

**EVALUATION OF CYTOTOXICITY AND ANTIBACTERIAL
EFFICACY OF VARIOUS CONCENTRATIONS OF 3MIX
ANTIBIOTIC PASTE WITH BARIUM SULFATE AS AN
ADDITIVE**

**By
Dr. NESLIN BASLEO.N**

A Dissertation submitted to the
Sathyabama Institute of Science and Technology
(Deemed to be University)



In partial fulfillment of the requirement for the Degree of
MASTER OF DENTAL SURGERY

PEDIATRIC AND PREVENTIVE DENTISTRY

**SATHYABAMA DENTAL COLLEGE AND HOSPITAL
CHENNAI 600 119**

2019 – 2022

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This is to certify that the dissertation work entitled **“EVALUATION OF CYTOTOXICITY AND ANTIBACTERIAL EFFICACY OF VARIOUS CONCENTRATIONS OF 3MIX ANTIBIOTIC PASTE WITH BARIUM SULFATE AS AN ADDITIVE”** is an original work done by **DR. NESLIN BASLEO.N** for the Master of Dental Surgery in Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital. This study has been carried out independently under my direct supervision and guidance in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital, Chennai. Study period 2019-2022.

Date: 25/02/2022

Place: Chennai



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Regenerative endodontics is the developing field in the treatment of immature teeth with infected root canals which is a fundamental shift in the management of these teeth and can result in continued root maturation and apical closure.

Regenerative endodontics has been defined as “biologically based procedures designed to replace damaged structures, including dentin and root structures, as well as cells of the pulp-dentin complex”.(1)

The promise and potential of regenerative endodontic therapies in necrotic teeth was first explored by Nygaard-Östby in 1961 who investigated the potential for repair when bleeding was induced by over-instrumentation beyond the apex prior to partial root filling of the canal with limited success.(2)

The clinical considerations for regenerative endodontic protocols are, disinfection of the root canal system, provision of a scaffold which often involves laceration of the periapical tissue to induce a blood clot and introduce stem cell activity within the root canal, and an adequate coronal seal to prevent reinfection.(3)

Disinfection of the root canal system is thought to be critical to the success of regenerative endodontic procedures as infection prevents regeneration, repair and stem cell activity.(4)

After forty years since Nygaard-Östby introduced regenerative endodontic therapy, in 2001 Iwaya et al reported a case using a procedure termed „revascularization“, on an infected necrotic immature premolar

tooth that showed continued root maturation and thickening of root canal walls with mineralized tissue.(5) Subsequent case reports also demonstrated the efficacy of this technique primarily in premolar teeth when using a triple-antibiotic paste (Jung IY et al 2008) (6) or calcium hydroxide (Cotti E et al 2008). (7)

The introduction of endodontic regeneration procedures has rejuvenated the use of various antibiotic mixtures as intracanal medicaments. The most important aspect of intracanal medicaments is that they should ideally have antibacterial properties and also must create a toxic free environment to help in regeneration and healing.

„3Mix“ antibiotic paste is the most widely used intracanal medicament in Regenerative endodontic procedures.(8) The use of ciprofloxacin, metronidazole and minocycline in regenerative endodontic procedures has experimental support in the work carried out by Hoshino et al. (1988), who studied the bactericidal efficacy of metronidazole against bacteria in carious dentine, concluding that metronidazole effectively disinfected the carious dentine.(9)

The first report of revascularization in an infected immature tooth in 2001 used a double antibiotic paste of metronidazole and ciprofloxacin.(5) The next report by Banchs et al used a combination of metronidazole, ciprofloxacin and minocycline often referred to as „3Mix“ or „triple antibiotic paste“. (10)

Triple antibiotic pastes using this combination of antibiotics have been shown to reliably disinfect dentine in infected root canals with

diffusion of the drugs throughout the entire root dentine.(9) The American Association of Endodontist protocol recommends triple antibiotic pastes at concentrations no greater than 0.1 mg/mL.(11) At this concentration, triple antibiotic paste is conducive with stem cell survival and proliferation(12) and also effective in eliminating microorganisms within the root canal.(13)

After the experimental confirmation of the efficacy of the combination of ciprofloxacin, metronidazole and minocycline against endodontic bacteria, Banchs & Trope (2004) used, for the first time, a paste containing these three antibiotics in a case of regenerative endodontics.(10) The three antibiotics have either been mixed with water, saline, or been mixed with propylene glycol until a dense and smooth mix was achieved that was deemed ideal by clinicians.(8)

Ciprofloxacin, metronidazole and cefixime which are used in the preparation of 3Mix antibiotic paste cannot be visualized radiographically. Hence, it poses a challenge for the clinicians in terms of insuring satisfactory application of the 3Mix antibiotic paste in root canals. To overcome this, radiocontrast agents can be incorporated to the 3Mix antibiotic paste, as a radiopaque material. This will be better appreciable by the clinicians in root canals and can result in better prognosis.

It is essential in regenerative endodontic procedures that, any antibiotic or combinations of antibiotics be biocompatible and has no toxic effect on host stem cells. The apical papilla contains various cell types including stem cells. These cells have been hypothesized to play a part in root formation(14). Therefore, maintaining cell survival and promoting cell

attachment of the recruited apical papilla cells seem to be the first step in achieving root continuation and also tissue generation after revascularization procedures. Therefore, deleterious effects from either medications or techniques that are used in all regenerative procedures should be limited.

Fibronectin, a high molecular-weight glycoprotein associated with a variety of cell functions, is one of the most common mediators for cell and extracellular matrix adhesion.(15) Moreover, fibronectin is also considered to function in terminal differentiation of odontoblasts.(16)

Fibroblasts are mesenchymal cells that form fibers of the connective tissue and contribute to their structural integrity. They originate from a multipotent mesenchymal stem cell that also gives rise to adipoblasts, chondroblasts, osteoblasts, and myoblasts. (17)

It has been demonstrated that both triple antibiotic paste and double antibiotic paste typically used in regenerative endodontic procedures are lethal in direct contact with stem cells of apical papilla (18). It is important to realize that this toxicity to stem cells is a concentration-dependent effect.(19)

The success of regenerative endodontic procedures is also largely dependent on the elimination of microbial flora from the root canal system. The use of triple antibiotic paste at certain concentrations has the potential to completely eliminate these microflora.

Enterococcus faecalis is one of the most commonly isolated species from failed root canal treatments, found in 4% - 40% of primary endodontic infections and it is involved in treatment-resistant periradicular lesions.(20) *E. faecalis* organisms contribute to the challenging nature of endodontic therapy, especially in the treatment of immature permanent teeth with necrotic pulps.

Enterococcus faecalis is a persistent organism that, despite making up a small proportion of the flora in untreated canals, plays a major role in the etiology of persistent periradicular lesions after root canal treatment. It is commonly found in a high percentage of root canal failures and it is able to survive in the root canal as a single organism or as a major component of the flora.(21)

The understanding of cytotoxicity and antimicrobial action of 3Mix antibiotic paste with barium sulfate is the first step in the optimal utilization of 3Mix antibiotic paste. With this goal, this work focuses on the cytotoxicity and antibacterial effect of different concentrations of 3Mix antibiotic paste with the addition of a radiopaque material, barium sulfate.

The null hypothesis proposed in this study is that there is no difference in cytotoxic and antibacterial efficacy between three different concentrations 1 mg/ml, 10 mg/ml and 25 mg/ml 3Mix antibiotic paste with barium sulfate as an additive.

The aim of this study was to assess the cytotoxicity and antibacterial efficacy of three different concentrations of 3Mix antibiotic paste of 1 mg/ml, 10 mg/ml and 25 mg/ml with 0.3 g/ml barium sulfate as a radiopaque material.

OBJECTIVES OF THE STUDY

- To assess the cytotoxic effect of 1 mg/ml 3Mix antibiotic paste with barium sulfate on L929 mouse fibroblast cells
- To assess the cytotoxic effect of 10 mg/ml 3Mix antibiotic paste with barium sulfate on L929 mouse fibroblast cells
- To assess the cytotoxic effect of 25 mg/ml 3Mix antibiotic paste with barium sulfate on L929 mouse fibroblast cells
- To compare the cytotoxic effect of 1 mg/ml with 10 mg/ml 3Mix antibiotic paste with barium sulfate
- To compare the cytotoxic effect of 1 mg/ml with 25 mg/ml 3Mix antibiotic paste with barium sulfate
- To compare the cytotoxic effect of 10 mg/ml with 25 mg/ml 3Mix antibiotic paste with barium sulfate
- To evaluate the antimicrobial efficacy of 1 mg/ml 3Mix antibiotic paste with barium sulfate on *E. faecalis*

- To evaluate the antimicrobial efficacy of 10 mg/ml 3Mix antibiotic paste with barium sulfate on *E. faecalis*
- To evaluate the antimicrobial efficacy of 25 mg/ml 3Mix antibiotic paste with barium sulfate on *E. faecalis*
- To evaluate the antimicrobial efficacy of 25 mg/ml 3Mix antibiotic paste without barium sulfate on *E. faecalis*
- To compare the antimicrobial efficacy of 1 mg/ml with 10 mg/ml 3Mix antibiotic paste with barium sulfate
- To compare the antimicrobial efficacy of 1 mg/ml with 25 mg/ml 3Mix antibiotic paste with barium sulfate
- To compare the antimicrobial efficacy of 10 mg/ml with 25 mg/ml 3Mix antibiotic paste with barium sulfate
- To compare the antimicrobial efficacy of 1 mg/ml with 25 mg/ml 3Mix antibiotic paste without barium sulfate
- To compare the antimicrobial efficacy of 10 mg/ml with 25 mg/ml 3Mix antibiotic paste without barium sulfate
- To compare the antimicrobial efficacy of 25 mg/ml with 25 mg/ml 3Mix antibiotic paste without barium sulfate

As early as the 1700s, treatments were attempted to search for relief from pulpal and periapical disease while maintaining one's natural tooth. These experimental treatments involved incomplete chemomechanical treatment of the pulp and obturation of the pulp chamber.

Antibiotic therapy has become an inseparable part of diverse medical and medical-related treatments, and acts as one of the main fronts against microorganisms. Various antibiotics with divergent formulas are used, for prevention and prophylaxis, to cure active and acute infections and diseases.

Odontogenic infections originate from the dental structure, and like other infections in our body they engage a huge number of different microbes and microorganisms. Therefore, combination of drugs particularly antibiotics are needed to combat the microbiota responsible for creating odontogenic derived infections and lesions. That is why many antibiotics have been investigated, studied, and used to control and defeat dental infections.

