EFFECT OF POLYLACTIC-CO-GYLCOLIC ACID NANOPARTICLES IN PHOTOACTIVATED DISINFECTION OF ROOT CANALS AGAINST ENTEROCOCCUS FAECALIS BIOFILM: AN IN VITRO STUDY

Dissertation submitted to

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In partial fulfillment for the Degree of

MASTER OF DENTAL SURGERY

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CERTIFICATE

This is to certify that this dissertation titled "**EFFECT OF POLYLACTIC-CO-GYLCOLIC ACID NANOPARTICLES IN PHOTOACTIVATED DISINFECTION OF ROOT CANALS AGAINST ENTEROCOCCUS FAECALIS BIOFILM: AN IN VITRO STUDY**" is a bonafide record work done by **Dr. ABHISEK DAS** under our guidance during his postgraduate period between **2010 - 2013.**

This dissertation is submitted to **THE TAMILNADU Dr. M.G.R.MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **MASTER OF DENTAL SURGERY – CONSERVATIVE DENTISTRY AND ENDODONTICS, BRANCH IV**. It has not been submitted (partial or full) for the award of any other degree or diploma.

Guided By:

Dr. M. Rajasekaran, M.D.S.,
Professor,
Department of Conservative
Dentistry & Endodontics,
Ragas Dental College & Hospital,
Chennai

PROFESSOR
Dept. of Conservative & Endodontics
Ragas Dental College & Hospital
CHENNAI - 600 113.

Dr. R. Indira, M.D.S.,
Professor& Head,
Department of Conservative
Dentistry & Endodontics,
Ragas Dental College &
Hospital,
Chennai.

PROFESSOR & HEAD.,
Dept. of Conservative & Endodontics
Ragas Dental College & Hospital,
CHENNAI - 600 113.

Dr. S. Ramachandran, M.D.S.,
Professor and Principal,
Department of Conservative Dentistry & Endodontics,
Ragas Dental College & Hospital, Chennai.

PRINCIPAL
RAGAS DENTAL COLLEGE & HOSPITAL
CHENNAI

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LIST OF ABBREVIATIONS

PAD	Photoactivated Disinfection
PDT	Photodynamic Therapy
PS	Photosensitizer
MB	Methylene blue
PLGA	Polylactic- co- glycolic Acid
NP	Nano particle
GP	Gutta Percha
US	Ultrasonic

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ABSTRACT

Background: The influence of PLGA nanoparticles in enhancing the photoactivated disinfection of root canals.

Aim & Objective: The objective of the study was to evaluate the in-vitro effects Polylactic-co- glycolic acid (PLGA) nanoparticles loaded with the Photosensitiser Methylene blue (MB) and light against E. faecalis biofilm.

Materials and methods: 50 extracted mandibular premolar teeth were decoronated and chemomechanically prepared. Biofilm was allowed to be formed in the root canals of specimens by incubating it for 2 weeks in BHI broth infected with E faecalis. Based on the mode of treatment, the specimens were divided into 5 groups (n=10): Group I- MB encapsulated with PLGA nanoparticles (MB+NP), Group II – MB with passive ultrasonic activation (MB+US), Group III- MB with GP activation (MB+GP), Group IV – MB without any activation and Group V- specimens without any treatment (control). The specimens were incubated with the Photosensitiser for 10 mins and subjected to Photoactivated disinfection using Diode laser (665nm), with a power density of 100mW/cm^2 and total energy fluence of 30 J/cm^2. The groups were subdivided, 8 specimens were subjected to microbiological analysis and 2 specimens were analysed under CLSM to evaluate the alive/dead bacteria.

Results: The MB+NP group showed least number of CFU's after PAD, and was followed by MB+US group. The control group exhibited maximum number of CFU's. All the groups showed significant difference when compared to the control group. The CLSM images mirrored and justified the results obtained by microbiological analysis.

Conclusion: The ultilisation of PLGA nanoparticles encapsulated with photosensitive dye proved to be effective against E faecalis biofilm and can be a promising adjunct in antimicrobial endodontic therapy.

Keywords: PLGA nanoparticles, Photoactivated disinfection, Methylene blue, *E.faecalis* biofilm, BHI broth, Diode lasers, Confocal laser scanning microscope.

INTRODUCTION

The success of endodontic therapy depends on the effective control of bacterial infection within the root canal system.[33] Accepted treatment procedures to eliminate the infection include chemo-mechanical preparation which comprises of removal of infected hard tissue, disinfection by one or more irrigants and intracanal medicaments, followed by obturation of the canal with an inert material to provide fluid impervious seal.[12] However, the main cause of treatment failures are the presence of persistent microorganism and recontamination of canals due to inadequate coronal seal.[29]

An ideal irrigant or combination of irrigants, kills bacteria, dissolves necrotic tissue, lubricates canal, removes smear layer and does not irritate healthy tissue. Most commonly used irrigants are 2.5% to 5.25% Sodium hypochlorite, 0.2% Chlorhexidine, 15% to 17% EDTA, Hydrogen peroxide. In addition, inter-appointment placement of intracanal medicaments such as Calcium hydroxide, has been suggested for disinfection of the root canal system.[29] In spite of plethora of new products and techniques used, achieving complete

disinfection has not been possible and poses a challenge to the endodontist.

The microorganisms present in the root canal system can be either in the form of planktonic species i.e. free floating or in the form of biofilm. Eradication of biofilm with conventional treatment has been challenging due to biological complexity of the biofilm structure and the variety of inanimate surface that microbial cells colonize.[8]

Incidence of treatment failures associated with non-surgical endodontic treatment are associated with high proportions of Gram positive anaerobic organisms, especially *Enterococcus faecalis*, and have been reported to show resistance to common intracanal medication.[37] *E. faecalis* has the ability to survive in the root canal as a single organism without the support of other bacteria, in harsh ecology of environment[34]. In addition, *E-faecalis* can form intra and extra radicular biofilms, which make it even harder to control them.[35]

Despite the improvements in instrumentation techniques and use of intracanal medicaments, failure of endodontic treatment has been cited in literature due to the persistence of biofilm. Moreover,

the use of antibiotics serves an alternative approach but its long term use can be rendered ineffective by the resistance developed in the target organisms.[10] Considering this, disinfection of root canal, including the most distant areas of the tubular system is a major challenge in endodontic treatment.

Contemporary approaches to disinfect root canal have been proposed that include the use of ultrasonics, negative pressure syringe and photodynamic theory.[12] Low power lasers within visible range along with dyes or Photosensitiser (PS) have been used for root canal disinfection, and is termed as photoactivated disinfection (PAD). It is based on the concept that non-toxic photosensitisers can be preferentially localised in certain tissues and subsequently activated by a light source of appropriate wavelength, to generate singlet oxygen and free radicals that are cytotoxic to the target tissue.[25]

Methylene blue (MB) a well established photosensitiser has been used in PAD for targeting various gram positive and negative oral bacteria, as well as in endodontic disinfection. However, studies have demonstrated incomplete destruction of oral biofilms using MB-mediated PAD. The reduced susceptibility of biofilms to PAD was

attributed to reduced penetration of dye.[20] In addition, it has been shown that dyes such as MB are substrates of multidrug resistance pumps in bacteria thus decreasing the effectiveness of the photosensitiser.[16] Therefore, to overcome these deficiencies a drug delivery system has to be developed that can improve the pharmacological characteristics of MB.

Recent studies on PAD have focused on the use of polymer-based nanoparticles for photosensitizer delivery and release systems.[20,30,1] Nanoparticles containing photosensitizers have exhibited several advantages over photosensitizing molecules not encapsulated in nanoparticles.[20]

FDA-approved biodegradable polymeric nanoparticle Poly lactic co-glycolic acid (PLGA), has been used as a drug delivery system for photosensitizers. Once encapsulated within PLGA, the excited state of the photosensitizer is quenched, which results in loss of phototoxicity. When the nanoparticles were incubated with the targeted cells, they showed a time-dependent release of the photosensitizer, which then regained its phototoxicity and resulted in an activatable PAD nanoagent.[20]

AIM:

The aim of the present study was to determine the efficiency of photoactivated disinfection against *E. faecalis* biofilm.

The hypothesis of the study was that, encapsulation of Methylene blue with PLGA nanoparticle may offer a novel design of nanoplatform for enhanced drug delivery in the root canal system and photodestruction of *E. faecalis* biofilm.

OBJECTIVE:

The objective of the study was to explore the:

i. Efficacy of photoactivated disinfection against *E. faecalis* biofilm using photosensitiser MB loaded with PLGA nanoparticle and blank MB, by Colony Forming Unit technique.
ii. Demonstrate the viable and dead bacteria in the dentinal tubules with the Confocal laser scanning microscope.

REVIEW OF LITERATURE

Siqueira et al (1999)[34] reviewed the function of calcium hydroxide as a routine intracanal medicament. He elaborated the physic-chemical properties of the substance that may limit its effectiveness in disinfecting the entire root canal system. Calcium hydroxide was not effective against all bacterial species found in root canal infections. Its association with other medicaments enhanced the efficacy of the intracanal medication in eliminating residual bacteria in the root canal system.

Lee et al (2004)[19] investigated the usefulness of Class IV lasers (such as Nd:YAG, diode, KTP and Er:YAG) for photo-thermal disinfection of the root canal using photosensitisers such as tolonium chloride. They concluded that while PAD can be undertaken as part of the routine disinfection of the root canal system, it also has potential use for eradicating persistent endodontic infections for which conventional methods have been unsuccessful.

Ugo Bilati et al (2005)[3] formulated and modified the process to improve the versatility of the nanoprecipitation technique for encapsulation of hydrophilic drugs using parameters such as the solvent

and the non-solvent nature, the solvent/non-solvent volume ratio and the polymer concentration. It was shown that the mean particle size was closely dependent on the type of non-solvent selected. High polymer concentration in the solvent prevented nanoparticle formation. Thus they concluded, both poly lactic acid (PLA) and polylactic-*co*-glycolic acid (PLGA) could be used as a efficient carrier by accurately choosing the polymer solvent.

Wei Tang et al (2005)[42] developed and characterised the nanoparticles loaded with methylene blue (MB), which are designed to be administered to tumor cells externally and deliver singlet oxygen for photodynamic therapy. Induced by light irradiation, the entrapped MB generated singlet oxygen which was measured quantitatively. Polyacrylamide nanoparticles showed the most efficient delivery of oxygen**,** but its loading of MB was low. In contrast, the sol-gel nanoparticles had the best MB loading but the least efficient oxygen delivery. The encapsulation of MB in nanoparticles diminished the interaction of this PS with the biological milieu, thus facilitating its systemic administration.

