x10^6$, $8.39x10^6$, $1.80x10^6$ cfu/ml respectively whereas the post-irrigation count was $3.55x10^4$, $1.152x10^3$, $2.68x10^3$cfu/ml respectively. On intragroup comparison between the pre and post-irrigation counts in all the three group A, group B, group C, using the paired t-test it was found that, there was statictically significant difference (table 5). The mean log reduction of p.gingivalis bacteria in group A was 96.3604%, in Group B was 96.9808% and in group C it was 98.6992%(table 6). On intergroup comparison by applying the Anova test no statistically significant difference (p-value- 0.069) was found between the irrigants for log reduction of P.gingivalis(table 6). All the three irrigants were effective in reducing the p.gingivalis count and there was no significant difference in their antimicrobial activity.

Antimicrobial efficacy of root canal irrigants against Prevotella (Prevotella sp.):

The mean pre-irrigation count for Prevotella in group A, group B and group C was $7.86x10^6$, $1.89x10^6$, $5.13x10^6$ cfu/ml respectively whereas the post-irrigation count was $1.15x10^5$, $2.98x10^3$, $3.61x10^3$ cfu/ml respectively. On intragroup comparison between the pre and post-irrigation counts in the group A & group B using the paired t-test there was statictically significant difference but no significant difference was found between the pre and post irrigation counts in group (table.7). The mean log reduction of Prevotella sp. bacteria in group A was 90.5621%, in Group B was 99.0900% and in group C it was 99.0479%(table.8). On intergroup comparison by applying the Anova test significant difference was found between the irrigants for log reduction of prevotella sp. On applying post-hoc Tukey test, a significant difference was observed between Group A & B, Group A & C (p- value <0.0001). But the difference between group B and group C was insignificant (p-value- 0.997) for reduction of Prevotella sp . (table.9) All the three irrigants were effective in reducing the Prevotella sp. count and on intergroup comparison 2.5% sodium hypochlorite and aqueous ozone were effective.

Antimicrobial efficacy of root canal irrigants against Peptostreptococcus micros (P.micros):

The mean pre-irrigation count for P.micros in group A, group B and group C was $8.47x10^5$, $7.8x10^5$, $1.33x10^6$cfu/ml respectively whereas the post-irrigation count was $1.10x10^4$, $1.30x10^3$, $1.62x10^3$cfu/ml respectively. On intragroup comparison between the pre and post-irrigation counts in the group A using the paired t-test there was statictically significant difference but no significant difference was found between the pre and post irrigation counts in group B and group C(table.10). The mean log reduction of P.micros bacteria in group A was 79.4401%, in Group B was 96.0567% and in group C it was 98.9420%(table.11). On intergroup comparison by applying the Anova test, significant difference was found between the irrigants for log reduction of P.micros. On applying post-hoc Tukey test, a significant difference was observed between Group A & B, Group A & C (p- value <0.0001). But the difference between group B and group C was insignificant (p-value- 0.577) for reduction of P.micros (table.12) All the three irrigants were effective in reducing the P.micros count and on intergroup comparison 2.5% sodium hypochlorite and aqueous ozone were effective.

Antimicrobial efficacy of root canal irrigants against Enterococcus Faecalis (E.faecalis):

The mean pre-irrigation count for E.faecalis in group A, group B and group C was $2.33x10^5$, $1.36x0^6$, $4.92x10^6$cfu/ml respectively whereas the post-irrigation count was $3.41x10^4$, $2.12x10^3$, $4.30x10^2$cfu/ml respectively. On intragroup comparison between the pre and post-irrigation counts in the group A using the paired t-test there was statictically significant difference but no significant difference was found between the pre and post irrigation counts in group B and group C(table.13). The mean log reduction of E.faecalis bacteria in group A was 93.7489%, in Group B was 99.1775% and in group C it was 99.8050%(table.14). On intergroup comparison by applying the Anova test, significant difference was found between the irrigants for log reduction of E.faecalis. On applying post-hoc Tukey test, a significant difference was observed between Group A & B, Group A & C (p- value <0.0001). But the difference between group B and group C was insignificant (p-value- 0.857) for reduction of E.faecalis (table.15). All the three irrigants were effective in reducing the E.faecalis count and on intergroup comparison 2.5% sodium hypochlorite and aqueous ozone were effective.

DISCUSSION

Endodontic in primary teeth can be challenging and biomechanical preparation is considered as one of the important step in root canal therapy. It helps to preserve and maintain the function of severely carious primary teeth. The success of pulpectomy in primary teeth relies on the combination of proper instrumentation, irrigation and obturation of the root canal.[65] Bacteria have long been recognized as the cause of pulpal and periradicular diseases. Irrigants can augment mechanical debridement by flushing out debris, dissolving tissue, and disinfecting the root canal system.[66] The ideal requisites of a root canal irrigant as given by **Zehnder** are:[67]

1. Broad antimicrobial spectrum
2. High efficacy against anaerobic and facultative microorganisms organized in biofilms
3. Ability to dissolve necrotic pulp tissue remnants

4. Ability to inactivate endotoxin

5. Ability to prevent the formation of a smear layer during instrumentation or to dissolve the latter once it has formed.

6. Systemically nontoxic when they come in contact with vital tissues, noncaustic to periodontal tissues, and with little potential to cause an anaphylactic reaction.

Ismail S et al[65] (2017) in her review article mentions that no single irrigant has these optimal properties, and hence the use of two or more solutions in a specific sequence or in combinations should be used for better results. She also concluded that the most commonly used root canal irrigants in pediatric endodontics are normal saline and sodium hypochlorite. Physiological saline has no effect on removing dentinal debris and smear layer.

Sodium hypochlorite (NaOCl) though well-known and considered as an gold standard for endodontic usage. **Vianna et al [29,30] (2004)** tested antimicrobial activity of 2.5% sodium hypochlorite against endodontic pathogens like Prevotella sp., P.gingivalis, E.fecalis and found the similar result when used intra-orally. Their results are in accordance with our study. **Siqueira J[68]** found that though 2.5% sodium hypochlorite was effective in reducing the bacteria in root canals it could not render the canals completely free of bacteria. From the present study it can be concluded that 2.5% sodium hypochlorite is an effective concentration for reducing the bacterial population and its effectivity is 99.99% against anaerobic black pigmented bacteria, Streptococcus sp. and E.faecalis but it cannot render the root canal completely free of the bacteria and the results are in accordance with the aforementioned studies.

But it cannot be used at required concentrations in children due to its limitations such as unpleasant taste, ineffectiveness in removing smear layer, and incompletely eradicating microbes from the infected canals. Also, upon inadvertent extrusion it may cause rather toxic damage to the periapical environment on passing beyond the tooth apical foramina.[65,69]

Ozone was discovered in 1840, and is found in nature consisting of three oxygen atoms(O3), a higher energetic form than normal atmospheric oxygen (O2). Edward Fisch (1932) was the first to demonstrate the use of ozone in dental practice. However only by the end of 1980s, medical ozone was used for dental research and practice.(13)

Ozone is generated artificially through the commercially available devices by UV system, Cold plasma system, Corona discharge system. The corona discharge system generates a high concentration of ozone as a direct result of power dissipation. Ozone gas is formed when an oxygen molecule is acted upon by an electric discharge splitting it into two individual oxygen atoms. These individual atoms combine with another oxygen molecule to form an O3 molecule. The handling of this design is easy and the ozone production rate can be controlled, hence most commonly used in the medical and dental field.(13) When administered in gaseous or aqueous form for endodontic purpose, ozone has a powerful antimicrobial property due to the high oxidation potential which is 1.5 times as compared to chloride. It acts on the cell wall and cytoplasmic membranes of microorganisms, by damaging it due to ozonolysis of dual bonds and ozone-induced modification of intracellular contents because of secondary oxidant effects.(14)

Hence, in the search for an ideal irrigant for pediatric root canal procedure, we evaluated normal saline, 2.5% sodium hypochlorite and ozonated water, for their antimicrobial efficacy on the basis of their ability to reduce the number of bacteria.

Dr. Blass was one of the pioneers who used 5% sodium hypochlorite (chlorinated soda) as organic material solvent as well as potent germicide. Trepagnier& et al, through their in-vitro study, concluded that 5% sodium hypochlorite had a potential for the use as tissue solvent and he showed that dilution of this solution with equal amount of water (2.55%) did not appreciably alter its solvent action. Through his study it was brought to light that frequent and abundant irrigation with 2.5% sodium hypochlorite solution, contains enough amount of free chlorine to eliminate significant amounts of bacterial cells thereby compensating the irritant effect caused by use of higher concentrations. [69] **Vianna ME et al (2004)[29,30],** **Rocas I and Siqueira JF[68] (2011)** proved that 2.5% is effective in significantly reducing the number of bacterial taxa and their levels in infected root canals. **M. Hülsmann & W. Hahn[10](1999)** in their review article on sodium hypochlorite complications mentioned that the hypochlorite accident may occur on inadvertent injection beyond the apical foramen, either with wide apical foramina or if the apical constriction has been enlarged during root canal preparation or by physiologic resorption. They also stated that extreme pressure during irrigation or binding of the irrigation needle tip in the root canal with no space for the irrigant to leave the root canal coronally may lead to large volumes of the irrigant to come in contact with the apical tissues which will result in tissue necrosis. tissue necrosis. Therefore, 2.5% was chosen for the study to achieve the antibacterial concentration and considering the safety of sodium hypochlorite in children.