3Mix antibiotic paste

Grossman (1945) recommended using non-aqueous penicillin as an intracanal medicament and later recommended the use of paper points impregnated with antibiotic inside the canal space. This sparked an interest in chemotherapeutic root canal treatment, but it was discovered that antibiotics alone would not sterilize the root canal space. (22)

Glick DH (1968) formed the chemomechanical root canal treatment, from which the concept of mechano-chemical preparation came about. This involved the mechanical preparation of a space which would allow the chemicals to penetrate and disinfect the canal space.(23)

Black et al. (1990) stated that synthetic fluoroquinolone ciprofloxacin inhibits DNA gyrase in bacterial nuclei, degrading the DNA by exonucleases and resulting in a bactericidal effect. Ciprofloxacin has very potent activity against gram-negative pathogens, but its activity is limited against gram-positive bacteria and most anaerobic bacteria are resistant to ciprofloxacin. Consequently, ciprofloxacin is often combined with metronidazole in the treatment of mixed infections. Although ciprofloxacin can cause side effects, in low doses, the drug is clinically safe. It can be assumed that when low doses of ciprofloxacin are applied as an intracanal medicament, insignificant adverse general side effects occur.(24)

Hoshino et al. (1993) reported that the clinical disinfection protocol in revascularization of permanent dentition and the concept of 'Lesion sterilization and tissue repair procedure associated with the triple antibiotic paste has been reported to show significant reduction of periapical lesions as it has greater antibacterial effect in root canals. (9)

Sato et al. (1993) demonstrated that different mixtures of ciprofloxacin, metronidazole, plus a third antibiotic (amoxicillin, cefaclor, cefroxadine, fosfomycin or okitamycin) were effective in sterilizing cultures of samples taken from carious dentine and infected pulpal tissues. It was

found that no bacteria could be recovered after treatment with 100 mg/ml of each medicament.(25)

Hoshino et al (1996) introduced 3-antibiotic combination (3Mix), a mixture of minocycline, ciprofloxacin, and metronidazole. An interesting issue is the concentration of 3Mix used because the clinical use of 3Mix has been empirical. Local application of 3Mix using an excessive concentration might affect the host tissue, causing cell death, which limits tissue regeneration. The 25 mg/mL 3Mix was able to eradicate all isolated bacteria.(9)

Roche & Yoshimori (1997) have investigated the activity of metronidazole against clinical isolates from odontogenic abscesses. They found that although it had no activity against aerobes, metronidazole had excellent activity against anaerobes isolated from odontogenic abscesses.(26)

Slots et al (2002) stated that the use of metronidazole has been advocated because of its low induction of bacterial resistance. Metronidazole, a nitroimidazole compound, is widely used for its broad-spectrum and strong antibacterial activity against anaerobic cocci, as well as gram-negative and gram-positive bacilli. Metronidazole permeates bacterial cell membranes, reaches the nuclei and binds to the DNA, disrupting its helical structure, and causing cell death.(27)

Cytotoxicity

Suzuki et al (2011) stated that in regenerative endodontic procedures growth factors such as stromal cell-derived factor-1 and basic fibroblast growth factor enhance dental pulp stem cell migration in 3D collagen gels.(28)

Ruparel et al (2012) stated that clinically used concentrations (500-1000 mg/mL) antibiotic mixtures were found to exert cytotoxic effects on stem cells from apical papillae. Published data concerning the cytotoxicity of 3Mix reported that 0.01-0.10 mg/mL did not have an effect on cultured stem cells from apical papilla. Low concentrations of double antibiotic paste or triple antibiotic paste ranging between 0.1-1 mg/mL provide an efficient antimicrobial medicament without jeopardizing the fate of pluripotent stem cells within the root canal system.(18)

Jamshidi et al (2013) stated that triple antibiotic paste showed cytotoxicity in 10 mg/ml and 100 mg/mL concentrations at all-time points and in 1, 10 and 100 mg/ml concentrations at 72 hours. In addition, its genotoxicity was significantly higher than that of other groups. Double antibiotic paste showed cytotoxic effects only in 100 mg/ml concentration at 24 hours and 10 mg/ml and 100 mg/ml concentrations at 48 and 72 hours. And also, its genotoxicity in these concentrations was significantly higher than that of control and calcium hydroxide groups.(29)

Yadlapati et al (2013) evaluated the cytotoxic effects of triple antibiotic paste, double antibiotic paste, minocycline and calcium hydroxide and their influence on cytokine mRNA expression levels on

human periodontal ligament fibroblasts and concluded that the triple antibiotic paste showed deleterious effects on human periodontal ligament fibroblast viability and increased expression of the pro-inflammatory cytokine IL6.(30)

Lundy et al (2016) found that fibroblasts are the most common cells of the pulp and can produce growth factors, including bFGF, VEGF, and PDGF aimed at mineralization, angiogenesis, and neurogenesis which are highly significant in the revascularization of pulp in infected premature teeth.(31)

Khoshkhounejad M et al (2019) evaluated triple and double antibiotic paste, a modified triple antibiotic paste (minocycline replaced by clindamycin), augmentin, and calcium hydroxide on stem cells of apical papilla and stated that triple antibiotic paste seems to be the safest drug for the stem cells of apical papilla, while augmentin may have some adverse effects.(32)

Antibacterial efficacy

Noble et al (1978) stated that *Enterococci* are gram-positive bacteria that occur singly, in pairs or short chains. They are present in the colon of all humans in numbers as high as 10⁸ colony-forming units per gram of faeces and are recognized as facultative anaerobes with an optimum growth temperature of 35°C.(33)

Jett BD et al (1994) found that *E. faecalis* is not limited to its possession of various virulence factors. It is also able to share these

virulence traits among species, further contributing to its survival and ability to cause disease.(34)

Love RM et al (2001) discovered that *E. faecalis* has shown to adhere to host cells, express proteins that allow it to compete with other bacterial cells, and alter host responses. *E. faecalis* is small enough to proficiently invade and live within dentinal tubules. It has the capacity to endure prolonged periods of starvation until an adequate nutritional supply becomes available. Serum, which originates from alveolar bone and the periodontal ligament, also helps *E faecalis* bind to type I collagen.(35)

Evans M et al (2002) reported that *E. faecalis* passively maintains pH homeostasis. This occurs as a result of ions penetrating the cell membrane as well as the cytoplasm's buffering capacity. It has a proton pump that provides an additional means of maintaining pH homeostasis. This is accomplished by "pumping" protons into the cell to lower the internal pH. At a pH of 11.5 or greater, *E. faecalis* is unable to survive.(21)

Gilmore et al (2002) stated that *enterococcus* species catabolize a variety of energy sources including carbohydrates, glycerol, lactate, malate, citrate, arginine, agmatine, and many keto acids. *Enterococci* survive very harsh environments including extreme alkaline pH and salt concentrations. They resist bile salts, detergents, heavy metals, ethanol and desiccation. (36) There are currently 23 *Enterococci* species and these are divided into five groups based on their interaction with mannitol, sorbose, and arginine. *E. faecalis* belongs to the same group as *E. faecium*, *E. casseliflavus*, *E. mundtii*, and *E. gallinarum*.(36)

Figdor D et al (2003) reported that once nutritional supply is available, the starved *E. faecalis* cells are able to recover by utilizing serum as a nutritional source.(39)

Hubble TS et al (2003) found *E. faecalis* possesses serine protease, gelatinase, and collagen-binding protein, which help it bind to dentin.(38)

Tendolkar et al (2003) reported that the *enterococci* are intrinsically rugged organisms endowed with traits such as growth in a temperature range between 10°C and 45°C and growth in broth containing 6.5% sodium chloride or pH of 9.6 and ability to survive at 60°C for 30 minutes.(37)

Lee W et al (2004) stated that *E. faecalis* is able to suppress the action of lymphocytes, potentially contributing to endodontic failure.(41)

Rocas IN et al (2004) stated that *E. faecalis* is associated with different forms of periradicular disease including primary endodontic infections and persistent infections. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen. In primary endodontic infections, *E. faecalis* is associated with asymptomatic chronic periradicular lesions significantly more often than with acute periradicular periodontitis or acute periradicular abscesses. *E. faecalis* is found in 4% to 40% of primary endodontic infections. *E. faecalis* also possesses certain virulence factors including lytic enzymes, cytolysin, aggregation substance, pheromones, and lipoteichoic acid. Because *E.*

faecalis is less dependent upon virulence factors, it relies more upon its ability to survive and persist as a pathogen in the root canals of teeth.(40)

Sedgley CM et al (2004) stated that *E. faecalis* overcomes the challenges of survival within the root canal system in several ways. It has been shown to exhibit widespread genetic polymorphisms.(42)

The present in vitro study was conducted in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital, Chennai.

Other facilities involved in the study

- Department of Biochemistry, Sathyabama Dental college and Hospital
- Life Teck Research Centre, Arumbakkam, Chennai

Materials for preparation of 3 Mix antibiotic paste with Barium sulfate

- Ciprofloxacin (Ciplox 500 mg - Cipla)
- Metronidazole (Metrogyl 400 mg - Unique's)
- Cefixime (Cefix 400 mg - Cipla)
- Barium sulfate (Nice Chemicals pvt ltd)
- Methylcellulose powder (Nice Chemicals pvt ltd)
- High Precision Electronic Balance (Mettler Toledo AT-201)

Materials & Equipment for evaluation of Cytotoxicity

- Scot duran media bottles
- Tissue culture flask - NUNC 25 cm²
- Serological pipettes - (Thermo scientific)
- Well plates - (Thermo scientific)

- L929 Mouse fibroblast cells (National Centre for Cell Sciences, Pune)
- Discarding jars
- Quartz Cuvettes
- Carbon dioxide incubator (HERA CELL 150i)
- Inverted microscope (Leitz Labovert FS)
- Fluorescence Microscope (Labomed LX-400)
- Spectrophotometer (Systronics Spectrophotometer 104)
- Dulbecco's Modified Eagle's medium
- 10% Fetal bovine serum
- 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT)
- Phosphate buffer
- Sodium bicarbonate
- Hydrochloric acid
- Trypsin