Garcez et al (2006)[9] in their invitro study proved that photosensitizer alone or laser alone did not have any bactericidal effect. Chemical solution (0.5% NaOCl) reduced viable bacteria in 93.25%. Laser photosensitization resulted in a reduction of 99.2%, a significantly higher bacterial reduction than NaOCl. Laser photosensitization was effective for reducing *E. faecalis* in root canals and could be an adjunct to endodontic treatment.

Willam et al (2006)[44] measured the antibacterial action of photoactivated disinfection (PAD) on endodontic bacteria in planktonic suspension and root canals using *Fusobacterium nucleatum, Peptostreptococcus micros, Prevotella intermedia and Streptococcus intermedius*. Bacteria in canals were sampled before and after light irradiation. In suspension, reductions in bacteria were highly significant for light/Tolonium chloride combinations compared to light or Tolonium chloride alone. Antibacterial action was increased by energy dose increase, but not by Tolonium chloride concentration. PAD killed endodontic bacteria at statistically significant levels and the kills varied with bacterial species.

Soukos et al (2006)[37] investigated the effects of photodynamic therapy on endodontic pathogens in planktonic phase as well as on Enterococcus faecalis biofilms in experimentally infected root canals of extracted teeth. Methylene blue fully eliminated all bacterial species with the exception of *E. faecalis* (53% killing). The same concentration of methylene blue in combination with red light was able to eliminate 97%, using an optical fiber with multiple cylindrical diffusers that uniformly distributed light at 360 degrees.

Tada et al (2007)[42] prepared and characterised methylene blue-containing silica-coated magnetic particles by transmission electron microscopy, light scattering, and X-ray diffraction. The immobilized drug can generate singlet oxygen, which was detected by its characteristic phosphorescence decay curve in the near-infrared and by a chemical method using 1, 3-diphenylisobenzofuran to trap singlet oxygen. The lifetime of singlet oxygen was determined to be 52 μsec (in acetonitrile) and 3μsec (in water). The release of singlet oxygen was affected by the encapsulation of MB in the silica matrix, which caused a reduction to 6% of the quantum yield of MB free in solution.

Saji et al (2007)[13] evaluated the cytotoxicity and selectivity of an advanced noninvasive light-activated disinfection against fibroblast cells. Simultaneous evaluation of cytotoxicity and antibacterial effect was also conducted to study the specificity of light activated therapy toward prokaryotic cells (*Enterococcus faecalis*). Data revealed that cytotoxicity was significantly less in LAT compared with NaOCl. E faecalis cells were killed at a faster rate than fibroblasts. An irradiation dose producing 97.7% bacterial killing showed only 30% fibroblast dysfunction.

Jason M. Duggan et al (2007)[6] tested the hypothesis that the ability of Enterococcus faecalis to form biofilms is related to the source of the strains. They evaluated the *E. faecalis* strains recovered from root canals, oral cavity, and non-oral/non-endodontic sources and concluded that there were no significant associations between biofilm formation and the presence of the virulence determinants asa, cylA, esp, and gelE.

Sulis et al (2007)[36] reviewed the use of Passive ultrasonic irrigation in endodontics. Irrigation with sodium hypochlorite is more effective than with water and ultrasonic irrigation is more effective than sonic irrigation in the removal of dentine debris from the root canal. The

role of cavitation during PUI remains inconclusive. The influence of irrigation frequency and intensity on the streaming pattern as well as the complicated interaction of acoustic streaming with the adherent biofilm has been clarified to reveal the underlying physical mechanisms of PUI.

Garcez et al (2008)[12] analyzed the antimicrobial effect of photodynamic therapy (PDT) in association with endodontic treatment and suggested that the use of PDT added to endodontic treatment leads to an enhanced decrease of bacterial load and may be an appropriate approach for the treatment of oral infections.

Bechet et al (2008)[1] described and compared the different individual types of nanoparticles that are currently in use for PDT applications. They highlighted on the recent advances in the use of nanoparticles, including inorganic oxide-, metallic-, ceramic-, and biodegradable polymer-based nanomaterials as carriers of photosensitizing agents. The nanoparticles were described in terms of stability, photocytotoxic efficiency, biodistribution and therapeutic efficiency. Finally, they summarized with new results concerning the improvement of the photophysical properties of nanoparticles by means of biphotonic absorption and upconversion.

Pinheiro et al (2008)[23] evaluated photodynamic therapy in deciduous teeth with necrotic pulp by means of fully quantifying viable bacteria, before and after instrumentation and after the use of photodynamic therapy. The instrumentation resulted in a reduction of 82.59% of viable bacteria, and, after PDT, the microbial reduction observed was 98.37%.

Fimble et al (2008)[8] investigated the photodynamic effects of methylene blue on multispecies root canal biofilms comprising *Actinomyces israeli*, *Fusobacterium nucleatum* subspecies nucleatum, *Porphyromonas gingivalis*, and *Prevotella intermedia* in experimentally infected root canals of extracted human teeth in vitro. Root canal systems were incubated with methylene blue (25 μg/mL) for 10 minutes followed by exposure to red light at 665 nm with an energy fluence of 30 J/cm^2 by diode laser via a 250m diameter polymethyl methacrylate optical fiber. Photodynamic therapy (PDT) achieved up to 80% reduction of colony-forming unit counts.

Saji et al (2008)[14] tested the hypothesis that the inclusion of an oxidizer and oxygen carrier in the photosensitization formulation would facilitate comprehensive disinfection of matured endodontic biofilm by

light activated disinfection. MB in emulsion was overall the most effective photosensitizer formulation for photooxidation, generation of singlet oxygen, and in disinfecting biofilm bacteria. Advanced noninvasive PAD using a photosensitizer formulation containing oxidizer and oxygen carrier disrupted the biofilm matrix and facilitated comprehensive inactivation of biofilm bacteria.

Souza et al (2009)[39] aimed to investigate the antibacterial effects of photodynamic therapy with methylene blue (MB) or toluidine blue (TB) as a supplement to instrumentation/irrigation of root canals experimentally contaminated with *Enterococcus faecalis*. PDT with either MB or TB did not significantly enhance disinfection after chemomechanical preparation using NaOCl as irrigant. No significant differences were observed between the two photosensitizers. Thus the results suggest that PDT with either MB or TB may not exert a significant supplemental effect to instrumentation/ irrigation procedures with regard to intracanal disinfection.

Reddy et al (2009)[27] reviewed Photodynamic therapy (PDT) as a powerful laser-initiated photochemical reaction and demonstrated the applications of photodynamic therapy in treatment of oral cancer,

bacterial and fungal infections and photodynamic diagnosis of malignant transformation of oral lesions. PACT (photodynamic antimicrobial chemotherapy) has been efficacious in the management of peri-implantitis, endodontic infections and oral biofilms such as plaque. The absence of genotoxic and mutagenic effects, no risk of developing resistance to its antimicrobial action and increased healing process favors its long-term safety and use.

Xu et al (2009)[45] assessed the in vitro synergistic effect of methylene blue (MB) and red light on human gingival fibroblasts and osteoblasts. Assessment of PDT-induced apoptosis was investigated. Light at 20 and 40 mW/cm^2 with MB had modest effects at 24 hours on osteoblasts in both assays, whereas sodium hypochlorite (NaOCl) completely eliminated cells. Western blot analysis revealed no signs of apoptosis in either cell type. The data suggest that there is a safe therapeutic window whereby PDT can inactivate endodontic pathogens without affecting host cell viability.

Avinesh Kumari et al (2010)[18] reviewed the biodegradable nanoparticles that have been used frequently as drug delivery vehicles. They highlighted the impact of nanoencapsulation of various disease

related drugs on biodegradable nanoparticles such as PLGA, PLA, chitosan, gelatin, polycaprolactone and poly-alkyl-cyanoacrylates.

Schlafer et at (2010)[29] evaluated the antimicrobial effect of photoactivated disinfection (PAD) using toluidine blue and an LED lamp on endodontic pathogens in planktonic suspension and after inoculation into extracted teeth. Photoactivated disinfection yielded significant reductions in the viable counts of all organisms in planktonic suspension as well as in root canals.

Anil Kishen et al (2010)[16] evaluated the efficacy of antimicrobial photodynamic inactivation of *Enterococcus faecalis* biofilms using a cationic, methylene blue (MB) and an anion, rose bengal (RB). The role of a specific microbial efflux pump inhibitor (EPI), verapamil hydrochloride in the MB-mediated PDT on *E. faecalis* biofilms was also investigated. PDT with cationic MB produced superior inactivation of *E. faecalis* strains in a biofilm along with significant destruction of biofilm structure when compared to anionic RB. The ability to inactivate biofilm bacteria was further enhanced when the EPI was used with MB.

Garcez et al (2010)[10] reported the antimicrobial effect of photodynamic therapy (PDT) combined with endodontic treatment. Endodontic therapy alone produced a significant reduction in numbers of microbial species but only 3 teeth were free of bacteria, whereas the combination of endodontic therapy with PDT eliminated all drug-resistant species and all teeth were bacteria-free.

Shinde et al (2011)[32] formulated nanoparticles for simvastatin drug used as a lipid lowering agent. Nanoparticles were prepared by precipitation-solvent deposition method and were found to be effective in sustained drug release for a prolonged period.

S Rajesh et al (2011)[27] have reviewed the use of therapy for management of periodontal disease. They have enumerated the need to develop an evidence based approach to the use of this therapy for management of periodontitis, periimpantitis and endodontic therapy.

Rios et al (2011)[28] evaluated the antimicrobial effect of PDT using toluidine blue O (TBO) and a low-energy light emitting diode (LED) lamp after the conventional disinfection protocol of 6% NaOCl. The bacterial survival rate of the NaOCl/TBO/light group was significantly lower than the NaOCl and TBO/light groups. PDT using

TBO and a LED lamp has the potential to be used as an adjunctive antimicrobial procedure in conventional endodontic therapy.

Steier et al (2011)[40] evaluated the bovine pulp tissue dissolution ability of photodynamic therapy and concluded that only NaOCl was able to promote complete dissolution of pulp tissue whereas photodynamic therapy does not show any ability to dissolve pulp tissue.