Ozone for dental use is supplied by Ozotop, Product photo (Prozone) by W and H, OzonyTron by MYMED, HealOzone by KaVo, Ozone generator by ADC Inclusives in India. The ozone generator by ADC Inclusives was used in this study which utilizes the corona discharge system to generate ozone gas. This ozone gas was then bubbled into distilled water to achieve aqueous ozone. The cost of the unit is Rs.18,000-20,000.[13]

In dentistry, ozone has been used in either gaseous or aqueous form as an antimicrobial agent while performing endodontic treatment. **Huth KC et al[16] (2006),** through his study which determined the effect of ozone on human oral epithelial (BHY) cells and gingival fibroblast (HGF-1) cells proved that Aqueous ozone is most compatible in comparison to established antiseptics like chlorhexidine digluconate (CHX) 2%, 0.2%; sodium hypochlorite (NaOCl) 5.25%, 2.25%; hydrogen peroxide (H2O2) 3%. They even investigated whether gaseous ozone ($4x10^6/gm^3$) or aqueous ozone (1.25–20 ug/ml) exert any cytotoxic effects and found that aqueous ozone is safer alternative to gaseous ozone. **Ajeti et al[70] (2018)** used gaseous ozone for root canal disinfection and found that it was most effective when combined with 2.5% sodium hypochlorite. Gaseous ozone has limitation being irritating to the respiratory system and even low concentrations may cause headache, and irritation or dryness of the nose, throat and eyes whereas the drawbacks reported with the use of higher concentration are lung congestions, oedema, haemorrhage, changes to the blood and loss of vital lung capacity. It is irritating to the eyes and can cause redness, pain and blurred vision. The aqueous ozone or ozonated water has its own advantages like its potency, lack of mutagenicity, rapid microbicidal effects and ease of handling. It has also been known to penetrate into the periapical tissue through the apical foramen, where it encourages tissue regeneration by oxygenating the tissue and increasing blood supply.[16] Hence, aqueous ozone was chosen for the study.

Aqueous ozone works best when there is less organic debris remaining. Therefore, the ozonated water or ozone gas should be used at the end of the cleaning and shaping process. It is better to use any conventional irrigants during the earlier phase and finally irrigate with ozonated water using ultrasonics for deeper penetration into dentinal tubules and to use ozonated oil as a medicament.[71]

Aqueous ozone also essentially shows no toxicity to oral cells as was observed *in vitro*. **Huth et al** conducted a study to evaluate the effect of aqueous and gaseous ozone on the specific endodontic pathogens and found that aqueous ozone completely eliminated E. faecalis when used in concentrations 5 microgram mL-1, whereas lower concentration reduced substantially but did not eliminate them totally and the other finding was that high-concentrated gaseous and aqueous ozone was dose, strain- and time-dependently effective against the tested microorganisms in suspension and therefore the biofilm test model.(42)

Ozone has other actions apart from bactericidal effect such as angiogenesis stimulation capability and high oxidising power. **Nagayoshi M et al .(39) (2004)** found that 4 mg/l concentration of ozonated water was effective against streptococcus sp., P.endodontalis, P.gingivalis and the bactericidal activity was maintained for 180 min when stored at 22oC and concluded that it should be useful in reducing the infections caused by oral microorganisms. **Estrela C et al.**(40) **(2007)** found that irrigation of infected human root canals with ozonated water, 2.5% NaOCl was not sufficient to eliminate E. faecalis. **Ajay SS**[45] **(2011)** found that the aqueous ozone and 3% sodium hypochlorite was more effective in reducing E. faecalis Strain in planktonic phenotype. **Gozitans et al**[43]**(2014)**compared the antimicrobial activity of 25 mg/L of ozonated water (O3aq),2.5% NaOCl and found that NaOCl and O3aq applications provide antibacterial effect in vitro conditions in primary root canals. Similar results were demonstrated by **Pinheiro et al** [18]**(2018)** concluded that ozonated water may be an option for microbial reduction in the root canal system. In the present study, 4mg/l concentration was used as documented in literature based on in-vitro studies. The results of our study are in accordance with the in-vitro studies. [45] The findings demonstrate that ozonated water is an effective root canal irrigant and has shown similar efficacy to 2.5% sodium hypochlorite and hence can be used as an safer alternative.

Limitations:-

1) A larger sample size is required to determine the efficacy of aqueous ozone as root canal irrigant in primary teeth.

2) High quality studies with different concentrations, strain- and time-dependent action of ozonated water need to be carried out in future to demonstrate the effectivity of aqueous ozone as root canal irrigant.

SUMMARY AND CONCLUSION

The present study was single blind, randomized controlled clinical study. 72 children aged 3-7 years, comprising of 32 boys and 40 girls were enrolled based on the inclusion criteria and were randomly divided into 3 groups of 24 each. The antimicrobial efficacy of aqueous ozone, 2.5% sodium hypochlorite, and normal saline as root canal irrigants was compared. The samples were collected from 30 maxillary teeth and 42 mandibular teeth. The bacterial count was measured twice in the single visit. The initial bacterial count [pre-irrigation] was determined after access opening and final bacterial count[post-irrigation] was collected after biomechanical preparation. This reduction in bacteria across the same visit was converted to percentage bacterial reduction. Thereupon, an appropriate statistical analysis was carried out.

Following conclusion were drawn from the study:

1. 2.5% sodium hypochlorite is an effective concentration for bacterial reduction of prevotella sp, S. mutans, E. faecalis, P.micros.

2. 2.5% sodium hypochlorite is 99.99% effective against these bacteria but it cannot render the canal, completely free of these bacteria.

3. Saline has the least Anti-microbial efficacy amongst all the tested irrigants and has reduced the bacterial by 90%

4. Aqueous ozone is an effective root canal irrigant for bacterial reduction of prevotella sp, S. mutans, E. faecalis, P.micros. and has shown reduction of these bacteria by 99.99 %

5. On comparison with 2.5% sodium hypochlorite the anti-microbial effectivity is not statistically significant. Hence it can be said that 2.5% sodium hypochlorite and Aqueous ozone are equally effective as root canal irrigants in infected teeth with non-vital pulp.

A larger sample size comparing different concentrations, strain- and time-dependent action of ozonated water need to be carried out in future to demonstrate the effectivity of aqueous ozone as root canal irrigant

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Tables

Table1: Distribution of sample collection across the three groups

Sr. no.	Groups	Maxillary teeth	Mandibular teeth	Total
1.	Group A [0.9% normal saline]	11	13	24
2.	Group B [2.5% sodium hypochlorite]	8	16	24
3.	Group C [aqueous ozone]	11	13	24

Table 2: intragroup comparison of S.mutans count between the pre and post irrigation counts across the three groups

Groups	Mean pre-irrigation count[CFU/ml]	Mean post-irrigation count[CFU/ml]	t-value	p-value
Group A [0.9% normal saline]	$1.89x10^{6}$	$2.52x10^{4}$	4.6494	0.0001*
Group B [2.5% sodium hypochlorite]	$1.49x10^{7}$	$9.20x10^{2}$	6.6851	0.00001*
Group C [aqueous ozone]	$1.25x10^{7}$	$4.10x10^{2}$	4.0986	0.0004*

Table 3: Intergroup comparison of S. mutans bacteria between the groups using Anova Test

Groups	N	Mean log reduction of s.mutans bacteria in percentage	F value	Significance (p)
Group A [0.9% normal saline]	24	90.3383%	7.369	**0.001***
Group B [2.5% sodium hypochlorite]	24	99.3771%		
Group C [aqueous ozone]	24	99.4054%		

Table 4: Intergroup comparison of S.mutans bacteria between the groups using post-hoc Tukey

(I) Groups	(J) Groups	Difference in mean log reduction of s.mutans(I-J)	Significance (p)
GroupA [0.9% normal saline]	GroupB [2.5% sodium hypochlorite]	9.03875*	**.004***
	GroupC [ozonated water]	9.06708*	**.004***
GroupB [2.5% sodium hypochlorite]	GroupA [0.9% normal saline]	9.03875*	**.004***
	GroupC [ozonated water]	.02833	1.000
GroupC [ozonated water]	GroupA [0.9% normal saline]	9.06708*	**.004***
	GroupB [2.5% sodium hypochlorite]	.02833	1.000

Table5: intragroup comparison of P.gingivalis count between the pre and post irrigation counts across the three groups

