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EVALUATING THE ANTIMICROBIAL EFFICACY OF BIO PURE MTAD IN COMBINATION WITH NISIN AND DAPTOMYCIN IN PREVENTING ROOT CANAL INFECTIONS

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ABSTRACT

Successful root canal therapy relies on the combination of proper instrumentation, disinfection and obturation of the root canal. Of these three essential steps of the root canal therapy, disinfection of the root canal is a major determinant in the healing of the periapical tissues[1] Microorganisms play an essential role in the development of periradicular diseases and are the major causative factors associated with endodontic treatment failures.[2] Hence, proper debridement of the root canal system prior to obturation is an important step in achieving a successful treatment. There is a strong relationship between the long-term treatment success and root canal filled after negative culture in teeth with apical periodontitis. Studies reveal complete periapical healing in 94% of teeth with apical periodontitis that yielded negative culture after root canal instrumentation. In contrast, the samples with positive culture before root canal obturation had only 68% of complete periapical healing.[3] The microbiology of pulp cavity is of the most complex nature predominated mainly by anaerobic species commonly

KEYWORDS: chemical irrigants, root canal infections, enterococcus.

INTRODUCTION

Successful root canal therapy relies on the combination of proper instrumentation, disinfection and obturation of the root canal. Of these three essential steps of the root canal therapy, disinfection of the root canal is a major determinant in the healing of the periapical tissues[1] Microorganisms play an essential role in the development of periradicular diseases and are the major causative factors associated with endodontic treatment failures.[2] Hence, proper debridement of the root canal system prior to obturation is an important step in achieving a successful treatment. There is a strong relationship between the long-term treatment success and root canal filled after negative culture in teeth with apical periodontitis. Studies reveal complete periapical healing in 94% of teeth with apical periodontitis that yielded negative culture after root canal instrumentation. In contrast, the samples with positive culture before root canal obturation had only 68% of complete periapical healing.[3] The microbiology of pulp cavity is of the most complex nature predominated mainly by anaerobic species commonly *the Streptococci, P.gingivalis, P. intermedia, Peptostreptococcus, E. faecalis, Actinomyces sps*. etc.

In infected and necrotic root canal systems, bacteria grow mostly in sessile biofilms, aggregates, and coaggregates in which they are embedded in an extracellular matrix material.[4] Factors that contribute to a persistent periradicular infection after root canal treatment include intraradicular infection ,extraradicular infection ,foreign body reaction and cysts containing cholesterol crystals.[5] Root canal morphology is complex and contains numerous ramifications and anatomical irregularities. The microorganisms in root canals not only invade theanatomic irregularities of the root canal system but are also present in the dentinaltubules.[6] Persistent endodontic disease after root canal therapy may be caused bybacteria in dentinal tubules. Primary endodontic infections are polymicrobial in nature and dominated by gram-negative anaerobic rods.[7] and the microorganisms involved in secondary infections are majorly *Enterococcus faecalis*.

Most persistent organism that, despite making up a small proportion of the flora in untreated canals, plays a major role in the aetiology 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 flora [(8)].*Enterococcus faecalis* are gram

positive cocci that occur singly, in pairs or in short chains. They are facultative anaerobes, possessing the ability to grow in the presence or absence of oxygen. Enterococcus faecalis is associated with different forms of pulpal and periradicular diseases including primary and persistent endodontic infections. It possesses certainVirulence factors such as lytic enzymes, aggregation substance, cytolysin, and lipotechoic acid. It has also been shown to survive and persist as a pathogen in the root canals of teeth.[9]

Sundquist et al demonstrated the presence of *E faecalis* in the root canal system of 58% of teeth with endodontic treatment failure Furthermore, they showed that only 33% of the teeth which harboured *E faecalis* when the canals were being refilled demonstrated endodontic success to a greater extent.[10]

It is unrealistic to assume that a linear streamlined instrument can reach out into the delicate intricacies of the pulp spaces to effectively debride its contents and the huge load of microorganisms along with their toxins. The webs, fins and anastomoses can be cleaned through the effective use of an irrigant. Hence, thorough flushing with intracanal irrigants goes hand in hand with mechanical cleaning of the root canal which is often referred jointly as "chemomechanicalpreparation"[11] of the root canal system.