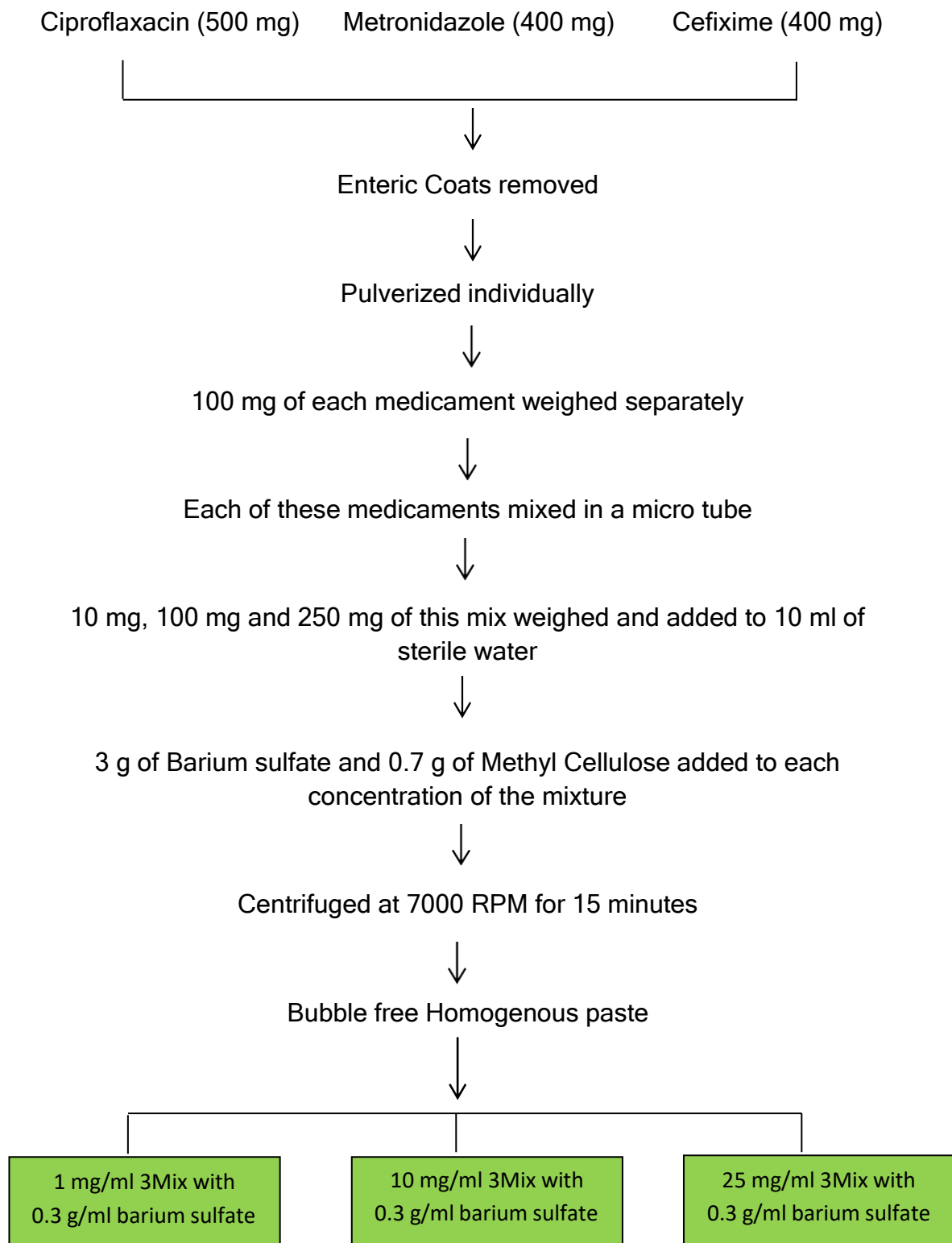
Materials used for the evaluation of antibacterial efficacy

- 88 non carious single rooted teeth extracted for orthodontic purposes
- E. faecalis (ATCC 29212)

- Sterile gloves
- Sterile tweezers
- Diamond disk (Xceldent 22 mm)
- Nail varnish
- Pipette
- Bio safety cabinet
- Fluorescence Microscope
- Micropipettes
- Anerobic Jar
- Incubator (HERA CELL 150i)
- Serological pipette
- 1.5 ml Eppendorf tubes
- Conical Flask
- Petri Dishes
- L- Rod
- Test tubes with stand
- Paper points (Diadent 6% taper)
- Sterile Gauze
- 5 ml Syringe with 23G needle

- Sterile Forceps
- Sterile Distilled Water
- Muller Hinton Agar
- Nutrient Broth
- 17% Ethylene Diamine Tetra Acetic acid (EDTA)
- Paraffin wax
- Sodium hypochlorite 5.25%
- Molecular grade water
- Slow speed handpiece (Marathon M3 Micromotor)
- Carbide bur (New Diamond, number L6)

Preparation of 3Mix Paste with Barium sulfate



Evaluation of Cytotoxicity

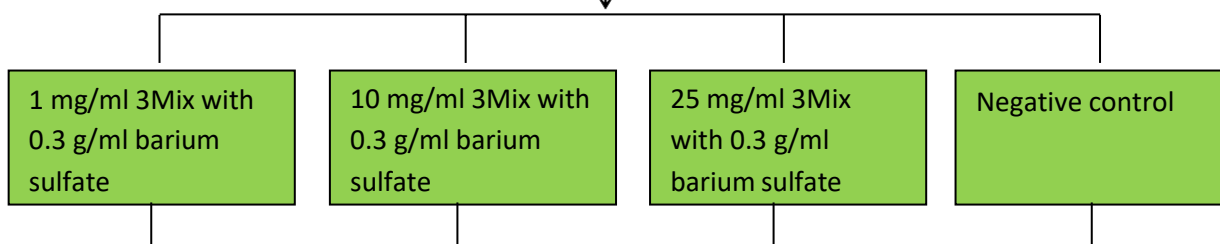
L929 Mouse fibroblast cells cultured in Dulbecco's Modified Eagle's medium with 10% Fetal Bovine Serum



Cultured fibroblast cells transferred to four well plates using triplicate method



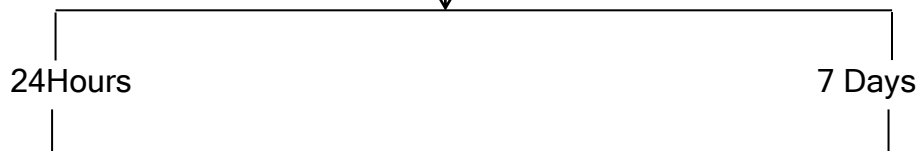
Experimental medicaments introduced into each well



Incubated at 37° C and 5% carbon dioxide



Incubated samples were examined at



200 µl of MTT and 0.5 mg per ml serum free media added to each well



Incubated for four hours at 37°C



200 ml of propanol added

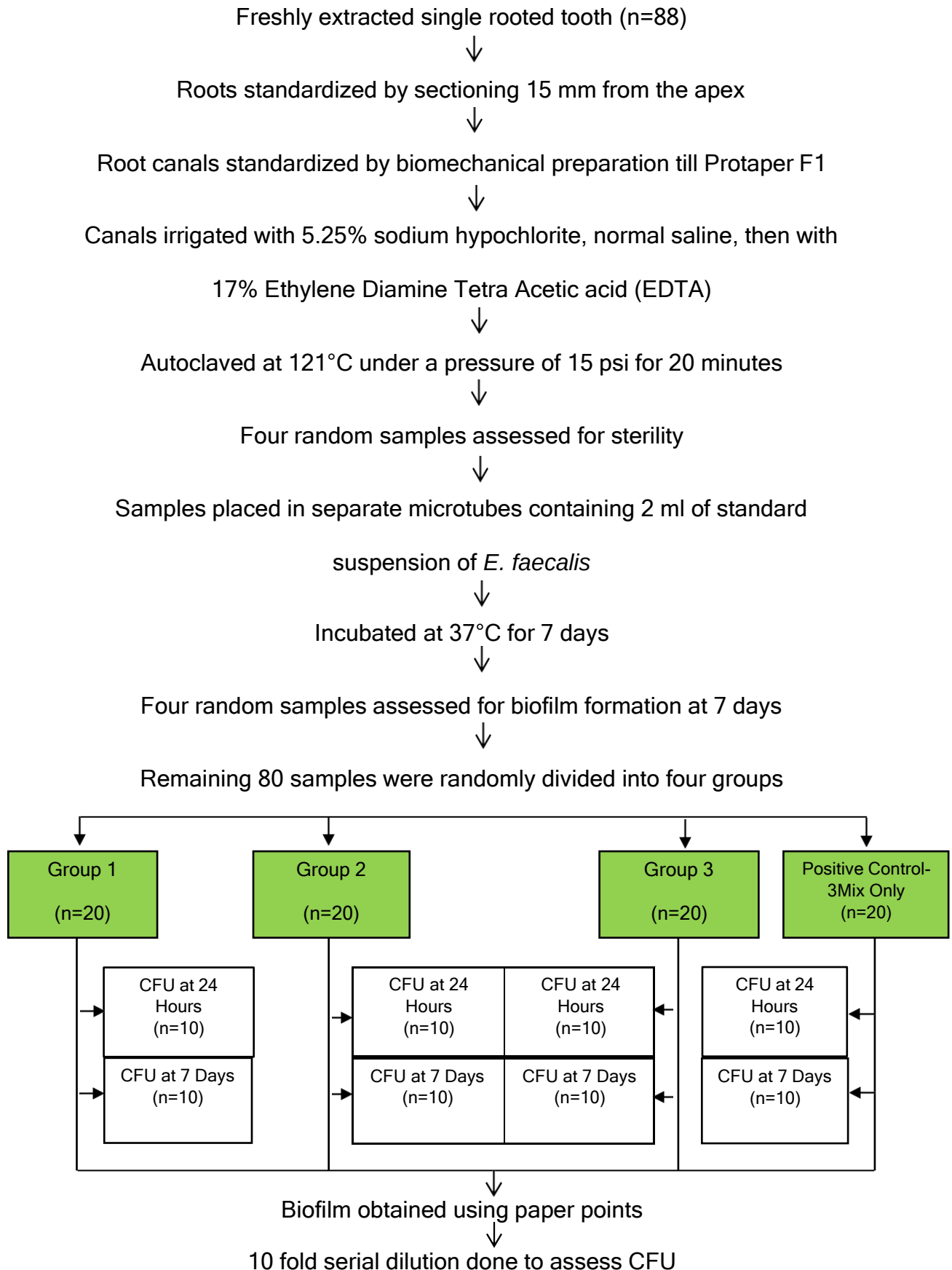


Spectrophotometric absorbance was measured at 570 nm wavelength using an UV spectrophotometer



Cell viability will be considered as the percentage of mean optical density values

Evaluation of antimicrobial efficacy



Preparation of 3Mix Paste

3Mix antibiotic paste used in this study consisted of a combination of ciprofloxacin, metronidazole and cefixime tablets. 3Mix paste was prepared using the technique described by Hoshino et al. The enteric coat of ciprofloxacin, metronidazole and cefixime tablets were removed using slow speed handpiece and carbide bur number L6. The medicaments were then pulverized using porcelain mortar and pestle to achieve a fine powder. 100 mg of each medicament was weighed using a high precision electronic balance and mixed in a 1:1:1 ratio. 10 mg, 100 mg, and 250 mg of this prepared 3Mix antibiotic powder were mixed with 10 ml of sterile water in microtubes to form a 3Mix antibiotic solution of 1 mg/ml, 10 mg/ml and 25 mg/ml respectively.