Groups	Mean pre-irrigation count[CFU/ml]	Mean post-irrigation count[CFU/ml]	t-value	p-value
Group A [0.9% normal saline]	$1.46x10^6$	$3.55x10^4$	3.8486	0.00083*
Group B [2.5% sodium hypochlorite]	$8.39x10^6$	$1.152x10^3$	3.3844	0.00225*
Group C [aqueous ozone]	$1.80x10^6$	$2.68x10^3$	3.2365	0.00365*

Table.6: Intergroup comparison of P.gingivalis bacteria between the groups using Anova Test

Groups	N	Mean log reduction of P.gingivalis bacteria in percentage	F value	Significance (p)
Group A [0.9% normal saline]	24	96.3604%	2.776	0.069
Group B [2.5% sodium hypochlorite]	24	96.9808%		
Group C [aqueous ozone]	24	98.6992%		

Table 7: intragroup comparison of Prevotella sp count between the pre and post irrigation counts across the three groups

Groups	Mean pre-irrigation count[CFU/ml]	Mean post-irrigation count[CFU/ml]	t-value	p-value
Group A [0.9% normal saline]	$7.86x10^6$	$1.15x10^5$	2.797	0.01023*
Group B [2.5% sodium hypochlorite]	$1.89x10^6$	$2.98x10^3$	4.4877	0.00017*
Group C [aqueous ozone]	$5.13x10^6$	$3.61x10^3$	1.6706	0.10834

Table 8: Intergroup comparison of Prevotella sp bacteria between the groups using Anova Test

Groups	N	Mean log reduction of Prevotella bacteria in percentage	F value	Significance (p)
Group A [0.9% normal saline]	24	90.5621	144.866	**<0.0001***
Group B [2.5% sodium hypochlorite]	24	99.0900		
Group C [aqueous ozone]	24	99.0479		

Table 9: Intergroup comparison of Prevotella Sp. bacteria between the groups using post-hoc Tukey

(I) Groups	(J) Groups	Difference in mean log reduction of prevotella (I-J)	Significance (p)
GroupA [0.9% normal saline]	GroupB [2.5% sodium hypochlorite]	8.52792*	**<0.0001***
	GroupC [ozonated water]	8.48583*	**<0.0001***
GroupB [2.5% sodium hypochlorite]	GroupA [0.9% normal saline]	8.52792*	**<0.0001***
	GroupC [ozonated water]	.04208	0.997
GroupC [ozonated water]	GroupA [0.9% normal saline]	8.48583*	**<0.0001***
	GroupB[2.5% sodium hypochlorite]	.04208	0.997

Table10: intragroup comparison of P.micros count between the pre and post irrigation counts across the three groups

Groups	Mean pre-irrigation count[CFU/ml]	Mean post-irrigation count[CFU/ml]	t-value	p-value
Group A [0.9% normal saline]	$8.47x10^{5}$	$1.10x10^{4}$	3.2171	0.00382*
Group B [2.5% sodium hypochlorite]	$7.8x10^{5}$	$1.30x10^{3}$	1.5958	0.12417
Group C [aqueous ozone]	$1.33x10^{6}$	$1.62x10^{3}$	2.0502	0.05191

Table 11: Intergroup comparison of P.micros bacteria between the groups using Anova Test

Groups	N	Mean log reduction of P.micros bacteria in percentage	F value	Significance (p)
Group A[0.9% normal saline]	24	79.4401	26.831	**<0.0001***
Group B[2.5% sodium hypochlorite]	24	96.0567		
Group C[aqueous ozone]	24	98.9420		

Table 12: Intergroup comparison of P.micros bacteria between the groups using post-hoc Tukey

Groups	Groups	Difference between the mean log reduction for P.micros	Significance (p)
Group A [0.9% normal saline]	GroupB [2.5% sodium hypochlorite]	16.61654*	**<0.0001***
	GroupC [ozonated water]	19.50192*	**<0.0001***
Group B [2.5% sodium hypochlorite]	GroupA [0.9% normal saline]	16.61654*	**<0.0001***
	GroupC [ozonated water]	2.88538	.577
Group C [ozonated water]	GroupA [0.9% normal saline]	19.50192*	**<0.0001***
	GroupB [2.5% sodium hypochlorite]	2.88538	.577

Table 13: Intragroup comparison of E.faecalis count between the pre and post irrigation counts across the three groups

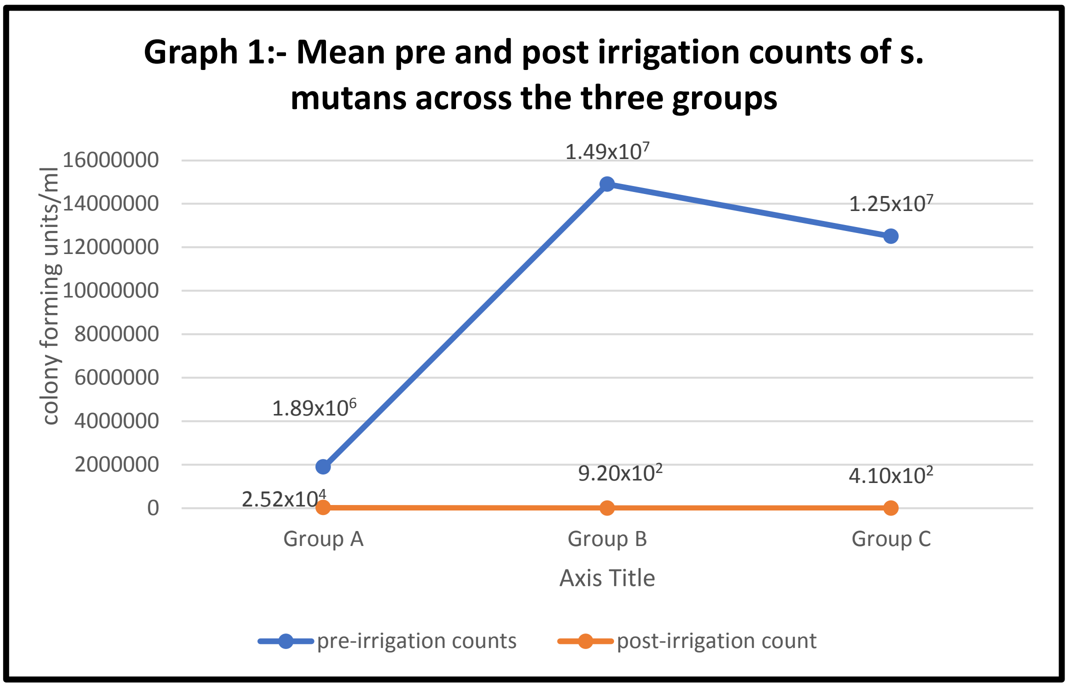
Groups	Mean pre-irrigation count[CFU/ml]	Mean post-irrigation count[CFU/ml]	t-value	p-value
Group A [0.9% normal saline]	$2.33x10^5$	$3.41x10^4$	6.4193	0.00001*
Group B [2.5% sodium hypochlorite]	$1.36x0^6$	$2.12x10^3$	2.7832	0.01057*
Group C [aqueous ozone]	$4.92x10^6$	$4.30x10^2$	2.4310	0.02326*

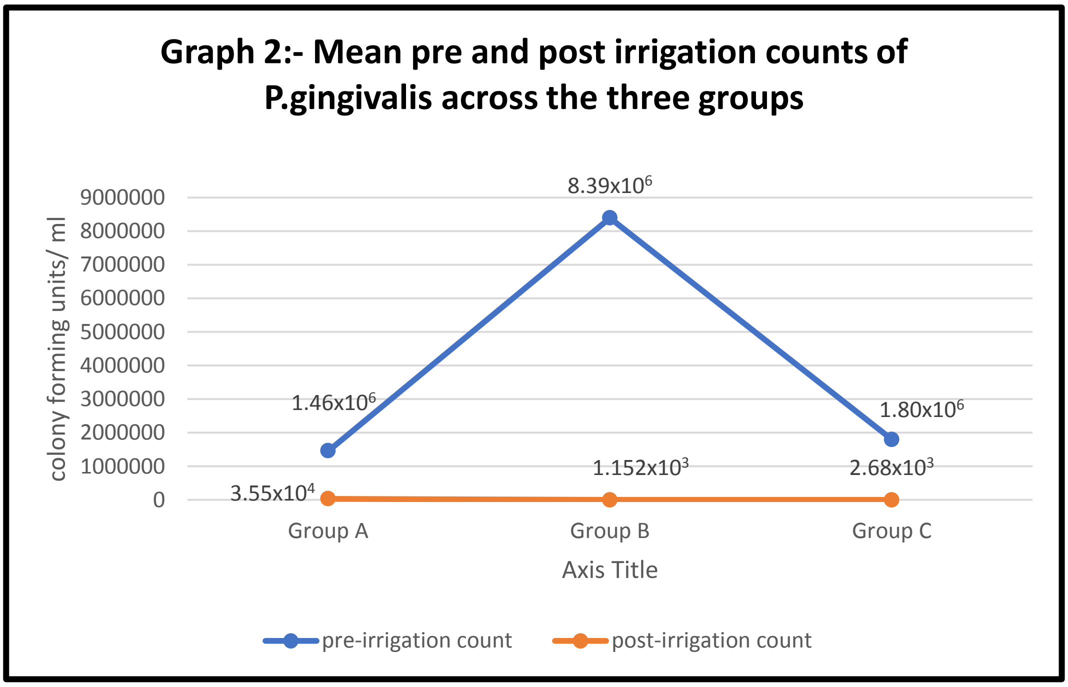
Table 14: Intergroup comparison of E.faecalis bacteria between the groups using Anova Test