Raymond et al (2011)[26] evaluated the anti-microbial effects of photodynamic therapy (PDT) on infected human teeth ex vivo. They divided the study into two groups- canals treated with chemomechanical debridement (CMD) and canals treated with CMD followed by treatment with Photoactivated disinfection (PAD) using Methylene blue. The results suggested that post-treatment detection levels for all species were markedly lower for canals treated by CMD+PDT than were for those treated by CMD alone.

Jarza et al (2011)[15] reviewed the application of nanotechnology for enhancement of PAD. In the article influence of silver-doped nanomaterials addition on the fluorescence intensity of photosensitizers immobilized in silica-titania (SiO2-TiO2) sol was examined via VIS

spectroscopy. Influence of sonication on the fluorescence enhancement was also investigated. It was demonstrated that the fluorescence enhancement of photosensitizers depends on the concentration of both: photosensitizer and silver-doped nanoparticles.

Shimab Shahin et al (2012)[30] reviewed the potential of nanotechnology to offer solutions to obstacles in cancer therapies. Nanoscale devices have impacted cancer biology at three levels: early detection, tumour imaging using radio contrast nanoparticles or quantum dots; and drug delivery using nanovectors and hybrid nanoparticles. Nanocrystals labeling with immune cells can act as a platform technology for nanoimmunotherapy.

Silva et al (2012)[33] evaluated the in vivo response of apical and periapical tissues of dogs teeth with apical periodontitis after one-session endodontic treatment with and without antimicrobial photodynamic therapy. In the PDT-treated groups, the periapical region was moderately/severely enlarged with no inflammatory cells, moderate neoangiogenesis and fibrogenesis, and the small periapical lesions was detected suggesting PDT to be a promising adjunct therapy to cleaning and shaping procedures.

MATERIALS

1. Extracted mandibular premolars
2. Enterococcus faecalis (ATCC 29212)
3. Blood agar
4. Sodium hypochloride – 6%, 0.5%
5. Methylene blue (Bio Lab systems)
6. Poly lactic-co-glycolic acid(PLGA) {Sigma Aldrich, Germany}
7. Pluronic (Sigma Aldrich, Germany)
8. Sodium oleate (Sigma Aldrich, Germany)
9. Diamond saw
10. Glyde(Dentsply, Tulsa)
11. Gutta Percha (Dentsply,Tulsa)
12. 17% EDTA (Tulsa,USA)
13. Micro centrifuge tube
14. Micro pipette (Eppendorf, Germany)
15. Phosphate buffered saline (PBS)
16. Nail polish
17. Canal clean 30 gauge irrigation needle

18. Rubber dam
19. SYTO-9 and Propidium iodide stains

ARMAMENTARIUM:

20. Hand piece (PAN AIR, NSK,Japan)
21. Incubator (Biotechniques, India)
22. Access opening bur (Mani Inc, Japan)
23. K- File- size 15, 25 (Mani inc, Japan)
24. ProTaper- S1,S2,F1,F2,F3,F4(Dentsply Maillefer,Tulsa)
25. X- smart (Dentsply Maillefer,Tulsa)
26. Ultrasonic instrument (Satlec, USA)

SPECIAL EQUIPMENTS:

27. Laser unit (Ezlase Diode laser, san Clemente, USA)
28. Magnetic stirrer (Remi Elektrotechnite Ltd)
29. Cryo-centrifuge (Rotor F-45)
30. Freeze dryer (Delvac)
31. Confocal Laser Scanning Microscope (Zeiss LSM 510 Meta)

METHODOLOGY

Preparation of the Poly lactic co-glycolic acid (PLGA) nanocarriers:

76mg of PLGA was mixed with 14mg of Pluronic in 5ml of acetone. The solution was heated and stirred until it became clear. 10mg of Methylene blue and 90mg of Sodium oleate was introduced into the acetone solution and kept for overnight vigorous magnetic stirring. The nanoparticle solution was centrifuged at 10000rpm for 20 minutes. The sediment part was collected, washed twice with water and freeze dried.

Preparation of the tooth specimens:

Fifty freshly extracted single-rooted mandibular premolar teeth with straight canals were stored in 0.5% sodium hypochlorite for two weeks. Specimens were decoronated to a standard 12 mm root segment length with a rotating diamond saw at 20,000 rpm under water-coolant. Patency of apical foramina was established by inserting a size 15K-file. A file measurement was taken at the point where the size 15 K-file became visible at the apical foramen and 0.5 mm was subtracted to set the working length. The instrumentation sequence consisted of ProTaper

S1, S2, F1, F2, F3 and F4 following a crown-down technique. X-smart hand piece was used, following a pecking motion. The final apical patency was established with a size 25 K-file in order to allow for an adequate apical aperture for flushing of microbial aggregates. Glyde was used as a lubricant and canals were irrigated with 6% sodium hypochlorite throughout the instrumentation sequence. The final irrigation consisted of 1 ml of 17% ethylene demine tetra acetic acid (EDTA) solution for 3 min for smear layer removal, deactivated with 1 ml of 6% NaOCl for 3 minutes. Each tooth specimen was then placed in a micro centrifuge tube containing 500 µl of phosphate buffered saline (PBS). Teeth were subsequently autoclaved at 121°C for 20 minutes. Following autoclave sterilization, PBS was aspirated from the microcentrifuge tubes under sterile conditions. The root surface was coated with nail polish to avoid external microbial contamination.

***E.faecalis* biofilm growth:**

Fifty root specimens were transferred into sterile microcentrifuge tubes. One millilitre of BHI broth containing 10^9 microorganisms of *E. faecalis* was injected into the prepared root canal system using a

Canal-Clean 30 gauge irrigation needle. After injection, each specimen was entirely submerged in BHI broth, and the tubes were incubated anaerobically for 10 days.

Grouping & Photodisinfection of root canals:

The specimens were randomly divided into 5 groups, each containing 10 specimens (n=10).

Group I - MB with Nanoparticle carrier + Laser (photoactivation)

Group II – MB + Ultrasonic activation + Laser

Group III – MB + GP activation + Laser

Group IV – MB +Laser

Group V – No PAD (control)

Parameters for Laser irradiation:

In the present study, the source was a diode laser (Ezlase), with an output power of 1 Watt and a central wavelength of 665 nm. The power density was 100mW/cm^2 and the total energy fluence dose was 30 J/cm^2. The system was coupled to a 250-μm diameter polymethylmethacrylate

optical fiber that was mechanically notched over a one-centimeter length at approximately one-millimeter intervals.

Group I

The root specimens were placed in microcentrifuge tubes under sterile conditions. The canals were loaded till the access cavity with Methylene blue (MB) encapsulated with PLGA nanoparticle at concentration of 50 μg/ml equivalent to MB, using Canal clean 30 gauge needle. The entire specimen was immersed in the nanoparticle solution for 10 minutes. Excess drug solution was aspirated and the root specimens were removed from the tubes. Canals were irradiated with laser for 5 minutes with a break at 2.5 minutes, for 2.5 minutes, placing the tip at 1mm short of the working length following a spiral outward motion.

Group II

The root specimens were placed in microcentrifuge tubes under sterile conditions. The canals were loaded till the access cavity with Methylene blue (MB), using Canal clean 30 gauge needle. The canals

containing MB were ultrasonically activated for 20secs. The entire specimen was immersed in MB for 10 minutes. Excess MB was aspirated and the root specimens were removed from the tubes. Canals were irradiated with laser for 5 minutes with a break at 2.5 minutes, for 2.5 minutes, placing the tip at 1mm short of the working length following a spiral outward motion.

Group III

The root specimens were placed in microcentrifuge tubes under sterile conditions. The canals were loaded till the access cavity with Methylene blue (MB), using Canal clean 30 gauge needle. The canals containing MB was activated using size #35 Gutta-percha following 20 up and down movements till the working length at a frequency of 3 per sec. The entire specimen was immersed in MB for 10 minutes. Excess MB was aspirated and the root specimens were removed from the tubes. Canals were irradiated with laser for 5 minutes with a break at 2.5 minutes for 2.5 minutes, placing the tip at 1mm short of the working length following a spiral outward motion.

Group IV

The root specimens were placed in microcentrifuge tubes under sterile conditions. The canals were loaded till the access cavity with Methylene blue (MB), using Canal clean 30 gauge needle. The entire specimen was immersed in MB for 5 minutes. Excess MB was aspirated and the root specimens were removed from the tubes. Canals were irradiated with laser for 10 minutes with a break at 2.5 minutes for 2.5 mins, placing the tip at 1mm short of the working length following a spiral outward motion.

Group V

The specimens of this group were not exposed to Photoactivated disinfection and served as the control for the study.

Following all treatments, the 8 specimens from each group were subjected to microbiological analysis and 2 specimens were subjected to confocal laser scanning microscopic analysis.

Microbiological analysis:

Specimens was aseptically mounted on a rubber dam, by utilizing a plastic u-shaped rubber dam frame attached to a rack and oriented parallel to the lab bench top. The coronal 4 mm of each specimen was above the surface of the dam. The contents of root canals were sampled by flushing the root canals with a coronal application of 1-ml of BHI broth with a Canal clean 30 gauge irrigation needle. The bacterial suspension was collected in a 1.5 ml microcentrifuge tube positioned below the apical foramen. After vortexing for 20 seconds, serial dilutions were prepared and 100 µl aliquots were inoculated onto blood agar and incubated anaerobically for 7 days. Observations were obtained from each treatment group and colony forming units (CFU's) were calculated.

Confocal Laser Scanning Microscopic analysis:

Tooth specimens were split longitudinally into two equal halves and rinsed with 10mL of PBS for three times. Sections were then stained immediately with the SYTO9 and Propidium iodide (PI) reagents which are marketed as the Live/Dead stain (Baclight; Invitrogen Corporation,

Carlsbad, CA, USA) and examined under the Confocal Laser Scanning Microscope.

Sections were scanned using Confocal Laser Scanning Microscope with illumination by a Krypton/Argon laser (488 nm). The border of the root canal was first located with the microscope, and five randomly selected places were scanned with the CLSM for each section. The mounted specimens were observed at Magnification of 63×. The dimension of each scanned field was 0.70 ×0.70 mm. A 477/543-nm double dichroic mirror was used as an excitation beam splitter and a 545-nm short-pass filter divided green (SYTO9) and red fluorescence (Propidium Iodide) between the photomultipliers. A 505- to 550-nm band-pass filter was used to visualize SYTO9 and a 650-nm long-pass filter for PI.