Barium sulfate was used as the radiopacifier in this study. 3 g of barium sulfate was incorporated into each of the three microtubes and stirred vigorously to form a radiopaque 3mix slurry composed of 0.3 g/ml radiopaque material. To this 0.7 g of methylcellulose powder was added to get a pasty consistency of 3Mix antibiotic paste.

These samples were centrifuged at 7000 rpm for 15 minutes to form a bubble-free homogenous injectable paste. The prepared 3Mix antibiotic pastes with barium sulfate were labeled as per their respective concentrations as follows:

- Group 1 - 1 mg/ml 3Mix antibiotic paste with 0.3 g/ml barium sulfate
- Group 2 - 10 mg/ml 3Mix antibiotic paste with 0.3 g/ml barium sulfate

- Group 3 – 25 mg/ml 3Mix antibiotic paste with 0.3 g/ml barium sulfate

Evaluation of Cytotoxicity

The cytotoxicity of different concentrations 1 mg/ml, 10 mg/ml and 25 mg/ml 3Mix antibiotic paste with barium sulfate were tested on cryopreserved L929 mouse fibroblast cells using direct contact method. Mouse fibroblasts were cultured in dulbecco's modified eagle's medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in an incubator with a humidified atmosphere of 50 µg/ml carbon dioxide at 37°C.

The cultured L929 fibroblast cells were transferred into four well plates consisting 24 wells each. Triplicate method was used to assess the cytotoxicity of 1 mg/ml, 10 mg/ml and 25 mg/ml of 3Mix antibiotic paste with 0.3 g/ml barium sulphate. The various concentrations of 3Mix antibiotic paste with 0.3 g/ml barium sulphate were introduced into the culture wells containing cultured L929 fibroblast cells using cell compatible plastic rings. The plates were then incubated for 24 hours and 7 days at 37°C and 98% humidity.

After the incubation period of 24 hours and seven days well plates were removed from the incubator and 200 µl of 3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT), and 0.5 mg per ml serum free media was added to each well. The plates were incubated for four hours at 37°C. After four hours of incubation, experimental samples from each well were

removed using serological pipette and placed in separate glass vials. 200 ml of propanol was added for the solubilisation of crystals.

During incubation, mitochondrial dehydrogenase in the cytochrome of the living cells can cleave the tetrazole ring of the MTT, and the yellow water-soluble MTT is reduced to produce a purple crystalline formazan. Propanol makes formazan crystals formed in viable cells dissolve and create a homogenous purple coloured solution. The purple coloured solution was then transferred to the ELISA plate and its spectrophotometric absorbance was measured at 570 nm wavelength using an UV spectrophotometer. The amount of crystals formed is directly proportional to the number of viable cells.

The cell viability will be considered as the percentage of mean optical density values of each medicament compared with the optical density value of the negative control. The percentage values of cell viability were interpreted as per Mosmann's (1983) formula.

$$\text{Percentage of viable cells} = \frac{\text{Optical density of test well}}{\text{Optical density of negative control}} \times 100$$

1. <30% cell viability = Severely cytotoxic
2. 30%-60% cell viability = Moderately cytotoxic
3. 60%-90% cell viability = Slightly cytotoxic
4. >90% cell viability = Non-cytotoxic

Assessment of Antibacterial Efficacy

88 extracted single-rooted permanent teeth were used to analyze the antibacterial efficacy. Intra oral periapical radiographs were taken for all these extracted teeth to exclude those with calcified canals, root caries, or open apices. The teeth were sectioned using a diamond disk at 15 mm from the apical region for standardization of the samples. The root canals were prepared using engine-driven protaper rotary system up to the size F1 to obtain a standard root canal system. The root canals were irrigated with 5.25% sodium hypochlorite solution, followed by normal saline, and then with 17% Ethylene Diamine Tetra Acetic acid (EDTA) and the final rinse was done using normal saline.

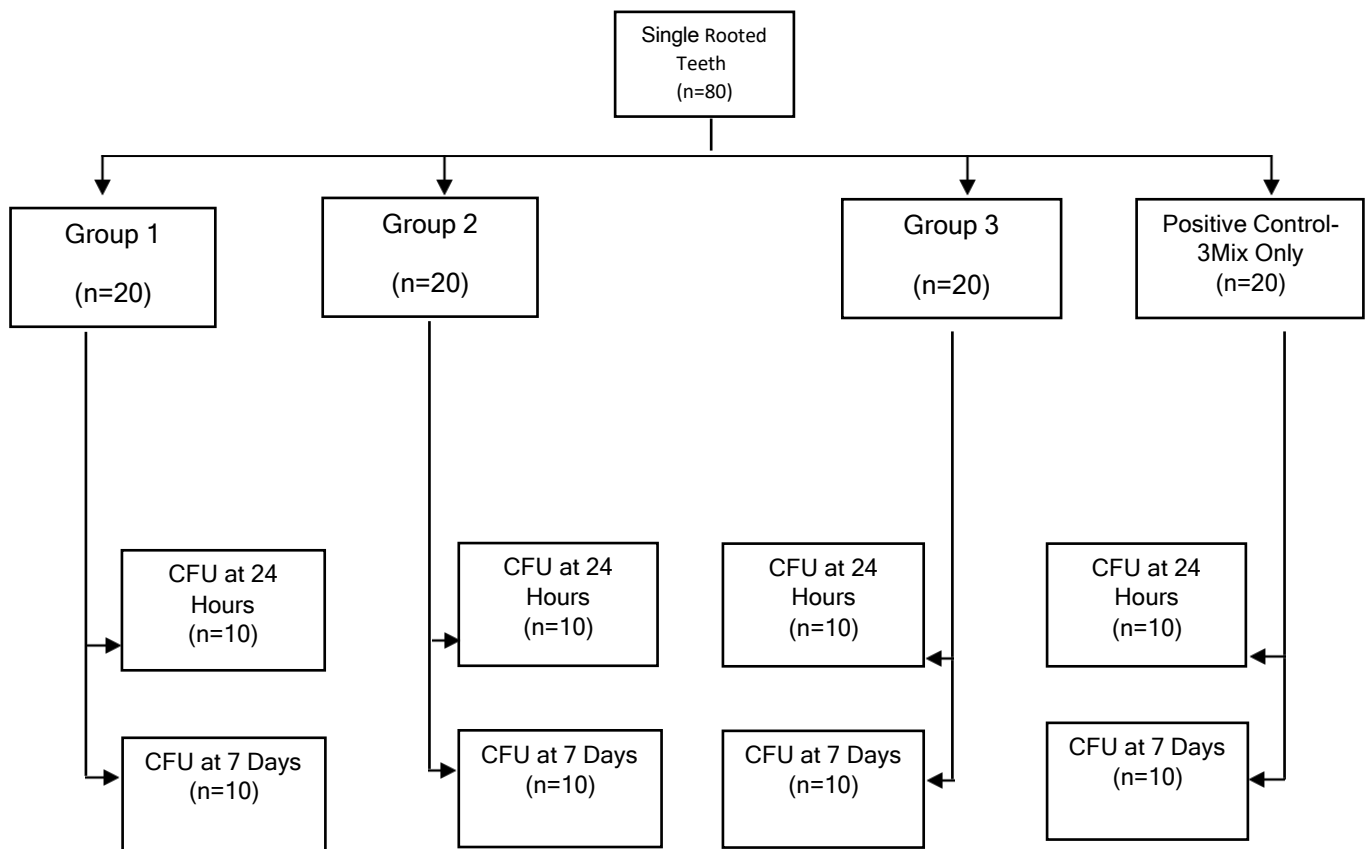
The irrigated samples were autoclaved at 121°C under a pressure of 15 psi for 20 minutes. Four samples were randomly selected and evaluated for sterility by placing them in the Mueller-Hinton Agar culture medium to observe for any bacterial growth. There was no bacterial growth in these four samples after 24 hours of incubation.

Absence of any bacterial growth indicates that the samples have been properly sterilized. All the samples were placed in separate sterile microtubes containing 2 ml of standard suspension of *E. faecalis*. These samples were incubated at 37°C for seven days. The *E. faecalis* suspension was changed every day on all seven days.

At the end of the seven day incubation period, four samples were randomly selected to check for the formation of *E. faecalis* biofilm on the root canal walls. The root canals of these four samples were irrigated with

normal saline. Biofilm samples were scraped off from the root canals using paper points. These paper points were stirred in separate test tubes containing 10 ml of distilled water and they were subjected to ten-fold serial dilution. 0.1 ml of this solution from each test tube was transferred into the nutrient agar plate and spread equally using an L-rod. The plates were incubated for 24 hours and then assessed for the growth of biofilm.

The remaining 80 samples were randomly divided into four groups as follows:



The prepared 3Mix antibiotic paste of various concentrations 1 mg/ml, 10 mg/ml and 25 mg/ml with 0.3 g/ml barium sulfate was transferred into the root canal by using a hand lentulo spiral. The coronal

orifice of the root canals were sealed with a thin layer of rose wax and it was covered with a layer of nail varnish. The apical foramina of the root canals were also sealed with a layer of nail varnish to prevent the leaching of drugs from the root canals. Each sample was wrapped in a piece of sterile gauze impregnated with saline and then placed in a sterile microtube. These microtubes with the samples were incubated at 37°C for 24 hours and seven days, respectively.

After 24 hours, 10 samples of each group were evaluated for antimicrobial efficacy. These samples were first irrigated with normal saline solution. The root canal walls were scraped using paper points. These paper points were added to separate microtubes containing 10 ml of sterile normal saline. 10-fold dilution was done using serial dilution method. Now, 50 µl of each diluted sample were inoculated into a Mueller-Hinton culture and incubated at 37°C for 24 hours, and the CFU/ml was calculated after 24 hours.

Statistical analysis

- The mean values of viable cells and antimicrobial efficacy of the three groups were analysed using the One-way ANOVA test for intergroup comparison.
- The mean difference in viable cells of each study group with the control were analysed using the independent sample t-test.
- Pairwise intragroup comparison was done using the paired sample t-test to analyse the difference of each group at two different time intervals.
- Post hoc Tukey-b test was done as a honest significance test, to compare all possible pairs of mean difference between the groups.