Groups	N	Mean log reduction of E.faecalis in percentage	F value	Significance (p)
Group A [0.9% normal saline]	24	93.7489	15.779	**<0.0001***
Group B [2.5% sodium hypochlorite]	24	99.1775		
Group C [aqueous ozone]	24	99.8050		

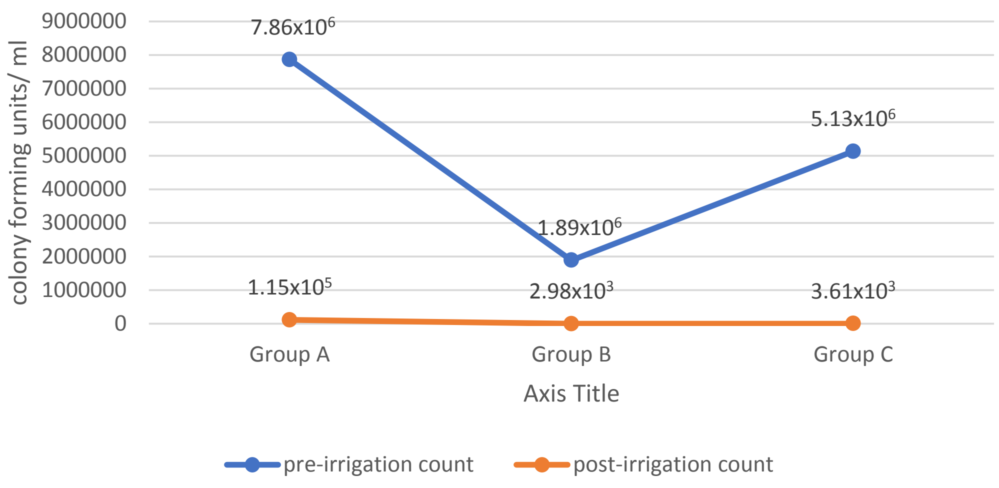
Table 15: Intergroup comparison of E.faecalis bacteria between the groups using post-hoc Tukey

Groups	Groups	Difference in mean log reduction of E. faecalis	Significance (p)
Group A [0.9% normal saline]	GroupB [2.5% sodium hypochlorite]	5.42858*	**<0.0001***
	GroupC [ozonated water]	6.05604*	**<0.0001***
Group B[2.5% sodium hypochlorite]	GroupA [0.9% normal saline]	5.42858*	**<0.0001***
	GroupC [ozonated water]	.62746	.857
Group C [ozonated water]	GroupA [0.9% normal saline]	6.05604*	**<0.0001***
	GroupB[2.5% sodium hypochlorite]	.62746	.857

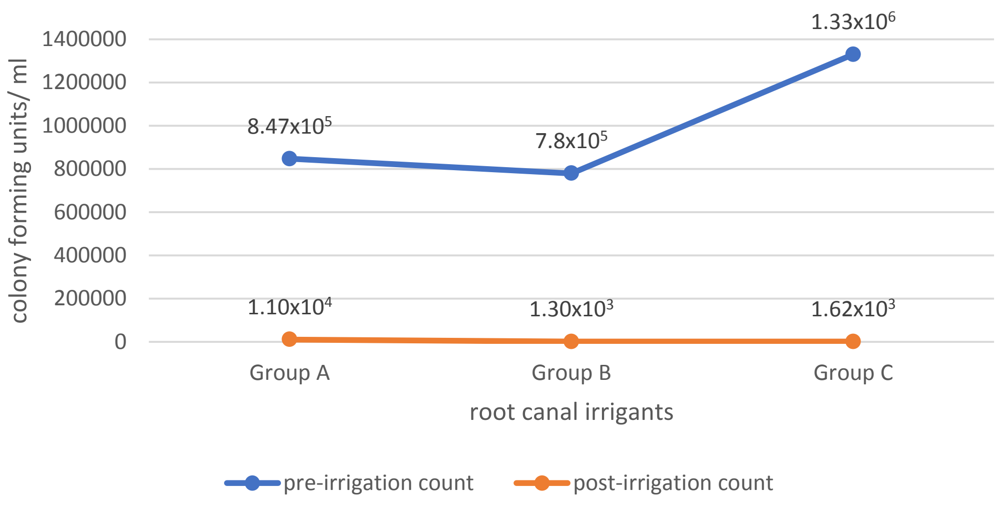

Graph 1:- Mean pre and post irrigation counts of s. mutans across the three groups
colony forming units/ml
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
1.49x10^7
1.25x10^7
1.89x10^6
9.20x10^2
4.10x10^2
2.52x10^4
Group A
Group B
Group C
Axis Title
pre-irrigation counts
post-irrigation count

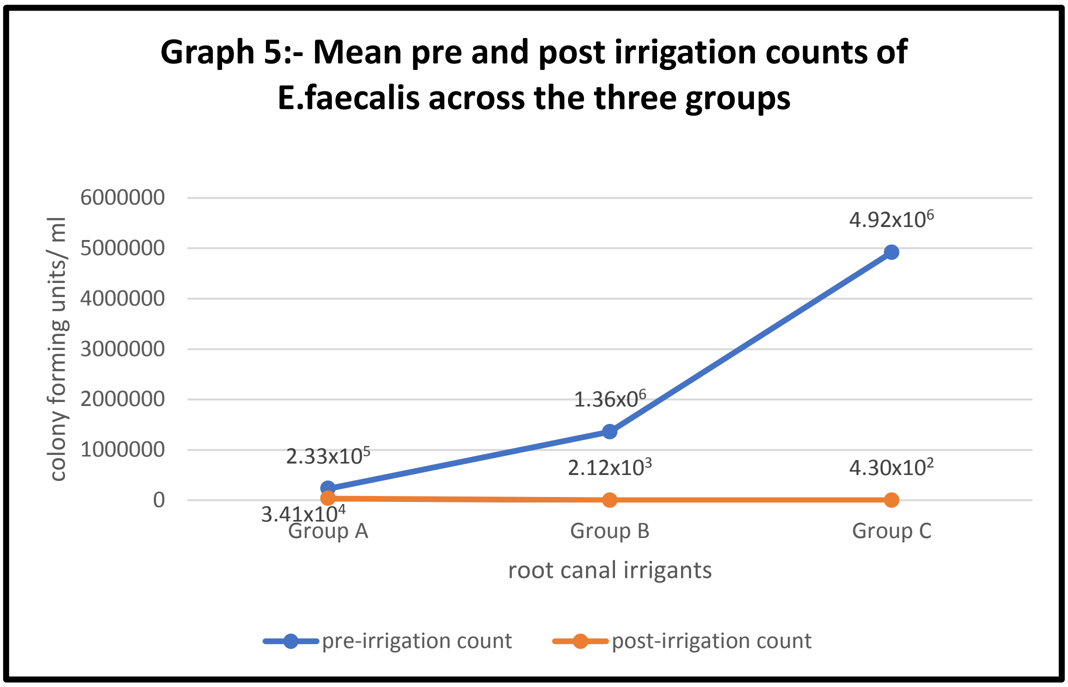

Graph 2:- Mean pre and post irrigation counts of P.gingivalis across the three groups
colony forming units/ ml
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
8.39x10^6
1.46x10^6
1.80x10^6
1.152x10^3
2.68x10^3
3.55x10^4
Group A
Group B
Group C
Axis Title
pre-irrigation count
post-irrigation count

Graph 3:- Mean pre and post irrigation counts of Prevotella sp. across the three groups



Graph 4:- Mean pre and post irrigation counts of P.micros across the three groups




Graph 5:- Mean pre and post irrigation counts of E.faecalis across the three groups
colony forming units/ ml
6000000
5000000
4000000
3000000
2000000
1000000
0
4.92x10^6
1.36x0^6
2.33x10^5
2.12x10^3
4.30x10^2
3.41x10^4
Group A
Group B
Group C
root canal irrigants
pre-irrigation count
post-irrigation count

DEPARTMENT OF PEDIATRIC AND PREVENTIVE DENTISTRY

Comparative evaluation of the anti- microbial efficacy of aqueous ozone and sodium hypochlorite as root canal irrigant in non-vital primary teeth- a randomized controlled study

EXAMINATION PROFORMA/CASE RECORD FORM

Date of examination

Identification No-

Day -

Date -

Group –

General information:

Name of child____ **Gender-**☐

1=M, 2=F

Day Month Year

Date of Birth- ☐☐☐☐☐☐☐☐

Age in years-☐☐

Name and Address of parent:
____

<u>Contact No. of Parent</u>: ………………………………….