The importance of root canal irrigation has widely been reported inendodontic literature. In a study conducted to evaluate the effect of mechanical shaping of root canals in endodontic treatment without the use of irrigants, it was concluded that, even after 5 visits, only 50% reduction in bacterial

Count could be possible. This clearly explains the insufficiency of mechanical instrumentation alone.

In elimination of endodontic pathogens.[12] Irrigants are used during root canal preparation to help lubricate canal walls, soften dentin, remove debris and smear layer, dissolve organic matter, kill microorganism and clean areas inaccessible to endodontic instruments. There has been a wide variety on the number and types of endodontic irrigants recommended for endodontic use. They include hot water, physiologic saline, anesthetic solution, 30% solution of urea, sodium hypochlorite in the concentrations of 0.5 to 5.2%, hydrogen peroxide in a concentration of 3%, chlorhexidine in a concentration of 0.2% to 2%, other smear layer modifying agents like EDTA, Citric acid, MTAD etc.

Various concentrations of sodium hypochlorite (NaOCl) have been used as root canal irrigant for many decades. The main advantage of NaOCl are its ability to dissolve necrotic tissues and its antibacterial properties against most microorganisms.[13] Combination of EDTA with NaOCl has also shown to be more effective at killing bacteria.

Sodium hypochlorite has a disadvantage of reaction with periapical tissues if extruded beyond the apex, to overcome these problems it lead to development of chlorhexidine gluconate that has been recognized as an effective antimicrobial agent. It is a cationic chlorophenylbisguanide with bacteriostatic and bactericidal action. Chlorhexidine possess many properties i.e. a broad spectrum antimicrobial agent, substantivity and relatively absence of toxicity, making it a potential endodontic irrigant.[14] The various concentrations of Chlorhexidine available are 0.12%, 0.2% and 2% of which 2% Chlorhexidine has shown to have maximum bactericidal effect, but couldn't eliminate the bacteria completely, which lead to development of new irrgants like MTAD, Nisin and Daptomycin.

MTAD, a common intracanal irrigant, consists of 3% doxycycline, 4.5% citric acid, and 0.5% polysorbate 80 detergent and is used to remove pathogenic bacteria and smear layers during root canal procedures. MTAD has many advantages in root canal irrigation, but its bactericidal activity remains to be improved upon as its antibacterial effect has been largely attributed to doxycycline, a tetracycline that is bacteriostatic rather than bactericidal.[15]

Nisin which is a naturally occurring antimicrobial peptide (discovered in 1928) is found to be effective against the *E. faecalis*. Nisin is produced by *Lactococcus lactis* and which is a class I bacteriocin. It is used as food preservative and found to be safe in human beings.[16] Daptomycin is a novel lipopeptide antibiotic with bactericidal activity against a wide range of clinically important Gram-positive bacteria, including vancomycin-resistant enterococci (VRE).

AIMS AND OBJECTIVES

The aim of this study is to evaluate the antimicrobial activity of Bio pure MTAD in combination with Nisin and Daptomycin, which is calculated by Zone of inhibition, Time killing study and Optical density using spectrophotometer.

OBJECTIVE

1. Irrigants that shows significant antimicrobial property against *Enterococcus faecalis.*
2. To check for the synergistic effect of MTAD in combination with Nisin and Daptomycin.
3. To check which irrigant shows a maximum zone of inhibition and reduction in bacterial count after the application to irrigants to inoculated solutions.

MATERIALS AND METHODOLOGY

This study was conducted in the Department of Conservative dentistry and Endodontics, Panineeya Institute of Dental sciences and Hospital and in the Department of Microbiology, NRI Medical College, Vijayawada during 2012-2015. The purpose of the study was to evaluate the antimicrobial efficacy of three root

canal irrigants used in combination – MTAD, MTAD with Nisin and MTAD with Daptomycin against Enterococcus faecalis. The various materials used in the present study were as follows.