Formulae used for analysis

$$\text{Mean} = \bar{X} = \sum X_i / n, i = 1, 2, \dots, n$$

$$\text{Standard deviation (SD)} = \sqrt{\sum (X_i - \bar{X})^2 / n - 1}$$

One Way ANOVA

$$F = \frac{\text{MST}}{\text{MSE}}$$

Post hoc Tukey-b test

$$\bar{y}_i - \bar{y}_j \pm \frac{Q(1 - \alpha; r, n_T - r)}{\sqrt{2}} s \sqrt{\frac{1}{n_i} + \frac{1}{n_j}}$$

Materials and equipment used for the preparation of 3Mix antibiotic paste with barium sulfate



Fig.1 Materials used for the preparation of 3Mix antibiotic paste with barium sulfate



Fig.2 High precision electronic balance



Fig.3 Different concentrations of prepared 3Mix paste



Fig.4 Centrifugation of 3Mix antibiotic paste with barium sulfate

Materials used for the evaluation of cytotoxicity



Fig.5 L929 fibroblast cells subcultured and stored in glass vials in an incubator



Fig.6 Subcultured fibroblast cells observed for confluence under inverted microscope



Fig.7 Fibroblast cells transferred to four well plates

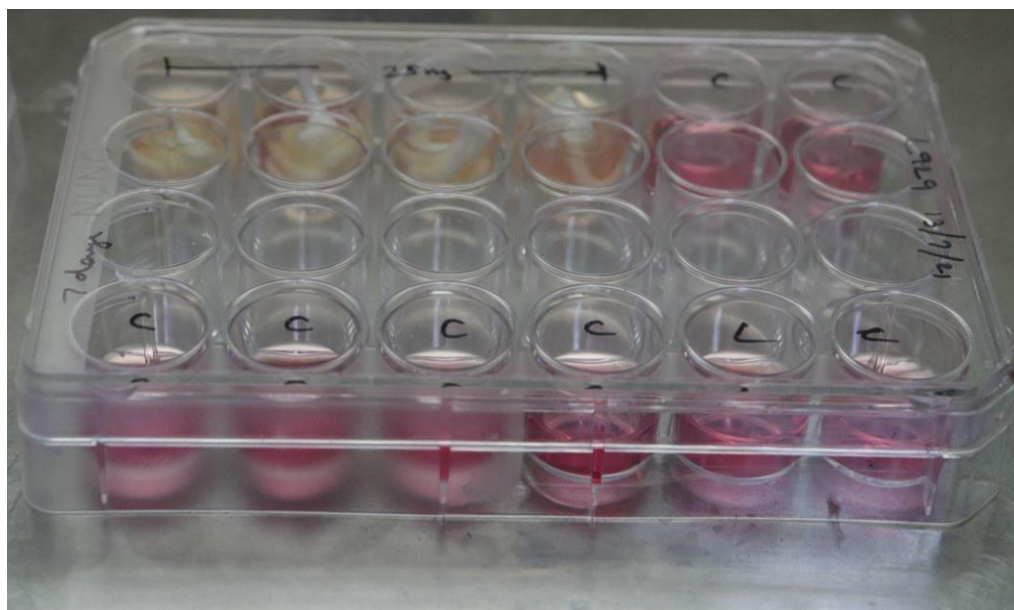


Fig.8 3Mix antibiotic paste with barium sulfate introduced in well plates



Fig.9 Optical density of MTT treated cells measured using UV spectrophotometer

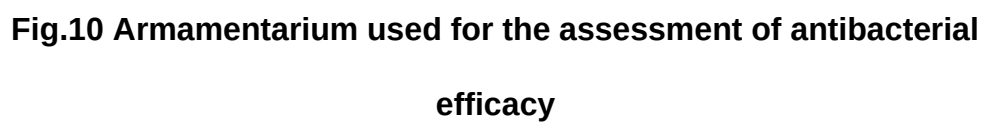




Fig.12 Standardisation of tooth samples of single rooted teeth to 15 mm



Fig.13 Sectioning tooth samples 15 mm from apex using slow speed handpiece



Fig.14 Sectioned teeth



Fig.15 Preparation of root canals using engine driven protaper system

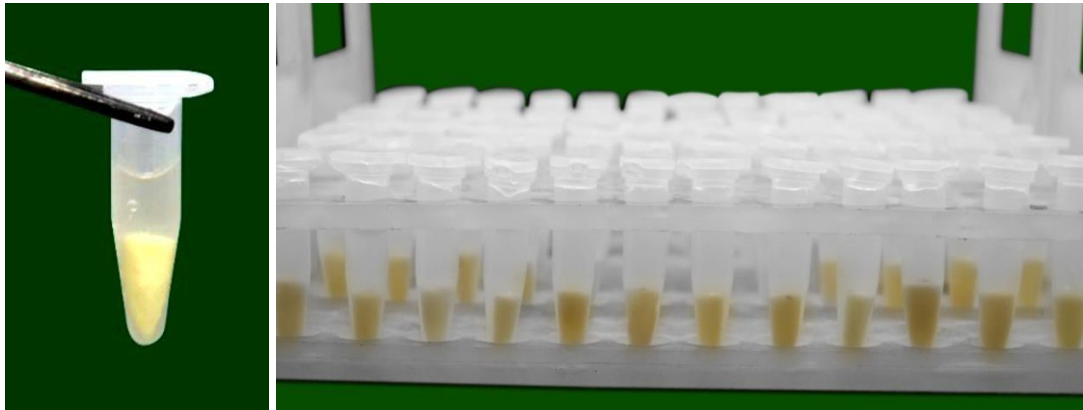


Fig.16 80 Tooth samples immersed in 2 ml of *E. faecalis* cell suspension

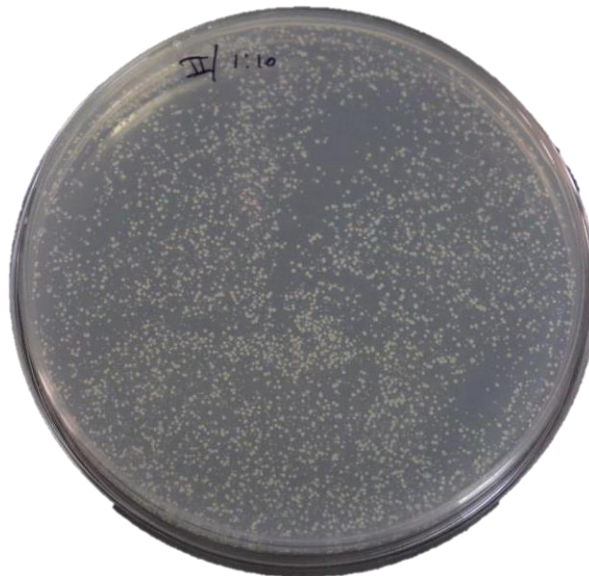


Fig.17 Formation of biofilm confirmed after 24 hours using Mueller-Hinton agar culture medium



Fig.18 3Mix antibiotic paste with barium sulfate introduced into the *E.faecalis* treated root canals using disposable tips



Fig.19 Sealed root canals using paraffin wax and nail varnish

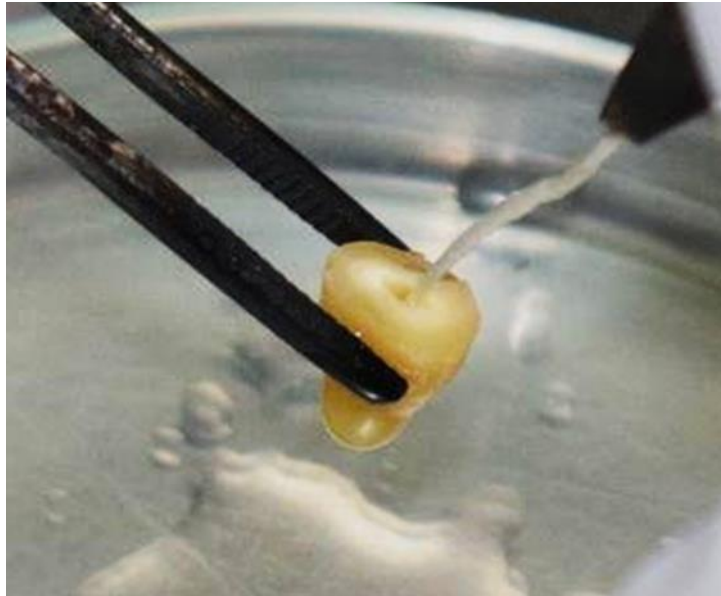


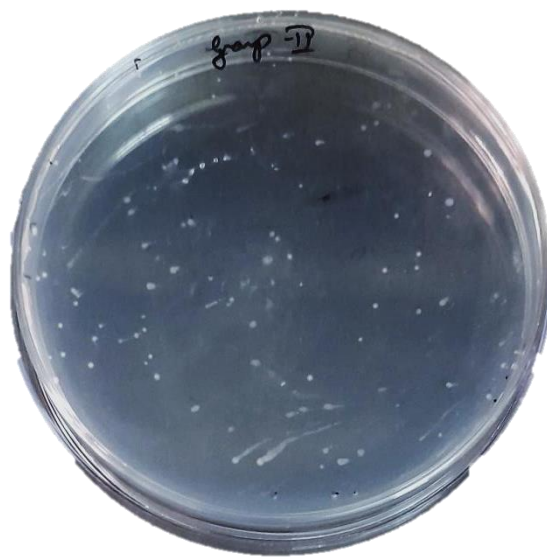
Fig.20 Extraction of *E.faecalis* samples from root canals using paper points



Fig.21 Ten fold serial dilution of samples, inoculated into Mueller-Hinton culture



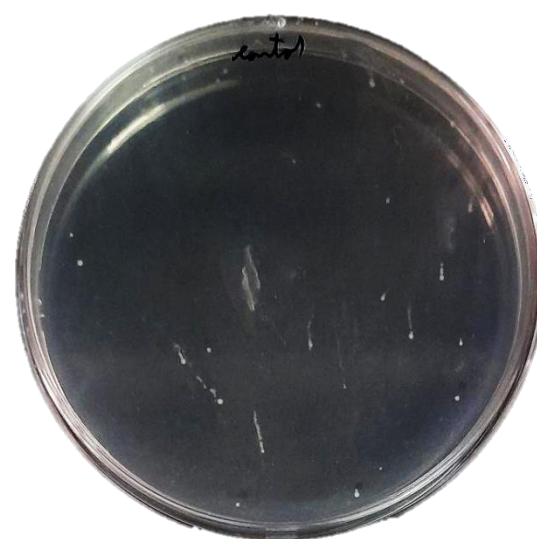
**Fig.22 Colony forming units of
1 mg/ml at 24 hours**