<u>Chief Complaint</u>:

<u>H/O Present illness</u>:

<u>Past Medical History</u>:

<u>Past Dental History</u>:

Frequency of tooth brushing per day:

1) Once

2) Twice

3) After every meal

Extra- Oral examination:

1. **Swelling on face:**

2. **Lymph nodes**:

Intra- Oral examination:

A. **Examination of soft tissues:**

1.Gingiva:

2.Floor of mouth

3. Tongue:

B. **Examination of hard tissues:**

1.Teeth present(FDI) –

2.Dental caries status –

- **<u>defs index</u>** –

16	**55**	**54**	**53**	**52**	**51**	**61**	**62**	**63**	**64**	**65**	**26**

46	**85**	**84**	**83**	**82**	**81**	**71**	**72**	**73**	**74**	**75**	**36**

Total score =

D	E	f	s	Defs score

<u>Radiographic interpretation:</u>

<u>Diagnosis:</u>

<u>Treatment plan:</u>

Information Sheet for Patient/Parent

Introduction

I, **Dr. Harsha Sawant,** Post Graduate in the Department of Pediatric and Preventive Dentistry, working on my Thesis titled **"COMPARATIVE EVALUATION OF THE ANTI- MICROBIAL EFFICACY OF AQUEOUS OZONE AND SODIUM HYPOCHLORITE AS ROOT CANAL IRRIGANT IN NON-VITAL PRIMARY TEETH- A RANDOMIZED CONTROLLED STUDY"** My study subjects would be children of 3-7 years of age group, visiting the department of Paediatric and Preventive dentistry.

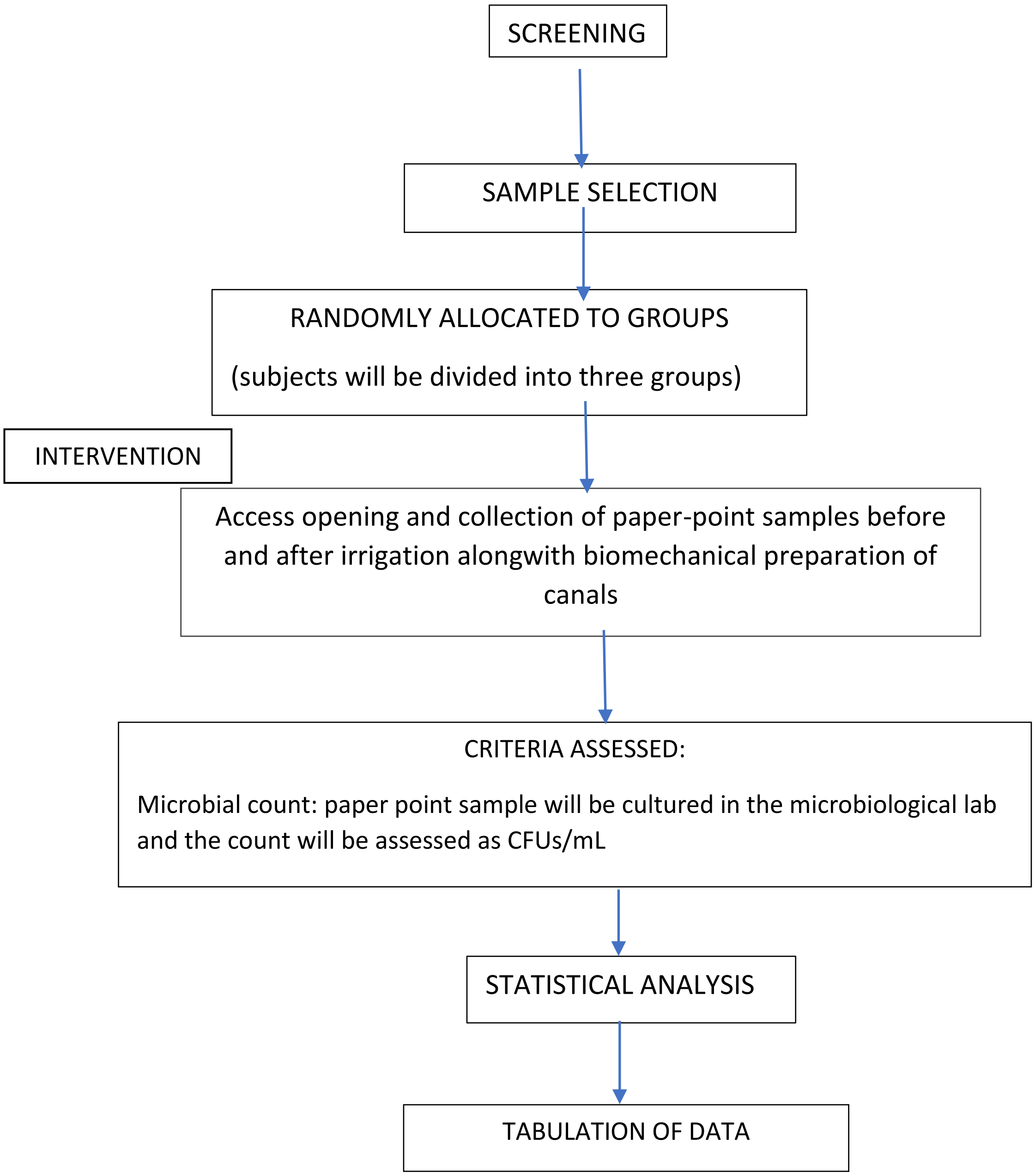
Purpose of the research

"comparative evaluation of the anti- microbial efficacy of aqueous ozone and sodium hypochlorite as root canal irrigant in non-vital primary teeth- a randomized controlled study

Voluntary Participation

Your participation in this research is entirely voluntary. You can withdraw from it any time you wish. This will no way adversely affect the subsequent outcome of the treatment of your relationship with the operating doctor on inclusion to the project. Any additional expense for the project will be borne by the project fund and will not be charged to you.

Procedures and Protocol



Confidentiality

The information that we collect from this research project will be kept confidential. Information about the patient that will be collected during the research will be put away and no-one but the researchers will be able to see it. Any information about the patient will have a number on it instead of your name.

DEPARTMENT OF PEDIATRIC & PREVENTIVE DENTISTRY

Certificate of Consent

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate as a participant in this research.

I acknowledge the "Specially designed proforma", and also the doctor has informed me about this research project suitably and sufficiently to my satisfaction. I agree to let my child's oral examination to be taken as required and also agree for the required treatment. I agree to take part in this project. I shall co-operate with the doctors, in all respects. I permit to publishing the results of my participation in this study. I shall not be given any reimbursement or compensation. I have been informed of my right to opt out of this research project at any time without giving any reason for doing so. I hereby record my consent for participation in the said project.

....	....	....	....
Parent's/guardian's name	Signature/thumbprint	Date	Time

....	....	....	....
Investigator's name	Signature	Date	Time

If illiterate a literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team). Participants who are illiterate should include their thumb-print as well.

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness_____________________ Thumb print of participant

Signature of witness ______________________

Date ________________________

Day/month/year

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

"Comparative evaluation of the anti- microbial efficacy of sodium hypochlorite and aqueous ozone as root canal irrigant in non-vital primary teeth.- A randomized controlled study"

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher/person taking the consent________________________

Signature of Researcher /person taking the consent__________________________

Date ___________________________

Day/month/year

<u>Informed Consent Form</u>

(Confidential)

"Comparative evaluation of the anti- microbial efficacy of sodium hypochlorite and aqueous ozone as root canal irrigant in non-vital primary teeth.- A randomized controlled study"

Mr./Master/Mrs./Miss.____

Resident of: ____

__________________________________ _________________aged _____ years,

Exercising my free will/choice, without any pressure/lure of incentive in any form, hereby give my consent for the project to be conducted by

I acknowledge the receipt of "patient's information sheet", and also that the doctor has informed me about this research project suitably and sufficiently to my satisfaction. I allow to perform the examination of my ward's oral cavity and thereby agree for the treatment. I agree to take part in this project and will not mix any other projects during the period of this trial. I permit to publishing the results of my participation in this study. I shall not be given any reimbursement or compensation. I hereby record my consent for participation in the said questionnaire.