The Confocal laser scanning microscopic (CLSM) images were recorded in the fluorescent mode. Fluorescence images were analysed with Amira 5.0 (Visage Imaging Inc., Andover, MA, USA), and image stacks were viewed with LSM Image Browser (Carl Zeiss Ltd). The initial stacks, comprising both green and red fluorescence, were split into

individual component colour channels and saved as grey-scale images. For each greyscale image, fluorescence was adjusted ('thresholded') such that signals of intensity less than 20% were regarded as background. The split greyscale images were then combined and calibrated to form a single fluorescent image which was qualitatively analyzed by three independent blinded observers to determine the proportion of green and red fluorescence denoting the presence of live and dead *E faecalis* cells in the mineralized human dentinal tubules.

LIST OF FIGURES

Fig 1 Autoclave

Fig 2 Incubator

Fig 3 Freeze Dryer

Fig 4 Magnetic stirrer

Fig 13 Teeth specimens

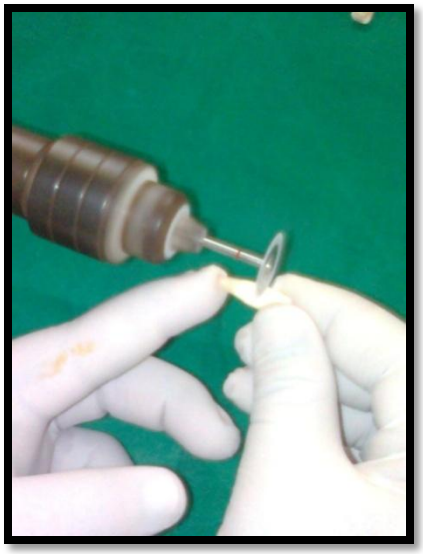


Fig 14 Decoronation

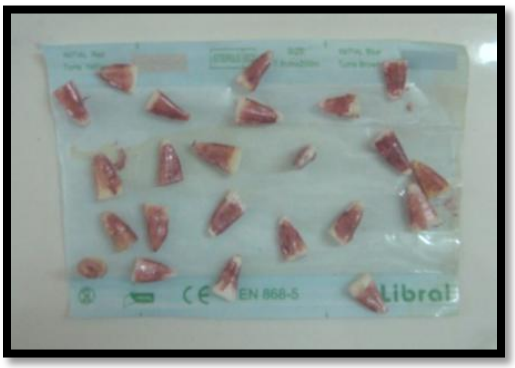


Fig 15 Autoclaved specimens

Fig 16 Infection of root canals

Fig 19 Incubated for 2 weeks

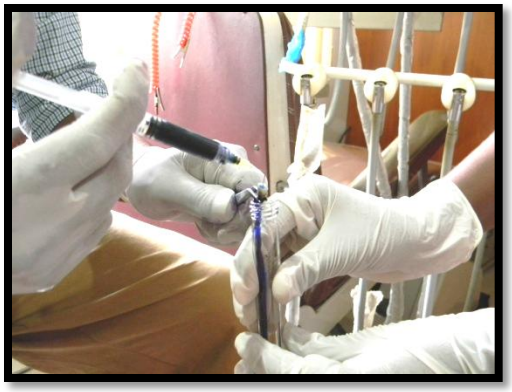


Fig 20 Incubation in PS

Fig21 Laser tip

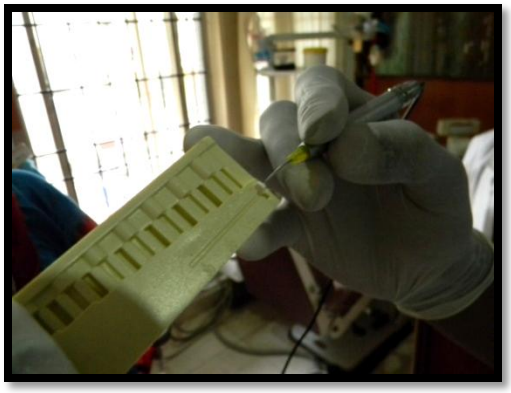


Fig22 W/L for Photo irradiation

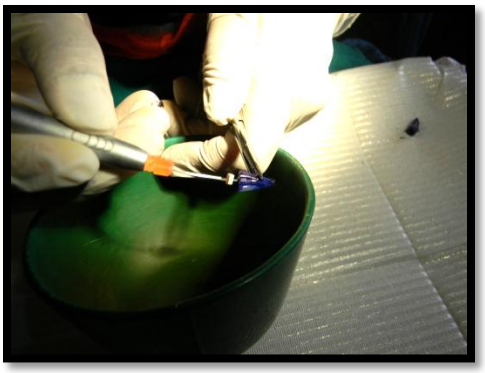


Fig 23 Photosensitization

Fig 24 Flushing of the root canals

Fig 25 Collection in Micro centrifuge tube

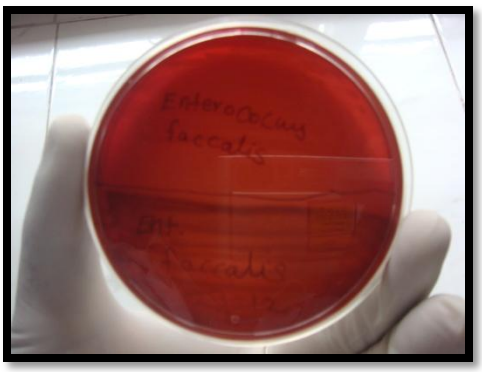


Fig 26 Aliquots plated on blood agar

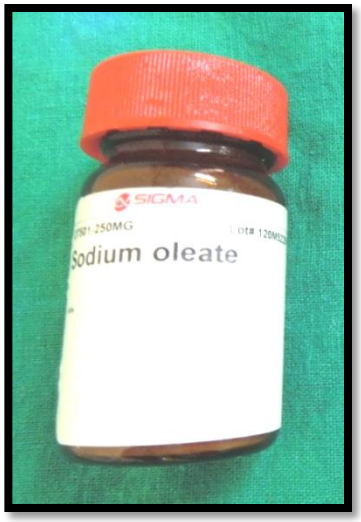


Fig. 9 Sodium Oleate

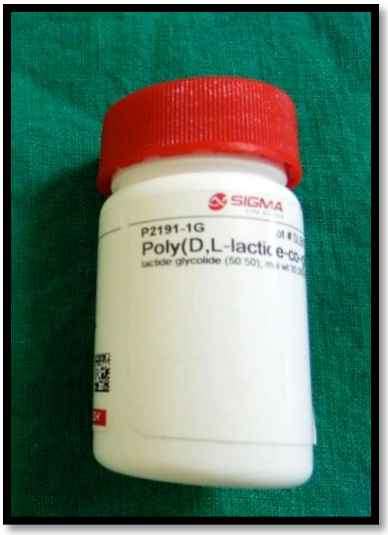


Fig. 10 Polylactic –co

-glycoic acid

Fig.11 Pluronic

Fig. 12 Methylene Blue

Fig 5 Diode laser

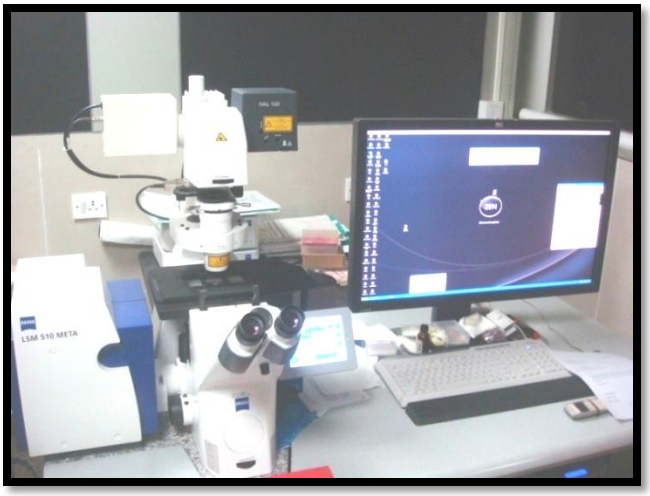


Fig.6 Confocal Laser Scanning Microscope (CLSM)