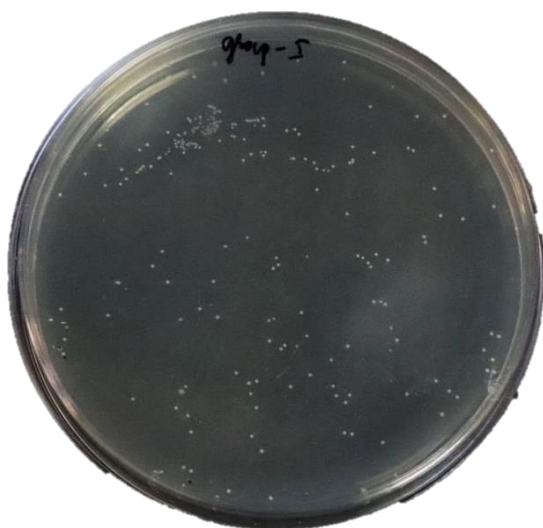
**Fig.23 Colony forming units of
10 mg/ml at 24 hours**



**Fig.24 Colony forming units of
25 mg/ml at 24 hours**



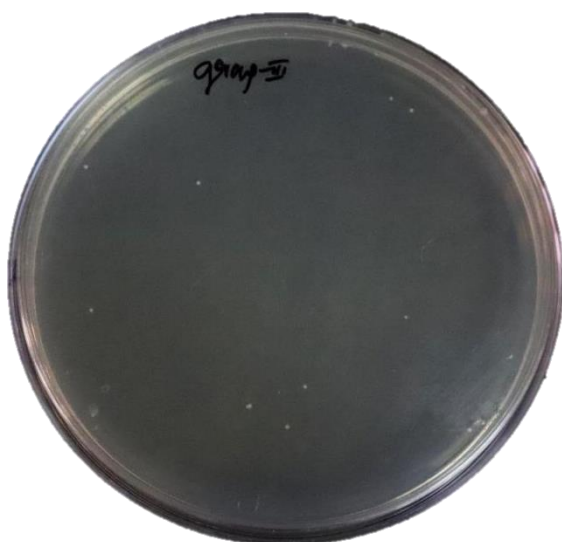
**Fig.25 Colony forming units of control
25 mg/ml at 24 hours**



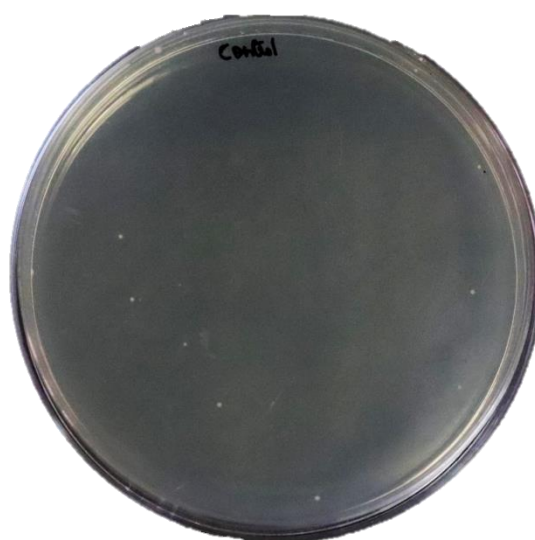
**Fig.26 Colony forming units of
1 mg/ml at seven days**



**Fig.27 Colony forming units of
10 mg/ml at seven days**



**Fig.28 Colony forming units of
25 mg/ml at seven days**



**Fig.29 Colony forming units of control
25 mg/ml at seven days**

The present in vitro study was conducted to compare and evaluate the cytotoxic effect and antimicrobial efficacy of three different concentrations 1 mg/ml, 10 mg/ml and 25 mg/ml of 3Mix antibiotic paste with 0.3 g/ml barium sulfate.

Evaluation of Cytotoxicity

Day	Experimental groups	Mean Viable Cells of Experimental groups		Mean Viable Cells of Control group		Significance
		Mean	SD	Mean	SD	p-Value
Day 1	Group 1 1 mg/ml	.813	.008	.893	.016	.003
	Group 2 10 mg/ml	.543	.016	.880	.018	<.001
	Group 3 25 mg/ml	.312	.007	.885	.016	<.001
Day 7	Group 1 1 mg/ml	.924	.008	1.215	.007	.001
	Group 2 10 mg/ml	.487	.049	1.054	.111	.004
	Group 3 25 mg/ml	.222	.015	1.123	.102	.003

Table 1 Mean viable cells in Group 1, Group 2 and Group 3 compared with that of the Control

Table 1 shows the mean values of the spectrophotometric absorbance of viable L929 fibroblast cells in different concentrations of 3Mix antibiotic paste with barium sulfate and control. Higher the absorbance level, lesser is its cytotoxic effect. Independent sample t-tests showed a statistically significant difference between the experimental groups and the control both on day one and day seven.

Experimental Groups	Mean Viable Cells	Day 1		Day 7		Paired samples t-test
		Mean	SD	Mean	SD	p-Value
Group 1 1 mg/ml	Experimental	.813↓	.008	.924↑	.008	.005
	Control	.893↓	.016	1.215↑	.007	<.001
Group 2 10 mg/ml	Experimental	.543	.016	.487	.049	.109
	Control	.880	.018	1.054	.111	.084
Group 3 25 mg/ml	Experimental	.312↑	.007	.222↓	.015	.003
	Control	.885↓	.016	1.123↑	.102	.045

**Table 2 Comparison of the cytotoxic effect of each concentration
between day 1 and day 7**

Paired sample t-test was done to compare the significance of the groups between day one and day seven. Statistically significant difference was observed in Group 1 and Group 3 between day one and day seven. There was no statistically significant difference in Group 2 between day one and day seven.

Day		Group 1 1 mg/ml		Group 2 10 mg/ml		Group 3 25 mg/ml		One way ANOVA
		Mean	SD	Mean	SD	Mean	SD	p- Value
Day 1	Experimental group – Viable cells	.813	.008	.543	.016	.312	.007	<.001
	Control - Viable cells	.893	.016	.880	.018	.885	.016	.645
Day 7	Experimental group – Viable cells	.924	.008	.487	.049	.222	.015	<.001
	Control - Viable cells	1.215	.007	1.054	.111	1.123	.102	.154

Table 3 Difference in viable cells between study groups

Intergroup comparison was made using one way ANOVA and it was observed that there was a statistically significant difference between the experimental groups.

Day 1				Day 7			
Concentration	Mean Viable cells			Concentration	Mean Viable cells		
	1	2	3		1	2	3
Group 3 25 mg/ml	.312			Group 3 25 mg/ml	.221		
Group 2 10 mg/ml		.543		Group 2 10 mg/ml		.487	
Group 1 1 mg/ml			.813	Group 1 1 mg/ml			.923

Table 4 Mean difference of viable cells between each experimental group

A pairwise comparison was done using Post Hoc Tukey b test to assess the superiority between the groups. The Post Hoc Tukey b Test clearly indicates that Group 1 showed the least levels of cytotoxicity with a mean value of 0.8133 viable cells on day one and 0.92367 of viable cells on day seven compared to Group 2 and Group 3. Group 3 showed the highest level of cytotoxicity with a mean value of 0.3120 viable cells on day one and 0.22167 of viable cells on day seven.

Evaluation of Antimicrobial Efficacy

Time Interval	Group								One way ANOVA
	Group 1 1 mg/ml		Group 2 10 mg/ml		Group 3 25 mg/ml		Control		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p-Value
Day 1	2.50	.10	2.12	.18	1.70	.07	1.29	.14	<.001
Day 7	2.11	.24	1.43	.12	1.15	.07	.69	.32	<.001

Table 5 Antimicrobial efficacy between Group 1, Group 2 and Group 3

Table 5 shows the mean colony forming units of the experimental groups and the control. Higher the mean value, lower is its antimicrobial efficacy. It has been observed that there was a statistically significant difference ($p < 0.005$) in antimicrobial efficacy between the experimental groups both on day one and day seven.

Day 1						Day 7					
Group	N	Mean Colony forming units				Group	N	Mean Colony forming units			
		1	2	3	4			1	2	3	4
Control	10	1.290				Control	10	.691			
Group 3 25 mg/ml	10		1.698			Group 3 25 mg/ml	10		1.150		
Group 2 10 mg/ml	10			2.122		Group 2 10 mg/ml	10			1.435	
Group 1 1 mg/ml	10				2.499	Group 1 1 mg/ml	10				2.110

Table 6 Mean difference of colony forming units between each experimental group

Pairwise comparison was done using Post Hoc Tukey b test to compare the mean difference of the colony forming units between the groups. Group 3 showed the least level of colony forming units with a mean value of 1.698 on day one and 1.150 on day seven. Group 1 showed the highest level of colony forming units with a mean value of 2.499 on day one and 2.110 on day seven.

Group	Time Interval				Dependent Samples t-test
	Day 1		Day 7		
	Mean	SD	Mean	SD	p-Value
Group 1 1mg/ml	2.50	.10	2.11	.24	<.001
Group 2 10 mg/ml	2.12	.18	1.43	.12	<.001
Group 3 25 mg/ml	1.70	.07	1.15	.07	<.001
Control	1.29	.14	.69	.32	<.001

Table 7 Antimicrobial efficacy between day one and day seven

The paired sample t-test compares the colony forming units of each group between day one to day seven. The experimental groups and the control showed statistically significant differences ($p < 0.005$) between day one and day seven.

Regenerative endodontic procedures have rejuvenated the ways of treating immature permanent teeth which undergo irreversible pathologic changes either due to dental caries or traumatic dental injuries. The American Association of Endodontists recommends performing a regenerative endodontic procedure when the patient presents with a tooth with a necrotic pulp and immature apex.

The systematic administration of antibiotics requires bio-sorption and biomodulation in the liver and is circulated via circulatory system to the infected area in the root canal system. In the infected root canal system and necrotic pulp there is hampered, blood supply and the bio modulated drug also get hampered at the infected site and hence the drug will no longer be effective in the enclosed canals, therefore local application of drugs is more effective.(43)

The traditional medicament used is calcium hydroxide. Other efficient medicaments are also used, of which the most recent is the triple antibiotic paste (the combination of ciprofloxacin, minocycline, and metronidazole), which acts as microbicidal and biocompatible for the stem cells that are present in the periapical area as compared with the traditional calcium hydroxide, which is toxic to the stem cells.(44)

The specific focus in this study was on the disinfection of the root canal system using a novel radiopaque triple antibiotic paste and to assess its cytotoxic properties on mouse fibroblast cells and also to evaluate its antimicrobial efficacy.

Recent in vitro studies have found that intracanal antibiotic medicaments such as triple or double antibiotic paste may offer superior root canal disinfection in comparison to traditional calcium hydroxide.(45)

However, unlike calcium hydroxide, clinically used concentrations (500 - 1000 mg/mL) of these antibiotic mixtures were found to exert cytotoxic effects on stem cells from apical papillae(18), dental pulp stem cells, and dental pulp fibroblast.(46).