______________ _____________________ __________ __________

Patient's name Signature/thumbprint Date Time

_____________________ _________ __________

Principal Investigator Signature Date Time

MASTER CHART

sr. no.	age	sex	tooth no.	canal	treatment	Irrigant	e. faecalis		bacterial reduction in %	s. mutans		bacterial reduction in %	p. gingivalis		bacterial reduction in %	prevotella		bacterial reduction in %	p. micros		bacterial reduction in %
							pre	post		pre	post		pre	post		pre	post		pre	post	
1	5	f	64	palatal	Group A	saline	3.45 X 10^{5}	3.0 X 10^{4}	90%	4.45 X 10^{6}	2.67 X 10^{4}	99	2.45 X 10^{5}	3.5X 10^{3}	99	1.5 X 10^{5}	0.2X 10^{4}	90	3.2 x 10^{4}	1.2X 10^{4}	60
2	5	f	54	mesial	Group B	sodium hypoch	5.8x10^{5}	3.5X 10^{3}	98.54	1.9 X 10^{7}	2X 10^{2}	99.87	3.5X 10^{3}	3x 10^{2}	90	1.45 X 10^{5}	1x 10^{2}	99.8	3.5X 10^{3}	1X 10^{2}	90
3	7	m	75	mesial	Group A	saline	1.45 X 10^{5}	3.5X 10^{3}	98%	3.45 X 10^{6}	2.2X 10^{4}	99	4.26 X 10^{5}	2.24X 10^{4}	90	3.76 X 10^{5}	5.2X 10^{4}	90	2.43 X 10^{5}	1.2X 10^{4}	90
4	7	m	75	distal	group C	aqueous ozone	1.9 X 10^{5}	100	99.99%	4.5 X 10^{5}	1x 10^{2}	99.9	4.26 X 10^{5}	1X 10^{2}	99.9	5.8x10^{5}	3.5X 10^{3}	99	1.45 X 10^{5}	1x 10^{2}	99.9
5	7	f	85	mesial	Group B	sodium hypoch	5.6x10^{5}	3.5X 10^{3}	99%	2.54 X 10^{7}	3.5X 10^{3}	98.3	1.1 X 10^{7}	1.4X 10^{2}	99.99	3.45 X 10^{5}	2.4x 10^{2}	99.2	3x10^{5}	1.5X 10^{3}	98.5
6	6	f	74	distal	group C	aqueous ozone	4.26 X 10^{5}	1x 10^{2}	99.90%	1.5 X 10^{7}	2X 10^{2}	99.99	7.26 X 10^{5}	3.5x 10^{2}	99.51	4.45 X 10^{6}	3x 10^{2}	99.99	2.9x10^{5}	3X 10^{3}	97.96
7	4	m	85	distal	Group B	sodium hypoch	3.3X 10^{4}	100	99	1.1 X 10^{7}	1.4X 10^{2}	99.98	5.8x10^{5}	3.5X 10^{3}	99.29	4.45 X 10^{6}	1.4X 10^{3}	99.58	5.26 X 10^{5}	3.5X 10^{3}	98.33
8	4	f	84	mesial	Group A	saline	4.5 X 10^{5}	3.5X 10^{3}	99	1.45 X 10^{5}	2.67X 10^{4}	90	4.45 X 10^{6}	3.5X 10^{3}	99.11	3.23 X 10^{5}	0.2X 10^{4}	89.93	3.3X 10^{4}	1.7X 10^{4}	51.51
9	7	m	84	mesial	Group B	sodium hypoch	4.26 X 10^{5}	1.4X 10^{2}	98.87	5.8x10^{5}	3.5X 10^{3}	98.29	1.5 X 10^{7}	3.5X 10^{3}	97.65	2.45 X 10^{6}	3.5X 10^{3}	98.47	3.76 X 10^{5}	1.4X 10^{2}	99.61
10	4	f	64	palatal	group C	aqueous ozone	3.45 X 10^{5}	1x 10^{2}	99.9	1.5 X 10^{7}	2X 10^{2}	99.99	5.45 X 10^{6}	2.75X 10^{3}	99.4	5.8x10^{5}	1.4X 10^{2}	99.7	1.45 X 10^{5}	1x 10^{2}	99.9
11	7	m	64	palatal	Group A	saline	3.23 X 10^{5}	2.23 X 10^{5}	67	2.45 X 10^{5}	2.67 X 10^{4}	90	4.26 X 10^{5}	5.8x10^{4}	88.63	4.23 X 10^{5}	3.67 X 10^{4}	89.13	3.3X 10^{4}	2.7X 10^{4}	81.81
12	5	f	85	mesial	group C	aqueous ozone	4.65x 10^{5}	2.3X 10^{2}	99.80%	3.45 X 10^{7}	1x 10^{2}	99.99	4.5 X 10^{5}	3x 10^{2}	99.23	1.1 X 10^{7}	1.4X 10^{2}	99.91	2.9x10^{5}	1X 10^{2}	99.5
13	5	f	55	palatal	Group A	saline	nil	nil		4.3X 10^{4}	1.7X 10^{4}	39.53	3.34 X 10^{5}	3.5X 10^{3}	97.95	4.5 X 10^{5}	4.7 X 10^{4}	88.95	3x10^{5}	1.5X 10^{3}	98.5
14	4	m	64	palatal	group C	aqueous ozone	1.1 X 10^{7}	1.4X 10^{2}	99.99	5.8x10^{5}	3.5X 10^{3}	99.6	5.45 X 10^{6}	1.4X 10^{2}	99.73	1.45 X 10^{5}	1x 10^{2}	99.21	3x10^{5}	2.7X 10^{4}	90
15	4	f	55	palatal	Group B	sodium hypoch	4.26 X 10^{5}	1x 10^{2}	99.87	7.5x10^{5}	1.5X 10^{3}	99.7	1.1 X 10^{7}	1.4X 10^{2}	98.72	4.65 X 10^{6}	3.5X 10^{3}	99.14	1.5X 10^{3}	1x 10^{2}	89.33
16	5	m	75	distal	Group A	saline	2.34 X 10^{5}	3.5X 10^{3}	99	4.45 X 10^{6}	4.3X 10^{4}	96.62	4.5 X 10^{5}	3x 10^{2}	99.23	3.45 X 10^{7}	5.45 X 10^{6}	87.42	3.3X 10^{4}	1.5X 10^{3}	89.54
17	6	f	74	mesial	Group B	sodium hypoch	5.6x10^{5}	3.5X 10^{3}	99	2.54 X 10^{7}	3.5X 10^{3}	99.86	1.1 X 10^{7}	1.4X 10^{2}	98.72	3.45 X 10^{5}	2.4x 10^{2}	99.21	3x10^{5}	1.5X 10^{3}	98.5
18	7	m	64	palatal	group C	aqueous ozone	3.5 X 10^{7}	2X 10^{2}	99.87	2.35 X 10^{6}	2X 10^{2}	99.14	4.53 X 10^{5}	3.5x 10^{2}	99.13	5.8x10^{5}	3.5X 10^{3}	99.9	3.5X 10^{3}	1X 10^{2}	99.61