Fig. 7 BacLight Viability Kit

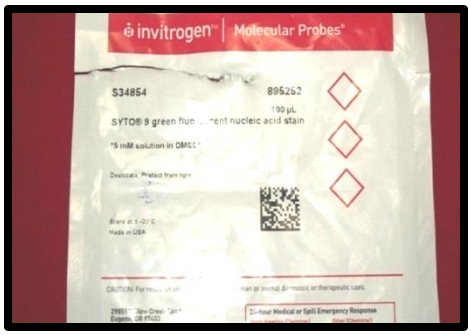


Fig.8 SYTO9 & Propidium iodide

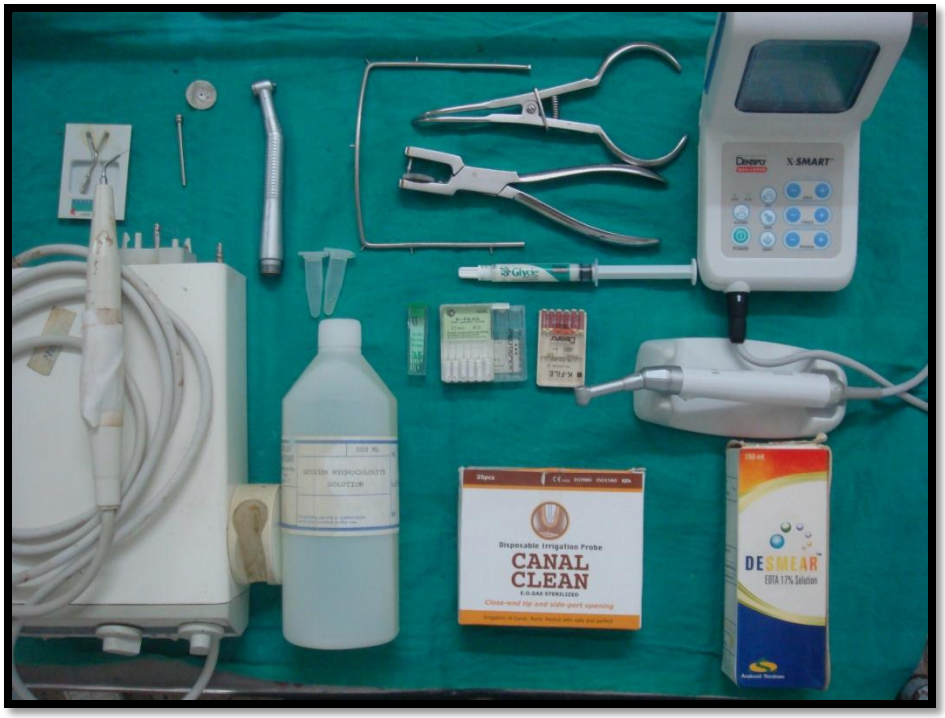


Fig. 17: Armamentarium

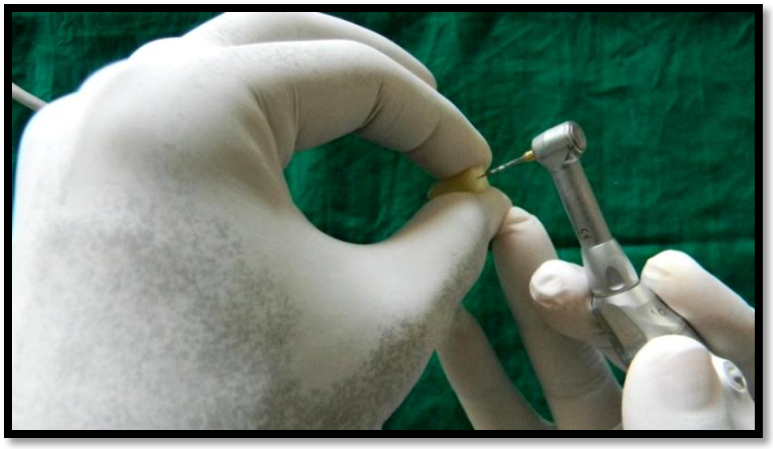
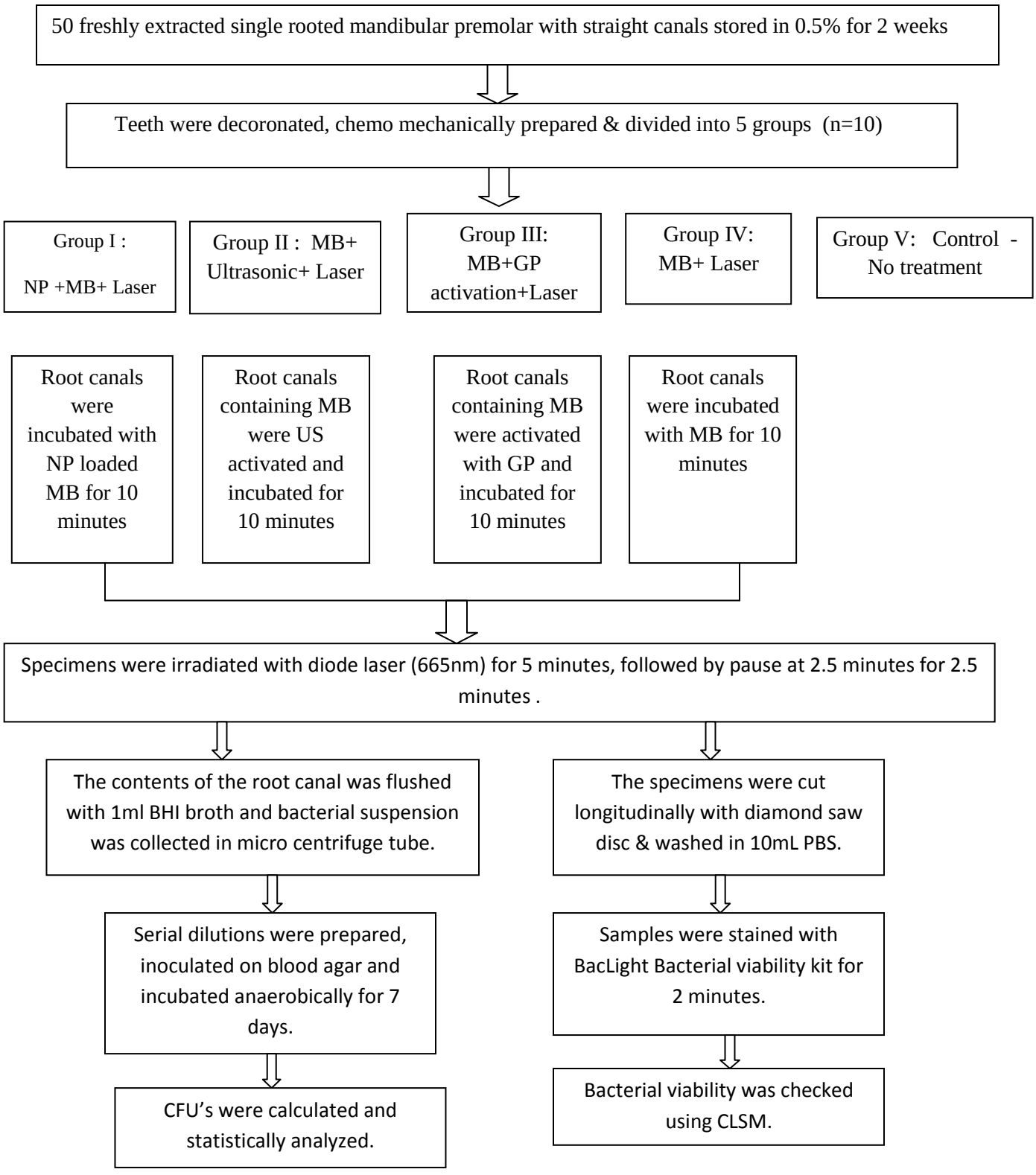


Fig. 18: Chemomechanical preparation

METHODOLOGY OVERVIEW



RESULTS

The colony forming units (CFU's) and survival fraction was calculated and statistically analyzed.

The data values of CFU's were log 10 transformed to reduce variance heterogeneity.

The survival fraction was calculated from CFU's counted on the plates:

Survival fraction = No. of CFU in the treated group/No. of CFU in the untreated group x 100

Cell death = 100 - Survival fraction

The untreated group (Group V) was used as a reference to calculate the survival fraction.

Cell death denotes exactly the efficiency of treatment or the reduction of bacteria, in terms of percentage, which gives better representation of bacterial killing.

The results of the present study were subjected to statistical analysis to interpret the significant differences among various treatment groups. One-Way ANOVA and post hoc Tukey HSD tests were used for statistical analysis in the present study.

One-way Analysis Of Variance (ANOVA) is used to study the overall variance within groups. It is the extension of the between groups t-test to the situation in which more than two groups are compared simultaneously. However, it is not possible to identify the difference between the various subgroups with the help of the P values obtained from ANOVA. Hence, the Tukey HSD is done in order to determine which groups differ from each other. The Tukey Test Honestly Significant Difference or HSD test is a post hoc test designed to perform a pair wise comparison of the means to identify the specific sub groups in which significant differential expression occurs.

In this study one way ANOVA followed by Tukey HSD test showed statistically significant difference among various subgroups concerning the discrepancy in the colony forming unit in each group.

I. Comparison of growth of CFU's in all the Groups:

Groups	Mean ± Standard deviation	P-value
I	2.45 ± 0.24	< 0.001**
II	3.05 ± 0.11	
III	4.07 ± 0.12	
IV	4.75 ± 0.16	
V	6.77 ± 0.08	

Note: ** denotes significance at 1% level

II. Comparison of growth of Colony forming units between each group:

Groups	Mean difference	Significance
Gr I x Gr II	-.6001*	.000
Gr I x Gr III	-2.2482*	.000
Gr I x Gr IV	-2.2962*	.000
Gr I x Gr V	-4.3203*	.000
Gr II x Gr III	-1.6480*	.000
Gr II x Gr IV	-1.6961*	.000
Gr II x Gr V	-3.7201*	.000
Gr III x Gr IV	-.0480	.964
Gr III x Gr IV	-2.0721*	.000
Gr IV x Gr V	-2.0241*	.000

Note: * denotes the mean difference is significant at the .05 level.

III. Comparison of the Survivability Fraction of all the groups:

Groups	Mean ± Standard deviation	P-value
I	0.06± .028	< 0.001**
II	5.46± .48	
III	9.13± 3.05	
IV	10.30± 3.41	

Note: ** denotes significance at 1% level.

IV. Comparison of Survivability Fraction in between the groups:

Groups	Mean difference	Significance
Gr I x Gr II	-5.4075*	.000
Gr I x Gr III	-9.0738*	.000
Gr I x Gr IV	-10.2512*	.000
Gr II x Gr III	-3.6663*	.018
Gr II x Gr IV	-4.8437*	.001
Gr III x Gr IV	-1.1775	.738

Note: * denotes the mean difference is significant at the .05% level.

Table I: denotes the Colony forming units in all the groups. Photoactivated disinfection with MB loaded with nanoparticles showed least value. It was followed by the group treated with MB activated with ultrasonic, MB+GP and MB. Group V (control) showed maximum CFU's. The P-value was <0.001, which implies statistically significant difference between the groups.

Table II demonstrates the significance of P values in all the groups. When the group treated with MB activated with GP was compared to the group treated with blank MB, no statistical significance was observed. The comparison of all the other groups were statistically significant (P < 0.5%).

Table III denotes the Survivability Fraction in all the groups. Photoactivated disinfection with MB loaded with nanoparticles showed least value. It was followed by the group treated with MB activated with ultrasonic and MB+GP. Group treated with blank MB showed maximum Survival Fraction. The P-value was <0.001, which implies statistically significant difference between the groups.

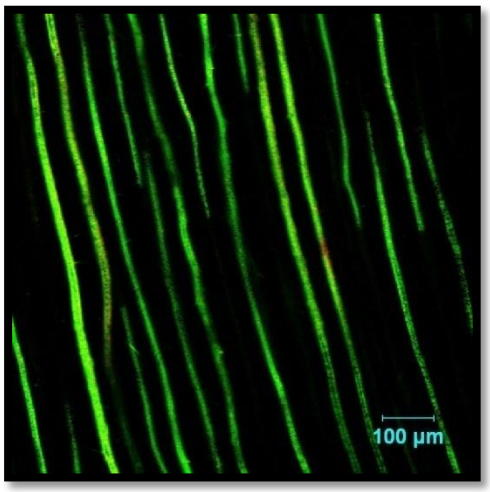
Table IV demonstrates the significance of P values in all the groups. When the group treated with MB activated with GP was

compared to the group treated with blank MB, no statistical significance was observed. The comparison of all the other groups were statistically significant ($P < 0.5\%$).