Although triple antibiotic paste is an established antibiotic paste, it has its own drawbacks. Triple antibiotic paste is radiolucent(9). Propylene glycol as a vehicle of triple antibiotic paste may be difficult to remove from the dentin surface. An additional appointment is required to remove triple antibiotic paste and re-opening the tooth to remove triple antibiotic paste introduces a risk of recontamination.

Hoshino et al in his preliminary study of the triple antibiotic paste used a concentration of 1 mg/ml, 10 mg/ml and 25 mg/ml and evaluated its antimicrobial efficacy on microbes isolated from infected root canals of primary teeth.(9)

Several studies have focused on assessing the triple antibiotic paste of similar concentrations of Hoshino et al as the lower concentrations are less cytotoxic compared to the clinically used 500 – 1000 mg/ml triple antibiotic paste.

Only few studies have since used a radiopaque agent with triple antibiotic paste. Rohan Verma et al in 2018 introduced a radiopaque

double antibiotic paste with concentrations similar to that of Hoshino et al with 1 mg/ml, 10 mg/ml and 25 mg/ml triple antibiotic paste and 0.3 g/ml of barium sulfate, hence in the present study a similar concentration of triple antibiotic paste was evaluated for cytotoxic and antibacterial effects.

The current study introduced three different concentrations of radiopaque 3 Mix antibiotic paste, with controlled concentrations using Food and Drug Administration approved radiocontrast agent barium sulfate. The significant difference with this new 3Mix paste in comparison to the original 3Mix antibiotic paste is the addition of barium sulfate. Barium sulfate was added to serve as a radiopacifier.

Barium sulfate is a commonly used radiopaque material in calcium hydroxide based root canal medicaments and zinc oxide eugenol based root canal sealers. Barium sulfate as a radiopaque material was also suggested to be biocompatible.(46)

Cytotoxocity

This study showed that group 1 (1 mg/ml) which had the lowest concentration of 3 Mix paste had the least cytotoxic effect and group 3 (25 mg/ml) which had the highest concentration of 3 Mix paste had the most cytotoxic effect on the fibroblast cells. Group 4 which acted as a negative control had no cytotoxic effect on the fibroblast cells.

This is in accordance with the results of the study done by Althumairy et.al which showed that the commonly used clinical concentration (approximately 1,000 mg/mL) of either triple antibiotic paste

or double antibiotic paste was completely lethal to stem cells of apical papilla after 7 days. For the 1 mg/mL concentration of either triple antibiotic paste or double antibiotic paste, there was no significant difference in stem cells of apical papilla survival compared with the control group.(19)

These findings are further supported by a recent study by Alguilan et al. (2017) who proposed the use of diluted triple antibiotic paste in a methylcellulose system as used in this study (1 mg/mL). Triple antibiotic paste caused significant increases in dental pulp stem cells attachment to dentine and did not negatively affect the dental pulp stem cells proliferation.(47)

Ruparel et al. evaluated the survival of stem cells of the apical papilla exposed to various concentrations of intracanal drugs demonstrating that when the cells were exposed to high concentrations of antibiotics, there was a significant decrease in stem cells of apical papilla survival. This was also in accordance to this present study as higher concentrations showed more cytotoxic effects.(18)

Jamshidi et al stated that triple antibiotic paste showed cytotoxicity in 10 and 100 mg/mL concentrations at all-time points and in 1, 10 and 100 mg/ml concentrations at 72 hours. In contrast to these results, 10 mg/ml 3Mix antibiotic used in this study with barium sulfate did not show significant amount of cytotoxicity.(29) This also indicates that the addition of barium sulfate have no direct cytotoxic effect to the fibroblast cells.

The addition of barium sulfate to the triple antibiotic paste did not show any change in cytotoxic effect. This is supported by a study done by Elizeu et al who assessed raw barium sulfate on L929 mouse fibroblast cells and found no cytotoxic effect.(48) Hence barium sulfate is safe to use as an adjunct to lower concentrations of triple antibiotic paste in regenerative endodontic procedures.

The cytotoxic effect of radiopaque 3Mix antibiotic pastes is concentration dependent and not associated with the radiopaque additives like barium sulfate. 3Mix antibiotic pastes are more cytocompatible when used in lower concentrations.

Antibacterial effect

The microbiota of root canal is polymicrobial, which includes aerobic and anaerobic bacteria, actinomyces, and other bacteria resistant to single antibiotic. Resistance to single antibiotic is most common, hence multiple antibiotics are required to overcome the resistance with all the polymicrobes.(44) To date, the most effective combined drug to overcome the resistance of bacterial strains is triple antibiotic paste.

E. faecalis was used in the present study because it has a major role in resistant infections of the root canal system. The biofilm model was used instead of the planktonic model to mimic the clinical scenario and better simulate the in vivo situation.(21)

To further eliminate any bias those teeth with calcified canals, root caries, or open apices were excluded since they could cause an error in

the study as calcified canals can restrict the placement of the medicament or in case of open apices the medicament can leach out of the opening.

Pai et al. studied the effect of calcium hydroxide and triple antibiotic paste. They found that 3 of 20 patients' with 15% calcium hydroxide developed inter-appointment flare-ups. However, with respect to the triple antibiotic paste none of them developed inter-appointment flare-ups.(49)

Adl A et al and Lakhani et al found that the triple antibiotic paste showed better disinfection properties as compared to calcium hydroxide.(50, 51)

Bolstad et al. evaluated the antibacterial efficacy on *E. faecalis* using triple antibiotic paste and calcium hydroxide with 2% chlorhexidine. They found that a combination of calcium hydroxide with 2% chlorhexidine is more potent than the triple antibiotic paste against *E. faecalis*. Triple antibiotic paste could eliminate bacteria to the depth of 400 μm , while calcium hydroxide could eliminate bacteria only from 200 μm depth of dentin.(52). This advantage possessed by triple antibiotic paste of diffusing further into the dentin compared to calcium hydroxide could be compromised with the use of insoluble barium sulfate as a radiopacifier.

Mohammad Frough Reyhani et al. in their study of different concentrations of 3Mix antibiotic pastes from 0.1 mg/ml to 1000 mg/ml assessed antibacterial efficacy against *E. faecalis* and concluded that triple antibiotic paste completely eliminated *E. faecalis* biofilms at all the intervals at concentrations of 1000, 100, and 10 mg/mL, whereas 1, 0.1, and 0.01 mg/mL triple antibiotic pastes resulted in significant reduction of

colony forming units. (53) This is in accordance with the present study where 25 mg/ml and 10 mg/ml 3Mix antibiotic paste with barium sulfate almost completely eliminated the *E. faecalis biofilm* whereas significant reduction of *E. faecalis* was seen in 1 mg/ml 3Mix antibiotic paste with barium sulfate.

Mohammad Frough Reyhani et al. also stated that there were no statistically significant differences of antibacterial efficacy against *E. faecalis* between the time intervals.(53) This is in contrast to the present study where a significant reduction of *E. faecalis* was seen from day one to day seven. But the 3Mix concentrations used in this study were not able to completely eliminate *E. faecalis* on day seven.

Based on a study by Chuensombat et al the cytotoxic effects and antibacterial effect of triple antibiotic pastes are time- and dose-dependent and the effects increase with an increase in exposure duration; therefore, it seems that, time, too, is an important factor in the use of this medication.(54) The results of the present study clearly indicate that the 24 hours use of 3Mix antibiotic paste showed significantly less effective antibacterial properties and was less cytotoxic compared to seven days which was in accordance with study done by chuensombot et al.

Hoshino et al reported that 25 µg/mL of this material can eliminate all the isolated bacteria. Also a study by Chuensombat et al showed that 25 µg/mL of triple antibiotic paste can completely eliminate the bacteria cultured on agar. (9) However in the present study even the highest concentration of 25 mg/ml 3Mix antibiotic paste was unable to entirely

eliminate the biofilm. This difference was probably due to the method in using mature biofilm in prepared root canals in this present study instead of an agar plate.

Rohan Verma et al evaluated a radiopaque double antibiotic paste on dentin slabs cultivated with bacterial biofilm from an infected immature tooth with pulpal necrosis. Radiopaque antibiotics of 1 mg/ml, 10 mg/ml and 25 mg/ml with barium sulfate and zirconium oxide as radiopaque additives were tested. Results of this study stated that the double antibiotic paste of 1 mg/ml and barium sulfate could completely eradicate the biofilm.(55) However in the present study the 1 mg/ml 3Mix antibiotic paste with barium sulfate was not able to completely eliminate the biofilm.

This decrease in antimicrobial efficacy in the present study could be because testing the 3Mix antibiotic paste on an isolated microbial biofilm like *E. faecalis* and the use of root canals instead of a dentinal slab where the diffusion of intracanal medicaments into the dentinal tubules could be harder which inevitably inhibits the complete elimination of biofilm.

The conclusions drawn from this in vitro study were as follows:

- Radiopaque 3Mix antibiotic paste of concentration 1 mg/ml exhibited the least cytotoxicity.
- Radiopaque 3Mix antibiotic paste of concentration 25 mg/ml exhibited the most cytotoxicity.
- Radiopaque 3Mix antibiotic paste of concentration 1 mg/ml exhibited the least antibacterial efficacy.
- Radiopaque 3Mix antibiotic paste of concentration 25 mg/ml exhibited the highest antibacterial efficacy of the experimental groups.
- 3Mix antibiotic paste without barium sulfate exhibited the highest antibacterial efficacy.

Based on the above findings it is evident that the least concentration of 3Mix antibiotic paste with barium sulfate (1 mg/ml) showed minimal cytotoxicity and the highest concentration (25 mg/ml) of 3Mix antibiotic paste with barium sulfate showed the maximum cytotoxicity.

In terms of antibacterial efficacy, the highest concentration (25 mg/ml) of 3Mix antibiotic paste with barium sulfate showed an improved antibacterial effect when compared to the lowest concentration (1 mg/ml) of 3Mix antibiotic paste with barium sulfate.

The addition of barium sulfate definitely showed a positive effect of radiopacity and did not alter the effectiveness of 3Mix antibiotic paste.

Encompassing the limitations of the study, the rat fibroblast cells show similar growth to that of stem cells of apical papilla, but the differentiation properties are not similar. This could limit the clinical implication of a radiopaque 3Mix antibiotic. The biofilm formation method assessed using paper points could also limit the retrieval of biofilm from deep dentinal tubules which further limits the understanding of diffusion properties of a radiopaque 3Mix antibiotic paste.