Materials Used

S. no.	Materials /Equipment Used	Manufacturer
1.	Biopure MTAD	Dentsply Tulsa Dental
2.	Nisin	Bimalpharma Pvt. Ltd
3.	Daptomycin	Aurobindo Pharma Ltd
4.	Brain heart infusion broth	Hi-media laboratories Pvt. Ltd, Mumbai
5.	Distilled water	Prepared at the department using double distillation glass apparatus
6.	Normal Saline	
7.	Autoclave	
8.	Biosafety cabinet	Imtech-Micro Safe
9.	Sterile micropipettes(tip)	
10.	10ml sterile test-tubes and test-tube holders	Hi-media laboratories Pvt. Ltd, Mumbai
11.	Culturing loops	Hi-media laboratories Pvt. Ltd, Mumbai
12.	Blood agar plates	Hi-media laboratories Pvt. Ltd, Mumbai
13.	Nutrient Broth	Hi-media laboratories Pvt. Ltd, Mumbai
14.	Bile Esculin Agar	Hi-media laboratories Pvt. Ltd, Mumbai
15.	Incubator	Tempo Instruments and Equipment Pvt Ltd, Mumbai
16.	*Enterococcus strains*	Department of Microbiology, NRI Medical College, Vijayawada

Methodology

Zone Inhibition Method

1. Preparation of blood agar medium plates

- The medium is prepared by adding sterile blood to sterile nutrient agar that has melted and cooled to 50^0c
- 10% concentration of sheep blood is used
- A thin layer of melted nutrient agar with blood about 14ml for a 9cm petri dish is poured and allowed to set. Sterility check is done by incubating 2 plates at 35^0c overnight.
- Quality check is done by using ATCC 25923*Staphylococcus aureus.*

2. Inoculum preparation of *Enterococcus faecalis*

- Inoculum is prepared using ATCC 29212 *Enterococcus faecalis*
- Inoculum of Mc Farland 0.5 turbidity is prepared using nutrient broth

3. Preparation of discs containing chemical irrigants MTAD, 3% MTAD with 3% Nisin and 3% MTAD with 3% Daptomycin

- 3% of each irrigant solution w/ v were prepared by mixing 3.0 g of each material in 100ml of water and combination irrigants were prepared by mixing them in equal proportions.
- Whattman no 1 filter paper is used
- 6mm diameter is cut and dipped in working solution of chemical irrigants of MTAD, MTAD with Nisin and MTAD with Daptomycin using sterile forceps.

4. Testing of chemical irrigant

- On a blood agar medium plates, ATCC 29212 *Enterococcus faecalis* is streaked using inoculating wire loops in a Biosafety cabinet level 2.
- The inoculum is streaked in four quadrants.
- The discs containing chemical irrigants of MTAD, MTAD with Nisin and MTAD with Daptomycin to be tested are placed on the streak line using sterile forceps
- One quadrant of inoculum is used for quality check
- The plates are incubated at 35^0c, overnight in an incubator
- Inhibition of growth is checked after incubation period

Optical Density and Time Kill Study

Optical density, measured in a spectrophotometer, can be used as a measure of the concentration of bacteria in a suspension. As visible light passes through a cell suspension the light is scattered. Greater scatter indicates that more bacterial or other material is present.

Time kill curves are procedure to check the amount of bacteria killed at different intervals of time.

Five 10ml test tubes are taken and one each labelled as

1. Saline
2. *Enterococcus faecalis*
3. Saline
4. MTAD
5. 3% MTAD with 3% Nisin
6. 3% MTAD with 3% Daptomycin

- The first test tube contains saline

- Second test tube contains only saline inoculated with *Enterococcus faecalis*
- Third test tube contains 3% MTAD irrigant solution which is inoculated with *Enterococcus faecalis*
- Fourth test tube contains 3% MTADN solution which Is inoculated with *Enterococcus faecalis*
- Fifth test tube contains 3% MTADd which is inoculated with *Enterococcus faecalis*
- All the test tubes inoculated with *enterococcus faecalis* were checked for reduction in the bacterial count at 30mins, 60mins,90mins intervals under spectrophotometer at 540nm
- The results were charted according to the percentage of reduction in bacteria.