19	5	f	54	mesial	group C	aqueous ozone	5.8x10^{5}	3.5X 10^{3}	99	4.3X 10^{4}	2.3x10^{1}	99.37	3.5X 10^{3}	1X 10^{2}	89.61	4.65 X 10^{6}	1.4x 10^{2}	99.68	1.45 X 10^{5}	1x 10^{2}	99.5
20	5	f	75	mesial	Group B	sodium hypoch	3.8x10^{5}	5.5X 10^{3}	99	1.1 X 10^{7}	1.4X 10^{2}	99.99	4.65 X 10^{6}	2.3x10^{1}	99.5	3.45 X 10^{5}	1.4x 10^{2}	99.49	1.5X 10^{3}	2.3x10^{1}	97.46
21	7	m	75	distal	Group A	saline	3.8x10^{5}	2.67X 10^{4}	90	5.8x10^{5}	3.5X 10^{3}	98.5	5.45 X 10^{6}	5.8x10^{5}	97.93	3.5X 10^{3}	1.5X 10^{3}	89.57	3.3X 10^{4}	1x 10^{2}	89.69
22	7	m	85	mesial	Group B	sodium hypoch	4.65x 10^{5}	2X 10^{2}	99.9	3.45 X 10^{7}	1x 10^{2}	99.99	4.3X 10^{4}	1.4X 10^{2}	98.67	3.45 X 10^{5}	2.4x 10^{2}	99.2	1.45 X 10^{5}	1.1x 10^{2}	99.14
23	7	f	74	distal	group C	aqueous ozone	1.1 X 10^{7}	2X 10^{2}	99.99	1.45 X 10^{5}	1x 10^{2}	97.32	4.7 X 10^{4}	2.3x10^{1}	99.41	5.8x10^{5}	3.5X 10^{3}	98.39	1.1 X 10^{7}	1.4X 10^{2}	98.71
24	6	f	85	distal	Group A	saline	2.34 X 10^{5}	3.5X 10^{3}	97	4.45 X 10^{6}	4.3X 10^{4}	96.62	4.5 X 10^{5}	3x 10^{2}	99.23	3.45 X 10^{7}	5.45 X 10^{6}	88.42	3.3X 10^{4}	1.5X 10^{3}	89.54
25	4	m	84	mesial	Group B	sodium hypoch	4.45 X 10^{6}	2.5X 10^{3}	99.89	5.8x10^{5}	3.5X 10^{3}	98.5	3.5 X 10^{5}	3x 10^{2}	99.04	2.14 X 10^{5}	1.4x 10^{2}	99.24	6.5 X 10^{5}	2.3x 10^{2}	99.55
26	4	f	84	mesial	Group A	saline	2.67X 10^{4}	2X 10^{2}	98.7	5.8x10^{5}	4.3X 10^{4}	88.67	3.2x10^{5}	3.5X 10^{3}	97.9	1.65 X 10^{6}	4.7 X 10^{4}	96.15	1.5X 10^{3}	1.4X 10^{2}	89.06
27	7	m	64	palatal	Group B	sodium hypoch	1.1 X 10^{7}	1.4X 10^{2}	99.99	1.1 X 10^{7}	2X 10^{2}	99.99	3.45 X 10^{7}	1x 10^{2}	99.7	4.3X 10^{4}	2.3x10^{1}	99.37	1.4x 10^{2}	1.3x10^{1}	89.07
28	4	f	64	palatal	group C	aqueous ozone	1.45 X 10^{5}	1x 10^{2}	99.9	6.45 X 10^{6}	2.3x 10^{2}	99.63	1.45 X 10^{5}	1x 10^{2}	99.2	3.2x10^{5}	3x 10^{2}	98.96	2.35 X 10^{6}	100	99.999
29	7	m	85	mesial	group C	aqueous ozone	5.8x10^{5}	3.5X 10^{3}	99	1.1 X 10^{7}	1.4X 10^{2}	99.99	3.45 X 10^{6}	2.25X 10^{3}	99.24	1.45 X 10^{5}	1x 10^{2}	99.2	4.3X 10^{4}	100	99.9
30	5	f	55	palatal	Group A	saline	4.5 X 10^{5}	3.5X 10^{3}	98.4	1.45 X 10^{5}	2.67X 10^{4}	89.81	4.45 X 10^{6}	3.5X 10^{3}	99.11	3.23 X 10^{5}	0.2X 10^{4}	89.93	3.3X 10^{4}	1.7X 10^{4}	67
31	5	f	64	palatal	group C	aqueous ozone	4.65x 10^{5}	1.67x 10^{2}	99.9	1.45 X 10^{5}	1x 10^{2}	97.32	3.45 X 10^{6}	1.2x 10^{2}	99.64	7.41 X 10^{7}	3.25X 10^{3}	99.55	4.45 X 10^{5}	1.1X 10^{2}	99.65
32	4	m	74	distal	Group B	sodium hypoch	1.45 X 10^{5}	1x 10^{2}	99.87	2.45 X 10^{6}	2.12x 10^{2}	99.99	4.3 X 10^{7}	2X 10^{2}	99.99	3.65x 10^{5}	1.23X 10^{2}	99.57	4.45 X 10^{5}	1x 10^{2}	99.67
33	4	f	65	palatal	group C	aqueous ozone	3.45 X 10^{7}	1x 10^{2}	99.999	3.3 X 10^{7}	2.3X 10^{2}	99.99	2.45 X 10^{6}	3.5X 10^{3}	98.47	4.65x 10^{5}	1.23X 10^{2}	99.64	5.8x10^{5}	3.5X 10^{3}	98.39
34	5	m	74	mesial	Group B	sodium hypoch	4.45 X 10^{6}	2.5X 10^{3}	99.7	4.65x 10^{5}	1.23X 10^{2}	99.63	5.8x10^{5}	3.5X 10^{3}	98.39	4.45 X 10^{6}	2.5X 10^{3}	99.33	4.65x 10^{5}	1.23X 10^{2}	99.64
35	6	f	64	palatal	Group A	saline	2.67X 10^{4}	2.5X 10^{3}	90	5.8x10^{5}	3.5X 10^{3}	99.29	4.45 X 10^{6}	4.3X 10^{4}	98.03	3.25 X 10^{6}	4.3X 10^{4}	98.57	4.45 X 10^{5}	4.3X 10^{4}	89.033
36	7	m	54	mesial	Group A	saline	2.67X 10^{4}	2.1x 10^{2}	99	1.45 X 10^{5}	1x 10^{2}	97.32	5.8x10^{5}	3.5X 10^{3}	99.29	3.25 X 10^{6}	3.23X 10^{4}	98.01	3.3X 10^{4}	1.5X 10^{3}	89.55
37	5	f	75	mesial	Group B	sodium hypoch	2.45 X 10^{6}	2.5X 10^{3}	99.87	1.1 X 10^{7}	1.4X 10^{2}	99.99	7.8x10^{5}	3.5X 10^{3}	98.55	3.25 X 10^{6}	4.3X 10^{4}	97.67	5.8x10^{5}	3.5X 10^{3}	98.39
38	5	f	75	distal	Group B	sodium hypoch	4.65x 10^{5}	2.5X 10^{3}	99	3.45 X 10^{7}	1x 10^{2}	99.99	1.2 X 10^{7}	1.1X 10^{2}	99.99	5.25 X 10^{6}	4.23X 10^{4}	98.19	5.32x10^{5}	3.25X 10^{3}	98.38
39	7	m	85	mesial	group C	aqueous ozone	1.45 X 10^{5}	1x 10^{2}	99.9	6.45 X 10^{6}	2.3x 10^{2}	99.63	1.45 X 10^{5}	1x 10^{2}	99.21	3.2x10^{5}	3x 10^{2}	99.91	2.35 X 10^{6}	100	99.99