The limitations of this study could be overcome by assessing cytotoxicity of radiopaque 3Mix antibiotic paste on human stem cells of apical papilla instead of rat fibroblast cells. Scanning electron microscopy or laser fluorescence microscopy can be used to assess the diffusion of 3Mix antibiotic paste with barium sulfate in deep dentinal samples and this could enhance its ability to eliminate the biofilm.

Furthermore the long term action of 3Mix antibiotic with barium sulfate on stem cells of apical papilla could be explored in future in-vivo studies and with the addition of different radiocontrast agents like zirconium oxide.

The present in vitro study was carried out in the Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital. The study intended to measure the cytotoxic effect and antibacterial efficacy of a novel 3Mix antibiotic paste with barium sulfate in three different concentrations.

Preparation of Novel 3Mix antibiotic paste with barium sulfate

A combination of ciprofloxacin, metronidazole and cefixime tablets, were used for the preparation of 3Mix antibiotic paste. 100 mg of each medicament was weighed using a high precision electronic balance and mixed in a 1:1:1 ratio. 10 mg, 100 mg, and 250 mg of this prepared 3Mix antibiotic powder were mixed with 10 ml of sterile water in microtubes to form a 3Mix antibiotic solution of 1 mg/ml, 10 mg/ml and 25 mg/ml respectively.

0.3 g of barium sulfate was incorporated into microtubes containing each of the three mixtures and stirred. 0.7 g of methylcellulose powder was added to get a pasty consistency of 3Mix antibiotic paste. These microtubes were centrifuged at 7000 rpm for 15 minutes to form a bubble-free homogenous injectable paste. The prepared 3Mix antibiotic paste contained 1 mg/ml, 10 mg/ml and 25 mg/ml 3Mix antibiotic paste with 0.3 g of barium sulfate.

Evaluation of Cytotoxicity

L929 mouse fibroblasts were cultured in Dulbecco's modified eagle's medium with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in an incubator with a humidified atmosphere at 37°C.

Cytotoxicity was assessed using MTT colorimetric assay through direct contact method. Triplicate method was done to assess the cytotoxicity of 1 mg/ml, 10 mg/ml and 25 mg/ml of 3Mix antibiotic paste with 0.3 g/ml barium sulphate.

The cultured L929 fibroblast cells were transferred into four well plates. Different concentrations of 3Mix antibiotic paste with 0.3 g/ml barium sulphate were introduced into the culture wells containing cultured L929 fibroblast cells using cell compatible plastic rings. The plates were then incubated for 24 hours and 7 days at 37°C, 5% carbondioxide, and 98% humidity.

After the incubation period 200 µl of MTT, and 0.5 mg per ml serum free media was added to each well. The plates were again incubated for four hours at 37°C.

After four hours of incubation the cultured L929 fibroblast cells, different concentrations of 3Mix antibiotic paste with barium sulfate along with the MTT solution was removed from the well plates using serological pipette and 200 ml of propanol was added.

Propanol makes formazan crystals formed in viable cells dissolve and create a homogenous purple coloured solution. The solution was then transferred to an ELISA plate and its spectrophotometric absorbance was measured at 570 nm wavelength using an UV spectrophotometer. The amount of crystals formed is directly proportional to the number of viable cells.

Antibacterial effect

88 freshly extracted single-rooted permanent teeth were used to analyze the antibacterial efficacy. For standardization the teeth were sectioned using a diamond disk at 15 mm from the apical region and the root canals were prepared using engine-driven protaper rotary system up to the size F1. Sterility was confirmed by placing four random samples in the Mueller-Hinton agar culture medium with no bacterial growth.

All the remaining samples were placed in separate sterile microtubes containing 2 ml of standard suspension of *E. faecalis*. These samples were incubated at 37°C for 7 days. At the end of incubation period, *E. faecalis* biofilm formation was confirmed by selecting another four random samples from which biofilm samples were scraped off using paper points from the root canals and placed in nutrient agar plate.

The prepared 3Mix antibiotic paste of various concentrations 1 mg/ml, 10 mg/ml and 25 mg/ml with 0.3 g/ml barium sulfate was then transferred into the root canals and sealed using nail varnish. Samples were incubated at 37°C for 24 hours and 7 days.

After the incubation period nail varnish was removed and canals were irrigated to remove the 3Mix antibiotic paste and biofilms were scraped off from the root canals using paper points. These paper points were added to separate microtubes containing 10 ml of sterile distilled water. 10-fold dilution was done using serial dilution method and 50 µl of each diluted sample was inoculated into a Mueller-Hinton culture and incubated at 37°C for 24 hours, and the CFU/ml was calculated after 24 hours.

Statistical analysis

The values obtained were subjected to statistical analysis using one-way ANOVA for intergroup comparison. Pairwise comparison was performed using Post hoc Tukey-b test, and intra group comparison was done using independent sample t-test and paired sample t-test.

Results

The cytotoxic effect was highest for 25 mg/ml of 3Mix antibiotic paste which was moderately cytotoxic on day one and severely cytotoxic on day seven and was significantly higher compared to both 1 mg/ml of 3Mix antibiotic paste and 10 mg/ml of 3Mix antibiotic paste. 10 mg/ml of 3Mix antibiotic paste was slightly cytotoxic on day one and moderately cytotoxic on day seven. 1 mg/ml of 3Mix antibiotic paste showed no cytotoxic effects on day one and slightly cytotoxic on day seven, however it was significantly lesser compared to 10 of mg/ml 3Mix antibiotic paste.

The antimicrobial efficacy against *E. faecalis* was highest for 25 mg/ml of 3Mix antibiotic paste among the experimental groups and lowest for 1 mg/ml of 3Mix antibiotic paste which was statistically significant. However the antimicrobial efficacy of 25 mg/ml of 3Mix antibiotic paste was significantly lesser compared to the control which indicates the addition of barium sulfate affects the antimicrobial efficacy of all different concentrations of 3Mix antibiotic pastes with barium sulfate. However the addition of barium sulfate did not affect the viability of fibroblast cells.

Conclusion

Based on the results of this study 1 mg/ml 3Mix antibiotic paste with 0.3 g/ml Barium sulfate had the least cytotoxic effects but has poor antibacterial properties, 25 mg/ml 3Mix antibiotic paste with 0.3 g/ml barium sulfate showed the highest antibacterial properties but was also the most cytotoxic, 10 mg/ml 3Mix antibiotic paste with 0.3 g/ml barium sulfate was only slightly cytotoxic and has good antibacterial efficacy.

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MASTER CHART

Compilation of data representing the mean viable fibroblast cells and colony forming units of the experimental and control groups.

Cytotoxic effect of 3Mix antibiotic paste with barium sulfate on L929 cell line on Day 1

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	1 mg/ml	0.820	0.905	90.60
2		0.804	0.875	91.88
3		0.816	0.900	90.66

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	10 mg/ml	0.542	0.871	62.22
2		0.528	0.869	60.75
3		0.559	0.901	62.04

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	25 mg/ml	0.315	0.882	35.71
2		0.304	0.871	34.90
3		0.317	0.902	35.14

MASTER CHART

Cytotoxic effect of 3Mix antibiotic paste with barium sulfate on L929 cell line on Day 7

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	1 mg/ml	0.915	1.220	75.00
2		0.926	1.207	76.71
3		0.930	1.218	76.35

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	10 mg/ml	0.461	1.003	45.96
2		0.457	0.977	46.77
3		0.543	1.181	45.97

S.NO	Concentration	Viable cells of Sample	Viable cells of Control	Cell Viability (%)
1	25 mg/ml	0.234	1.183	19.78
2		0.205	1.006	20.37
3		0.226	1.181	19.13

MASTER CHART

Antibacterial efficacy of 3Mix antibiotic paste with barium sulfate on *E. faecalis*

Colony count after 24 hours of 3Mix antibiotic paste treatment

SL NO.	Dilution: 10 ⁻¹ or 1:10				
	Sample	Group 1	Group 2	Group 3	Control
1	1	436	212	55	21
2	2	368	79	52	26
3	3	264	80	55	22
4	4	430	77	54	23
5	5	272	110	67	28
6	6	292	176	38	18
7	7	236	212	41	19
8	8	256	184	47	11
9	9	364	160	50	11
10	10	308	132	46	25

MASTER CHART 4**Colony count after 7 days of 3Mix antibiotic paste treatment**

SL NO.	Dilution: 10 ⁻¹ or 1:10				
	Sample	Group 1	Group 2	Group 3	Control
1	1	256	18	17	9
2	2	200	23	17	7
3	3	203	24	15	8
4	4	133	23	15	4
5	5	161	24	14	9
6	6	213	27	17	9
7	7	64	28	12	5
8	8	55	41	12	5
9	9	87	42	13	1
10	10	87	31	11	2



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15th September 2020

Ref: 163/IRB-IBSEC/SIST Dated 15th September 2020

Dr. A. Wilson Aruni, PhD,
FAMPV
Pro Vice chancellor,
Chair Person,
Institutional Biosafety &
Ethical Committee
(IBEC), SIST

Dr. B. Thayumanavan,
MDS
Dean, Sathyabama
Dental College
Secretary & Convener
Institutional Review
Board &
Institutional Biosafety &
Ethical Committee
(IBEC), SIST

To,

Dr. N. Neslin Basleo

II MDS

Department of Pedodontics

Sathyabama Dental College & Hospital

Chennai -119

This is to inform you that the Institutional Bio-Safety & Ethical Committee has approved your study proposal entitled “**Evaluation of Cytotoxicity and Antibacterial Efficacy of various Concentrations of 3Mix Antibiotic paste with Barium Sulfate as an additive**”, based on the presentation of the proposal made and recommendations of the Institutional Bio-Safety & Ethical Committee/IRB held on 10th August 2020.

The validity of the Ethical clearance is for one year and you are expected to submit six-month progress and the final report of the study to IHBSEC/IRB

Dr. Thayumanavan
Secretary & Convener
IBEC, SIST

Dr. A. Wilson Aruni
Deputy Chair Person
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To whomsoever it may concern

This is to inform that Dr. Neslin Basleo. N, post graduate student, Department of Pediatric and Preventive Dentistry, Sathyabama Dental College and Hospital has done his research on evaluation of cytotoxicity and antibacterial efficacy of various concentrations of 3mix antibiotic paste with barium sulfate as an additive using our facility from 05 – 07 – 2021 to 07 – 11 – 2021 and have made use of equipments present at Life Teck Research Centre.

List of equipments:

- High Precision Electronic Balance (Mettler Toledo AT-201)
- Carbon dioxide incubator (HERA CELL 150i)
- Inverted microscope (Leitz Labovert FS)
- Fluorescence Microscope (Labomed LX-400)
- Spectrophotometer (Systronics Spectrophotometer 104)



Regards
Dr. I. Seethalakshmi
Director