CFU's & CLSM IMAGES

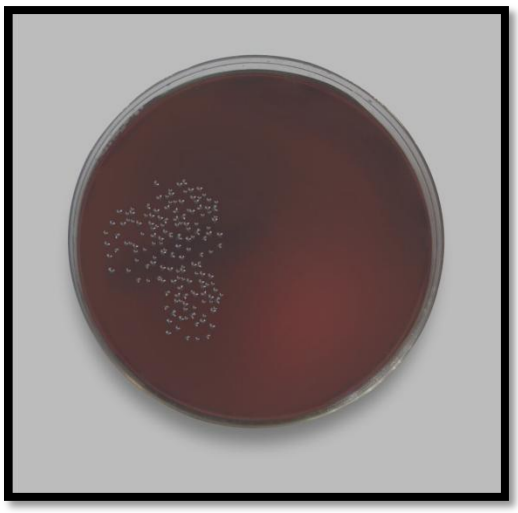
Image 1: Group V (Control)

Colony Forming Unit's



CLSM image (63x)

Image 2: Group I



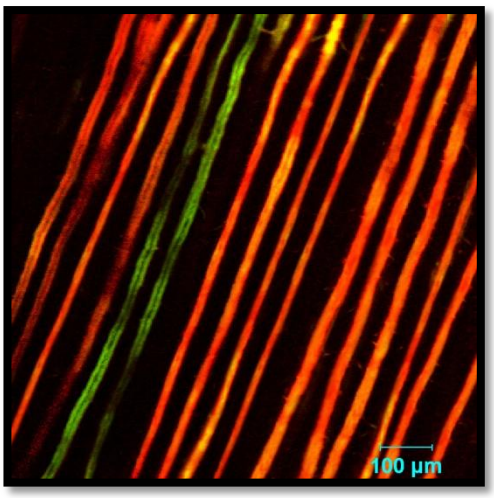
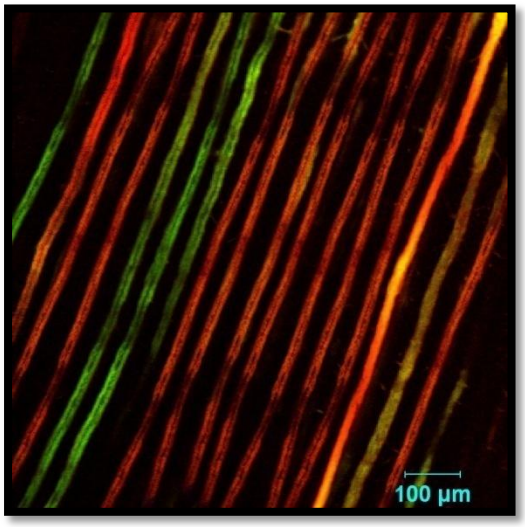


Image 3: Group II

Colony Forming Unit's



CLSM image (63x)

Image 4: Group III

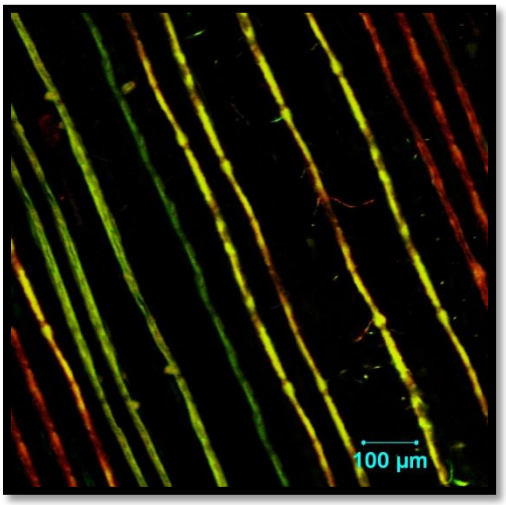
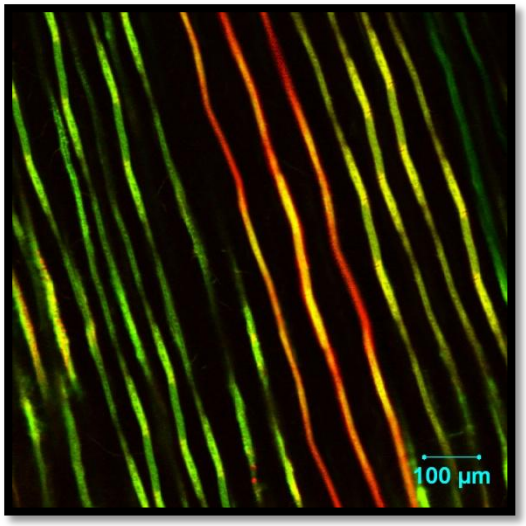


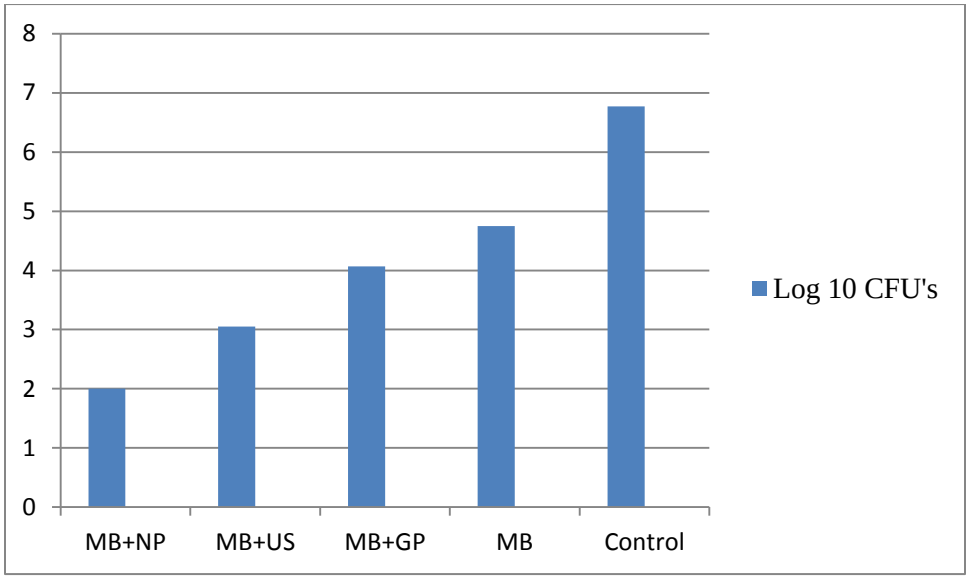
Image 5: Group IV

Colony Forming Unit's

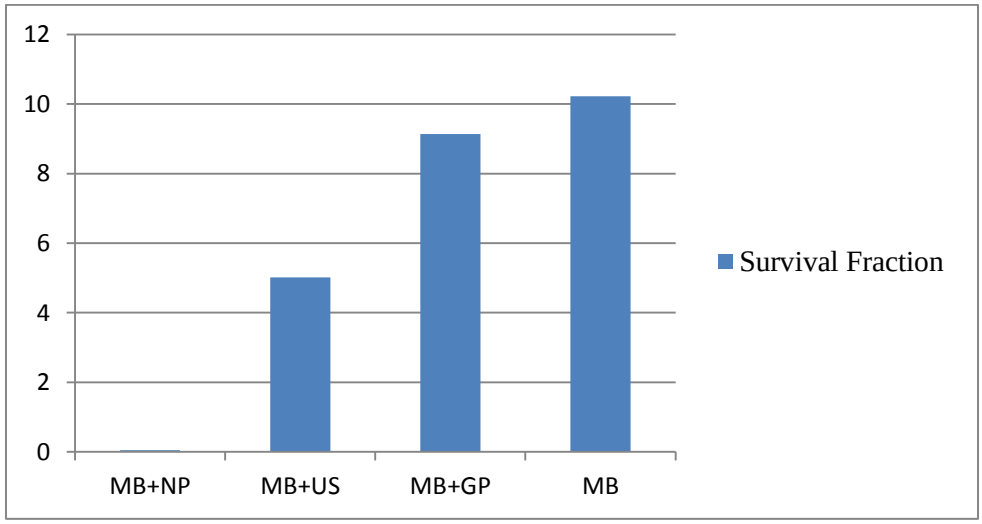


CLSM image (63x)

Graph I: Comparison of mean CFU's in all the groups:



Graph II: Comparison of mean Survivable fraction in all the groups:



DISCUSSION

The main goal of endodontic treatment is effective control of bacterial infection within the root canal system by elimination of pathogenic microflora, toxins and tissue debris.[33] Literature reports that endodontic therapy will have a 94% success rate when a negative microbiological culture is obtained from the root canal at the time of obturation. Whereas, when obturation is performed and the cultures are positive, the success rate is reduced to 68%.[11, 12] Studies have shown the shoddier healing of periapical lesions in cases where obturation has been performed with positive cultures.[12] Undoubtedly, the major factors associated with endodontic failure are the persistence of microbial infection in the root canal system and in the periradicular area.[33, 35]

Chemo-mechanical preparation can be considered to be an essential step in the root canal disinfection. It includes debriding of the infected dentinal walls of the root canal system using files in a sequence and use of irrigants. Most commonly used irrigants are hydrogen peroxide, sodium hypochlorite (2.5-5.2%), EDTA (15-17%), Chlorhexidine 0.2%.[28] The depth of penetration of irrigants such as Sodium hypochloride is approximately 130 µm into dentinal tubules

whereas tubular infection may occur closer to cementum-dentin junction at 1000 µm.[28] Moreover, studies have demonstrated that part of root canal space often remains untouched during chemomechanical preparation, regardless of the technique or instruments employed.[35] Thus complete elimination of the bacteria was difficult to accomplish. To overcome this, intrancanal medicaments such as Calcium Hydroxide were introduced.