40	7	m	74	distal	group C	aqueous ozone	$2.45 X 10^{5}$	$1x 10^{2}$	99.9	$3.45 X 10^{7}$	$1x 10^{2}$	99.97	$3.45 X 10^{5}$	$2.1x 10^{2}$	99.3	$6.45 X 10^{6}$	$3.5X 10^{3}$	99.35	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
41	7	f	85	distal	Group A	saline	$2.34 X 10^{5}$	$3.5X 10^{3}$	98.34	$4.45 X 10^{6}$	$4.3X 10^{4}$	99	$4.5 X 10^{5}$	$3x 10^{2}$	99.23	$3.45 X 10^{7}$	$5.45 X 10^{6}$	88.42	$3.3X 10^{4}$	$1.5X 10^{3}$	89.54
42	6	f	84	mesial	Group A	saline	$2.67X 10^{4}$	$2.1x 10^{2}$	98.21	$1.45 X 10^{5}$	$1x 10^{2}$	99	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$3.25 X 10^{6}$	$3.23X 10^{4}$	98	$3.3X 10^{4}$	$1.5X 10^{3}$	89.54
43	4	m	84	mesial	group C	aqueous ozone	$1.2 X 10^{7}$	$1.1X 10^{2}$	99	$3.45 X 10^{7}$	$1x 10^{2}$	99.99	$2.45 X 10^{6}$	$4.3X 10^{4}$	97.24	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
44	4	f	64	palatal	group C	aqueous ozone	$1.45 X 10^{5}$	$1x 10^{2}$	99.9	$5.8x10^{5}$	$3.5X 10^{3}$	99.9	$2.45 X 10^{6}$	$1.1X 10^{2}$	99.54	$4.65x 10^{5}$	$1.23X 10^{2}$	99.63	$1.2 X 10^{7}$	$1.1X 10^{2}$	99.97
45	7	m	64	palatal	Group B	sodium hypoch	$2.45 X 10^{6}$	$2.5X 10^{3}$	98.92	$1.1 X 10^{7}$	$1.4X 10^{2}$	99.32	$7.8x10^{5}$	$3.5X 10^{3}$	98.55	$3.25 X 10^{6}$	$4.3X 10^{4}$	97.67	$5.8x10^{5}$	$3.5X 10^{3}$	98.39
46	4	f	85	mesial	Group B	sodium hypoch	$4.65x 10^{5}$	$2.5X 10^{3}$	98.46	$3.45 X 10^{7}$	$1x 10^{2}$	99.99	$1.2 X 10^{7}$	$1.1X 10^{2}$	99.07	$5.25 X 10^{6}$	$4.23X 10^{4}$	98.19	$5.32x10^{5}$	$3.25X 10^{3}$	98.37
47	7	m	55	palatal	Group A	saline	$4.5 X 10^{5}$	$3.5X 10^{3}$	99	$1.45 X 10^{5}$	$2.67X 10^{4}$	88.15	$4.45 X 10^{6}$	$3.5X 10^{3}$	99.11	$3.23 X 10^{5}$	$0.2X 10^{4}$	89.93	$3.3X 10^{4}$	$1.7X 10^{4}$	51.52
48	5	f	64	palatal	Group A	saline	$3.23 X 10^{5}$	$2.23 X 10^{5}$	90	$2.45 X 10^{5}$	$2.67 X 10^{4}$	88.91	$4.26 X 10^{5}$	$4.3X 10^{4}$	88.99	$4.23 X 10^{5}$	$3.67 X 10^{4}$	89.13	$3.3X 10^{4}$	$2.7X 10^{4}$	81.81
49	5	f	54	palatal	group C	aqueous ozone	$1.9 X 10^{5}$	100	99.9	$4.5 X 10^{5}$	$1x 10^{2}$	99.76	$4.26 X 10^{5}$	$1X 10^{2}$	99.66	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
50	4	m	75	distal	Group A	saline	$4.3X 10^{4}$	$1.7X 10^{4}$	85	$4.3X 10^{4}$	$1.7X 10^{4}$	39	$3.34 X 10^{5}$	$3.5X 10^{3}$	97.95	$4.5 X 10^{5}$	$4.7 X 10^{4}$	88.95	nil	nil	
51	4	f	74	mesial	group C	aqueous ozone	$4.45 X 10^{6}$	$3.75X 10^{2}$	99.99	$3.45 X 10^{7}$	$1x 10^{2}$	99.99	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
52	5	m	64	palatal	Group B	sodium hypoch	$5.8x10^{5}$	$3.5X 10^{3}$	99	$1.9 X 10^{7}$	$2X 10^{2}$	98.93	$3.5X 10^{3}$	$3x 10^{2}$	89.14	$1.45 X 10^{5}$	$1x 10^{2}$	99.21	$3.5X 10^{3}$	$1X 10^{2}$	89.71
53	6	f	54	mesial	Group A	saline	$1.45 X 10^{5}$	$3.5X 10^{3}$	94.58	$3.45 X 10^{6}$	$2.2X 10^{4}$	98.36	$4.26 X 10^{5}$	$2.24X 10^{4}$	89.47	$3.76 X 10^{5}$	$5.2X 10^{4}$	88.61	$2.43 X 10^{5}$	$1.2X 10^{4}$	89.51
54	7	m	75	mesial	Group B	sodium hypoch	$4.65x 10^{5}$	$1.4X 10^{3}$	98.69	$3.45 X 10^{6}$	$4.2x 10^{2}$	99.99	$3.2 X 10^{7}$	$1.1X 10^{2}$	99.65	$4.65x 10^{5}$	$1.23X 10^{2}$	99.64	$1.2 X 10^{7}$	$1.1X 10^{2}$	99.07
55	5	f	75	distal	group C	aqueous ozone	$7.65x 10^{5}$	$1.1X 10^{2}$	99.9	$1.45 X 10^{5}$	$1x 10^{2}$	99.21	$1.2 X 10^{7}$	$1.1X 10^{2}$	99.07	$3.25 X 10^{6}$	$4.3X 10^{4}$	97.67	$5.8x10^{5}$	$3.5X 10^{3}$	98.39
56	5	f	85	mesial	Group A	saline	$4.5 X 10^{5}$	$3.5X 10^{3}$	98.43	$6.85 X 10^{5}$	$2.67X 10^{4}$	89.61	$4.45 X 10^{6}$	$3.5X 10^{3}$	99.12	$3.23 X 10^{5}$	$0.2X 10^{4}$	89.93	$3.3X 10^{4}$	$1.7X 10^{4}$	51.51
57	7	m	74	distal	Group B	sodium hypoch	$3.3X 10^{4}$	100	99	$1.1 X 10^{7}$	$1.4X 10^{2}$	98.62	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$4.45 X 10^{6}$	$1.4X 10^{3}$	99.58	$5.26 X 10^{5}$	$3.5X 10^{3}$	98.33
58	7	m	85	distal	Group B	sodium hypoch	$5.8x10^{5}$	$3.5X 10^{3}$	99	$1.9 X 10^{7}$	$2X 10^{2}$	98.93	$3.5X 10^{3}$	$3x 10^{2}$	89.14	$1.45 X 10^{5}$	$1x 10^{2}$	99.21	$3.5X 10^{3}$	$1X 10^{2}$	89.71
59	7	f	84	mesial	Group A	saline	$3.23 X 10^{5}$	$2.23 X 10^{5}$	0	$2.45 X 10^{5}$	$2.67 X 10^{4}$	88.91	$4.26 X 10^{5}$	$2.2X 10^{4}$	89.48	$4.23 X 10^{5}$	$3.67 X 10^{4}$	89.13	$3.3X 10^{4}$	$2.7X 10^{4}$	81.81
60	6	f	84	mesial	group C	aqueous ozone	$1.9 X 10^{5}$	100	99.9	$4.5 X 10^{5}$	$1x 10^{2}$	99.21	$4.26 X 10^{5}$	$1X 10^{2}$	98.76	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
61	4	m	64	palatal	Group B	sodium hypoch	$5.8x10^{5}$	$3.5X 10^{3}$	99	$1.9 X 10^{7}$	$2X 10^{2}$	98.93	$3.5X 10^{3}$	$3x 10^{2}$	89.14	$1.45 X 10^{5}$	$1x 10^{2}$	99.21	$3.5X 10^{3}$	$1X 10^{2}$	89.71
62	4	f	64	palatal	Group B	sodium hypoch	$5.8x10^{5}$	$3.5X 10^{3}$	99	$1.9 X 10^{7}$	$2X 10^{2}$	98.93	$3.5X 10^{3}$	$3x 10^{2}$	89.14	$1.45 X 10^{5}$	$1x 10^{2}$	99.21	$3.5X 10^{3}$	$1X 10^{2}$	89.71
63	7	m	85	mesial	group C	aqueous ozone	$4.45 X 10^{6}$	$3.75X 10^{2}$	99.99	$3.45 X 10^{7}$	$1x 10^{2}$	98.71	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
64	4	f	55	palatal	Group A	saline	$1.45 X 10^{5}$	$3.5X 10^{3}$	97	$3.45 X 10^{6}$	$2.2X 10^{4}$	98.36	$4.26 X 10^{5}$	$2.24X 10^{4}$	89.47	$3.76 X 10^{5}$	$5.2X 10^{4}$	88.61	$2.43 X 10^{5}$	$1.2X 10^{4}$	89.5
65	7	m	64	palatal	group C	aqueous ozone	$1.9 X 10^{5}$	100	99.9	$4.5 X 10^{5}$	$1x 10^{2}$	99.21	$4.26 X 10^{5}$	$1X 10^{2}$	98.76	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
66	5	f	74	distal	Group A	saline	$3.45 X 10^{5}$	$3.0 X 10^{4}$	90	$4.45 X 10^{6}$	$2.67 X 10^{4}$	98.4	$2.45 X 10^{5}$	$3.5X 10^{3}$	97.57	$1.5 X 10^{5}$	$0.2X 10^{4}$	89.86	$3.2 x 10^{4}$	$1.2X 10^{4}$	37.5
67	5	f	75	distal	Group B	sodium hypoch	$5.6x10^{5}$	$3.5X 10^{3}$	99	$2.54 X 10^{7}$	$3.5X 10^{3}$	98.61	$1.1 X 10^{7}$	$1.4X 10^{2}$	98.73	$3.45 X 10^{5}$	$2.4x 10^{2}$	99.2	$3x10^{5}$	$1.5X 10^{3}$	98.5
68	4	m	74	mesial	group C	aqueous ozone	$1.9 X 10^{5}$	100	99.9	$4.5 X 10^{5}$	$1x 10^{2}$	99.21	$4.26 X 10^{5}$	$1X 10^{2}$	98.76	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$1.45 X 10^{5}$	$1x 10^{2}$	99.21
69	4	f	85	distal	Group A	saline	$2.34 X 10^{5}$	$3.5X 10^{3}$	93	$4.45 X 10^{6}$	$4.3X 10^{4}$	98.03	$4.5 X 10^{5}$	$3x 10^{2}$	99.23	$3.45 X 10^{7}$	$5.45 X 10^{6}$	88.42	$3.3X 10^{4}$	$1.5X 10^{3}$	89.55
70	5	m	65	palatal	Group B	sodium hypoch	$3.3X 10^{4}$	100	98.69	$1.1 X 10^{7}$	$1.4X 10^{2}$	98.73	$5.8x10^{5}$	$3.5X 10^{3}$	98.39	$4.45 X 10^{6}$	$1.4X 10^{3}$	99.58	$5.26 X 10^{5}$	$3.5X 10^{3}$	98.29
71	6	f	55	palatal	group C	aqueous ozone	$4.65x 10^{5}$	$2.3X 10^{2}$	99.9	$3.45 X 10^{7}$	$1x 10^{2}$	98.71	$4.5 X 10^{5}$	$3x 10^{2}$	99.23	$1.1 X 10^{7}$	$1.4X 10^{2}$	98.73	$2.9x10^{5}$	$1X 10^{2}$	99.56
72	7	m	54	palatal	Group A	saline	$2.34 X 10^{5}$	$3.5X 10^{3}$	92.314	$4.45 X 10^{6}$	$4.3X 10^{4}$	98.03	$4.5 X 10^{5}$	$3x 10^{2}$	99.23	$3.45 X 10^{7}$	$5.45 X 10^{6}$	88.42	$3.3X 10^{4}$	$1.5X 10^{3}$	89.54