Action of Calcium hydroxide is mainly by increase in pH in the root canals by release of hydroxyl ions. But certain bacteria such as enterococci tolerate very high pH value, ranging from 9-11.Several studies have attested the inefficiency of calcium hydroxide in eliminating the bacterial cells inside dentinal tubules.[22,34] *Haapasalo and Orstavik (1987)* reported that Calcium hydroxide paste failed to eliminate even superficial *E faecalis* in the tubules.[35]

E.faecalis is a gram positive bacterium, member of the commensal human flora and an opportunistic pathogen implicated as one of the leading causes of nosocomial infection. It is mostly associated with persistent endodontic infection and is highly associated with endodontic failures. They are resistant to common intracanal medication

when present in the form of biofilms.[16] A biofilm can be defined as microbial population attached to an organic or inorganic substrate, surrounded by microbial extracellular products, which form an inter microbial matrix.[35]

According to *George et al, 2008* the phenotypic and genotypic variation of biofilm bacteria when compared to their free flowing counterpart, complemented by the structure and composition of biofilm matrix contribute to their high microbial resistance.[16] The surface protein (Esp), the transcriptional regulator (BopD), the quorum sensing locus fsr and gelatinase (GelE) have been reported to be involved in promoting biofilm formation in *E faecalis*.[14]

Inability to completely eradicate biofilm structures in proximity to host immune cells will result in persistent infection and subsequent re-establishment of infection, with probably more treatment-resistant resident bacteria. Epidemiological studies have reported that 30%- 50% of root canal treatments fail from residual infection.[33] This has led to the quest of novel disinfection procedures that can be an adjunct to standard endodontic antimicrobial procedure, increasing the effectiveness of

orthograde endodontic treatment and retreatment procedures. *(Fimble 2008)*[8].

Contemporary approaches to disinfect root canal include the use of ultrasonics and lasers. Ultrasonics in endodontics was introduced by *Richman* in the year 1955.This technique appeared promising and files had the potential to prepare as well as debride root canals mechanically. Unfortunately, it proved to be difficult to control the cutting of dentine during ultrasonic preparation, which resulted in irregularly shaped root canals and also apical perforations *(Sluis 2007)*[36].Passive ultrasonic irrigation has recently led to a renaissance in the use of ultrasonics during root canal treatment. The technique utilizes an ultrasonically activated file or smooth wire within the root canal space following the completion of canal preparation.[36]

Laser is a device which transforms light of various frequencies into a chromatic radiation in the visible, infrared, ultraviolet regions with all the waves in phase capable of mobilizing immense heat and power when focused at close range. *Stern and Sognnaes (1964)* and *Goldman et al (1964)* were first to investigate the potential use of ruby lasers in dentistry. After initial experiment with ruby lasers, clinician started

using other high power lasers such as Argon, Carbon dioxide, Nd: YAG and Er: YAG. The first lasers used in endodontics were reported by *Weichman and Johnson, 1971*.

Most of the lasers used for root canal disinfection were high power laser which were dose dependent and generated heat. Thus in addition to killing microorganisms they caused collateral damage such as charring of dentin, ankylosis of root, melting of cementum, root resorption and periradicular necrosis.[28] To overcome these problems a new antibacterial strategy that involves the combination of a non toxic photo sensitizer and a laser light source within visible region (400 - 700nm), has been adopted and was termed as Photoactivated disinfection (PAD) [27]. *Dickers et al, 2009*[5] demonstrated that after 150s of PAD irradiation, the average temperature rise was 0.16±0.08ºC, the recorded values were lower than 7ºC which was within the safety level for periodontal injury.

The origin of light as therapy in medicine and surgery can be traced from antiquity to modern day. It began in ancient Egypt, Greece and India but disappeared for many centuries only to be rediscovered by the Western Civilization in beginning of 20th century.[25] German

physician *Friedrich Mayer-Betz* performed the first study, with what was first called photoradiation therapy (PRT) with porphyrins in 1913 on humans. But it was *John Toth*, who acknowledged the photodynamic chemical effect of the therapy with early clinical argon dye lasers and renamed it as photodynamic therapy (PDT). It received even greater interest as *Thomas Dougherty* formed the *International Photodynamic Association*. Its use first started in dermatology (1992), then oncology (1995), and recently in microbiology (1996).[27]

Photoactivated disinfection can be defined as an oxygen dependent photochemical reaction that occurs upon light mediated activation of the photosensitizing compound, leading to generation of cytotoxic reactive oxygen species, predominantly reactive oxygen. The treatment procedure involves three basic components: a photosensitizer, a light source and tissue oxygen. A photosensitizer (PS) is a chemical compound (usually a dye) that can be excited by light of specific wavelength (visible or infra-red light). The photosensitizer is administered (injected or applied externally) to the patient and gets accumulated in the targeted tissues. The tissue is then exposed to the light activating the dye from its ground singlet state to an excited singlet

state which then undergoes an intersystem crossing forming a longer lived excited triplet state. In the presence of endogenous oxygen, energy transfer then takes place from this activated agent to the oxygen molecule forming excited singlet state oxygen or other reactive oxygen species **(ROS),** causing a rapid and selective destruction of the target tissues.[25,27]

During PAD, cytotoxic reactive oxygen species (ROS) are formed by two mechanisms. Type I reaction involves electron transfer directly from the photosensitizer producing ions, or electron/hydrogen removal from a substrate molecule to form free radicals. These radicals react rapidly with oxygen, resulting in the production of highly reactive oxygen species (superoxide, hydroxyl radicals, and hydrogen peroxide). Type II reactions produces the electronically excited and highly reactive state of oxygen known as singlet oxygen. Usually the procedure involves a contribution from both the mechanisms.[27]

PAD requires a source of light that activates the photosensitizer by exposure to low-power visible light at a specific wavelength. Various light sources for PAD are Diode laser systems, Non-coherent light sources and Light emitting diodes (LED).[27]

Diode laser is the most widely used laser. It emits a bandwidth of laser (Central wavelength: 665nm) which matches with the required wavelength for photosensitization.[8,11,39] Thus, in the present study diode laser has been used.

An optimal Photosensitizer (PS) should possess photophysical, chemical, and biological characteristics. The properties of an ideal photosensitizer are, stable composition, minimal self aggregation tendency, nontoxic in the absence of light, target specificity and can be easily cleared from the body.[1] Most of the sensitizers used for medical purposes belong to the following basic structures: a) Tricyclic dye i.e. Methylene Blue. b) Tetrapyrroles i.e. Porphyrins c) Furocoumarins i.e. Xanthotoxin.[25]

Methylene blue (MB) has been used as a photosensitizing agent for almost nine decades and is used routinely as a marker dye in surgery. The clinical use of MB for photodynamic therapy of bladder and oesophageal cancer along with its use in case of methaemoglobinamea suggests the local use of MB is safe.[26]

Methylene blue (MB) is a well-established photosensitizer and has been used in PAD for targeting endodontic bacteria. The hydrophilicity

of MB, along with its low molecular weight and positive charge, allows passage across the porin-protein channels in the outer membrane of gram-negative bacteria. MB predominantly interacts with the anionic macromolecule lipopolysaccharide, resulting in the generation of MB dimers, which participate in the photosensitization process.[8] MB has been successfully used in PAD for targeting various gram-positive and gram-negative oral bacteria and has the ability to infiltrate dentinal tubules.[46]

However, studies by *Soukos et al 2000*[38], have demonstrated incomplete destruction of oral biofilms using MB-mediated PAD. The reduced susceptibility of biofilms to PAD was attributed to reduced penetration of dye. In addition, *Kishen et al, 2010*[16] have shown that dyes such as MB are substrates of multidrug resistance pumps in bacteria thus decreasing the effectiveness of the photosensitiser. These resistant microorganisms have microbial efflux pumps that have the ability to extrude or expel antibiotics. Thus the penetrability of drugs into the microorganism biofilm is impaired, and as a result disinfectant action is compromised. To overcome these deficiencies, a drug delivery system

has to be developed which can improve the pharmacological action of MB.

Nanoparticles represent an emerging photosensitiser carrier that show great promise for PAD. *Bechet et el 2008*[1], have enumerated the overcoming of drawbacks of classic photosensitisers by use of nanoparticles and explained the improved pharmacokinetic properties of the drug. It can be achieved by either surface bounding of photosensitizer particles to a nanoparticle or confinement of photosensitizer in nanocapsules. The advantages of a nanoparticle carrier for PS has been described by *Pagonis 2010*[20], i.e. they include a larger critical mass (concentrated package of photosensitizer) for the production of reactive oxygen species (ROS) that destroy cells, it limits the target cell's ability to pump the drug molecule back out thus reducing the possibility of multiple-drug-resistance, selectivity of treatment by localized delivery agents, and having a non-immunogenic nanoparticle matrix.

In this study PLGA nanoparticles was used, which are synthetic and biodegradable carriers. Synthetic polymers have the advantage of sustaining the release of the encapsulated therapeutic agent over a period

of days to several weeks.[30] *Bechet et al 2008*[1] have enumerated that biodegradable carriers, in addition of being non immunogenic and non inflammatory, are aqueous in composition and can disintegrate readily to release the photosensitisers when light is irradiated.

PLGA polymers have a number of advantages over other polymers used in drug and gene delivery, such as their biodegradability, biocompatibility, and approval by the FDA for human use. PLGA polymers degrade in the body through hydrolytic cleavage of the ester linkage to lactic and glycolic acid. These monomers are easily metabolized in the body via Krebs' cycle and eliminated as carbon dioxide and water. Biodegradation products of PLGA are formed at a very slow rate, and they therefore do not affect normal cell function. Furthermore, these polymers have been tested for toxicity and safety in extensive animal studies and are currently used in humans for resorbable sutures, bone implants and screws, contraceptive implants, and also as graft materials for artificial organs and supporting scaffolds in tissue engineering research.[30]

Confocal Laser Scanning Microscopy (CLSM) has become an invaluable tool for a wide range of investigations in the biological and medical sciences for imaging thin optical sections in living and fixed specimens ranging in thickness up to 100 micrometer. CLSM works on the principle of fluorescence. The advantage of fluorescence for microscopy is that fluorescent dye molecules can be attached to specific parts of any sample, so that only those parts are the ones seen in the microscope. Therefore, it is possible to distinguish two different parts of a particular sample. The CLSM can determine the viable and dead microorganism immobilized in the dentinal tubules.[14,16]

The objective of the present study was to investigate the efficacy of photoactivated disinfection, by MB-loaded PLGA nanoparticles against E. Faecalis biofilm, using CFU technique, and to assess the dead and viable bacteria by Confocal laser scanning microscopy.

In the present study fifty freshly extracted single-rooted human mandibular premolar teeth with straight canals were used. Teeth were decoronated to a standard 12 mm root segment length and chemo mechanically prepared. Teeth were subsequently autoclaved at 121°C for 20 minutes *(Pagonis et al, 2010)*[20]. The root surface was coated with nail

polish to avoid external microbial contamination.[22] Biofilm was allowed to be formed on the specimens by the procedure described by *Rios et al, 2011*.[28]

The nanocarriers were prepared following the solvent displacement procedure as described by *Shenoy et al 2005*[31]. Nanoprecipitation technique or solvent displacement method for nanoparticle was first developed and patented *by Fessi et al* in 1989.Its advantages are that it is straight forward, rapid and easy. It enables production of small nanoparticles i.e. 100-300nm. Nanoprecipatition occurs by rapid dissolvation of the polymer when the polymer solvent is added to the nonsolvent. As soon as the polymer containing solvent has diffused into the dispersing medium, the polymer precipitates involving immediate drug entrapment.

The specimens were randomly divided into 5 groups (n=10) from which 8 specimens were subjected to microbial analysis and 2 specimens were used for confocal laser scanning microscopy. Group I [MB+NP] comprises of specimens being photo activated with Methylene blue (MB) loaded PLGA nanoparticle (NP).Group II [MB+US] comprises of Methylene blue (MB) being ultrasonically (US) activated for 20 secs,

followed by photoactivation. Group III [MB+GP] comprises of Methylene blue(MB) being activated using size #35 Gutta-percha following 20 up and down movements till the working length at a frequency of 3 per sec., followed by photoactivation. Group IV [MB] comprises of specimens being photoactivated using only Methylene blue. Group V [C] serves as the control, and is not treated with photoactivation.

The incubation time in photosensitiser used in the present study was in accordance to *Xu et al, 2009*[45], were he described short incubation times up to 10 minutes with low concentrations of photosensitizer led to bacterial killing while human cells (fibroblasts and keratinocytes) were spared.

The parameters for diode laser specifications in this study were a power density of 100 mW/cm^2 and the total energy fluence dose of 30 J/cm^2. These values were in accordance to the specifications described by *Xu et al,2009*[45] and provides a safe therapeutic window without affecting the normal periapical cells. The output power used is 1 Watt and a central wavelength of 665 nm.

Canals were irradiated with laser for 5 minutes with a pause at 2.5 minutes, for 2.5 minutes. Fractionating the exposure to light may enhance the efficacy of the PAD treatment (*Fimble et al,2008*)[8]. The PAD effects are abolished under anoxic conditions, and the dark interval of 2.5 minutes might allow time for oxygen to diffuse back into the anoxic root canal system.

The laser tip was placed at 1mm short of the working length following a spiral outward motion. The spiral movements, from apical to cervical, were manually performed to ensure even diffusion of the light inside the canal lumen (*Garcez,2010*).[10]

In the present study notched optical fibre was used as it has been enumerated by *Fimble et al, 2008*[8] that notches provided a mechanical interruption to light propagation through the fiber and was able to uniformly distribute light over 360 degrees in the entire root canal system.

Following all treatments, the 8 specimens from each group were subjected to microbiological analysis and 2 specimens were subjected to confocal laser scanning microscopic analysis.

CFU is a primary microbial technique allowing determination of the number of viable bacteria per sample. The microbiological analysis was performed by aseptically mounting the specimens on a rubber dam and sampling the contents of root canals by flushing with 1-ml of BHI broth. The bacterial suspension was collected, aliquots from the experimental groups were plated on blood agar and CFU were counted to check for surviving bacteria. The results of the present study was tabulated and subjected to statistical analysis analysis to interpret the significant difference for CFU's between the groups.

Confocal Laser Scanning Microscopic (CLSM) analysis was performed by longitudinally splitting the tooth specimens into two equal halves and rinsing with 10mL of PBS for three times. Specimens were then stained immediately with the SYTO9 and Propidium iodide (PI) and examined under the Confocal Laser Scanning Microscope (CLSM).

The nucleic acid–binding fluors, SYTO9 and propidium iodide (PI), have been widely applied in environmental studies, food microbiology and dental research including endodontic investigation. These reagents were introduced by Invitrogen Corporation as the Baclight – Live/Dead stain, as they differentiate between viable and non-

viable bacteria. Fimble et al (2008)[8] evaluated the viability of *E faecalis* biofilm using combination stains, SYTO9 and propidium iodide (PI) and demonstrated that SYTO9 and PI are reliable vital stains that may be used to investigate under the Confocal Laser Scanning Microscope. Thus, the use of the fluorescent dyes to assess the viability of *E faecalis* biofilms on the root canal dentin has been confirmed in this study. SYTO 9 penetrates intact biological membranes, whereas PI penetrates only bacteria with compromised plasma membranes and quenches the SYTO 9 fluorescence on binding the nucleic acid. Thus, simultaneous application of the stains generates red-fluorescing dead bacteria and green-fluorescing live bacteria, and these can be visualized by fluorescence microscopy.

The CLSM determines the viable and dead bacteria immobilized in the dentinal tubules and is thus the appropriate tool of choice in this investigative study. Hence the CLSM method serves as a confirmatory guide and reflects the validity of the results obtained by the CFU method.

According to the results in the present study, MB when encapsulated with PLGA nanoparticles showed a mean CFU of

2.45±0.24, with P value <0.001 and the cell survivability fraction was 0.05%. The results were in accordance to *Pagonis et al,2010*[20], who has attributed similar results due to better penetrability of the MB into the *E faecalis* biofilm. PLGA nanoparticles were majorly concentrated on the bacterial cell walls. This may have rendered the cell wall permeable to MB released by nanoparticles, resulting in improved phototoxicity.

The results in the present study showed, when passive ultrasonic activated MB is photosensitized, CFU's were more than that of PLGA particles loaded with MB (CFU=3.05±0.11). The cell survivability fraction was 5.09%. The findings of this study were in agreement with the results obtained by *Bhuva et al, 2010*[2] and proves the efficacy of ultrasonics in disintegrating the biofilm.

MB with gutta percha activation showed high amount of viable cells (CFU= 4.70±0.12). Increased survivability fraction (SF= 9.13%) in this group could be due to lack of activation of MB by gutta-percha, resulting in poor penetration of the dye into the dentinal tubules. The results were in accordance to the findings of *Pragolia et al (2010)*[24].

According to the present study, Group IV (MB) showed maximum CFU's (4.75± 0.16) and had a survival fraction of 10.06%,

which was higher than all other PAD treated groups. The results corroborated with results obtained by *Soukos et al, 2000*[38] in their study regarding PAD against *E faecalis* biofilm.

The CLSM evaluation results mirrored the results obtained by the CFU method and demonstrated least number of *E faecalis* in MB+NP group. It was followed by the MB+US group. The two groups which performed best by the CFU analysis contained significantly fewer viable *E faecalis* in root canals as evidenced by the scanty green fluorescence. Most of the tubules were patent and empty with little or no bacterial penetration. Control specimens which were untreated showed almost 96 – 98% of viable bacteria in the group confirming the formation of *E faecalis* biofilm.

Literature has showed that it is safe to use PAD against microorganisms near normal cells, i.e. cells from apical region. According to *George et al, 2009*[13] the increased killing of bacterial cells by PAD could be because of the selective accumulation of MB and reduced level of singlet oxygen required to kill prokaryotic cells compared with mammalian cells. PAD produced 97.7% bacterial killing and only 30% fibroblast dysfunction.

Kishen et al, 2010[16] have explained the efficacy of PAD against Multiple Drug Resistant bacteria. These multi-drug resistant bacteria consisted of facultative and obligate anaerobic species. The aerobic microorganisms can deal better with reactive oxygen species, whereas the anaerobes have a greater susceptibility to the reactive oxygen species produced during PAD. This could explain the 100% reduction of multi-drug resistant bacteria after PAD.

According to *Reddy et al, 2009*[27] development of resistance to PAD appears to be unlikely, since, in microbial cells, singlet oxygen and free radicals interact with several cell structures and different metabolic pathways. Singlet oxygen produced during PAD has a direct effect on extracellular molecules thus the polysaccharides present in bacterial biofilm were also susceptible to photodamage.

During PAD, light acted as low level laser therapy, stimulating the healing process. *Silva et al, 2012*[33] demonstrated moderate fibrogenesis and neoangiogenesis and confirmed the absence of inflammatory cells in the groups in which PAD was used. *Reenstra et al* speculated that there must be an increase in oxygen diffusion through the tissues during the application of PAD, which might have favored the repair process

because collagen secretion by fibroblasts in extracellular spaces occurs only in the presence of high rates of oxygen pressure.[33]

The results of this study confirmed the hypothesis that photo activated disinfection by PLGA mediated Methylene Blue was found to be effective against *E.faecalis* biofilm.

SUMMARY

The purpose of the present study was to investigate the efficiency of Photoactivated disinfection against *E faecalis* biofilm, by Methylene blue loaded with and without PLGA nanoparticles. 50 extracted mandibular premolars were used for the study. Chemo-mechanical preparation was done using ProTaper files, and *E faecalis* biofilm was allowed to be formed in the canals. Teeth were divided into five groups Group I [MB+NP] comprises of specimens being photo activated with Methylene blue (MB) loaded PLGA nanoparticle (NP).Group II [MB+US] comprises of Methylene blue(MB) being ultrasonically(US) activated, followed by photoactivation. Group III [MB+GP] comprises of Methylene (MB) blue being activated with Gutta percha (GP), followed by photoactivation. Group IV [MB] comprises of specimens being photoactivated using only Methylene blue. Group V [C] serves as the control, and is not photo-treated. Aliquots from the experimental groups were plated on blood agar and CFU were counted to check for surviving bacteria. In the CLSM method, bacterial viability was demonstrated using special dyes SYTO 9 and Propidium iodide.

CONCLUSION

Within the limitations of the present study it can be concluded:

i. Photoactivated disinfection by using MB loaded PLGA nanoparticles was effective against *E faecalis* biofilm and reduced the bacterial load by 100%.

ii. Photoactivated disinfection using standard Methylene blue alone could reduce the bacterial load only by 90%.

iii. PLGA nanoparticles serve to be an efficient drug delivery system for the photosensitiser MB, and have enhanced its penetration into the *E faecalis* biofilm.

iv. Confocal laser scanning microscopic evaluation to demonstrate *E. faecalis* viability has been explored and confirmed in this investigation.

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