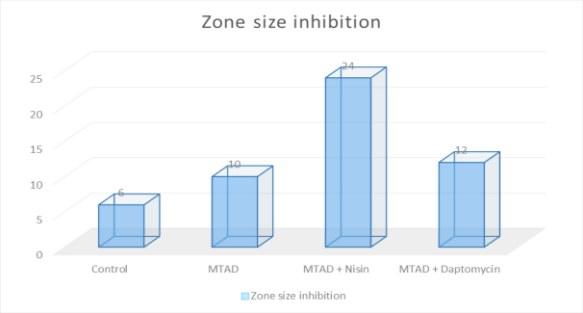
Scanning Electron Microscopy

Morphologic changes in *E.faecalis* were observed by scanning electron microscope (SEM) after treatment with MTAD, MTADN, or MTADd. Four Eppendorf tubes containing 1ml of overnight culture of the supernatant was discarded .After the bacterial pellets were lightly washed twice with 0.01 mol/L phosphate-buffered saline, the depositions were treated with 1ml MTAD, MTADN or MTADd at 37^0c for 24hours. *E faecalis* was treated with PBS as a negative control. After removing the supernatant by centrifugation, all of the depositions were immersed in fixative containing 1% osmium tetroxide for 2hours. The bacterial depositions were washed twice with sterile water and then dehydrated in a series of acetonitrile solutions. The specimens were then dried and spread with gold. The bacterial morphology was observed by using a field emission scanning electron microscope.

RESULTS

Table 1: Zones of inhibition by MTAD, MTAD+ Nisin, MTAD + Daptomycin and Control.

Chemical irrigant	Zone of inhibition
Control	6mm
MTAD	10mm
MTAD + Nisin	20mm
MTAD + Daptomycin	12mm



Graph 1: Showing Zones of inhibition.

Zone size of inhibition

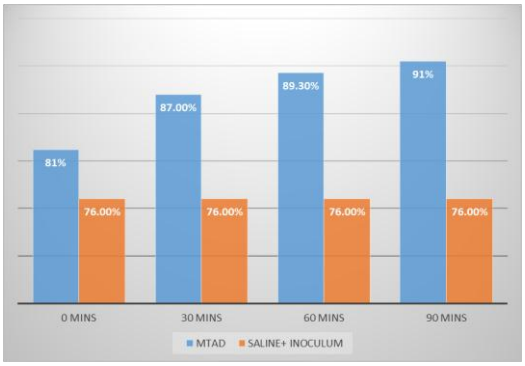
The results of this in vitro study showed that the combination of MTAD with Nisin showed a relative larger zone of inhibition for the growth of *Enterococcus Faecalis* ,when compared to the zones by MTAD alone MTAD in combination with Daptomycin.

The Zones of inhibition produced by MTAD alone and MTAD in combination with Daptomycin were not different from each other.

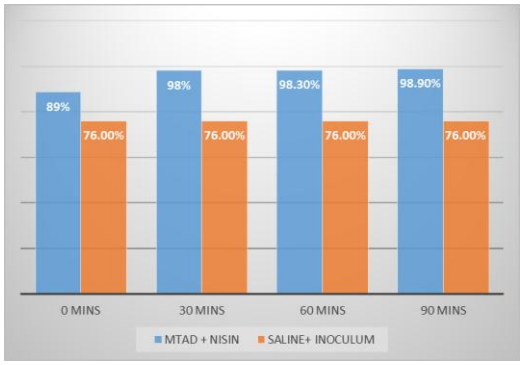
The control did not show any inhibition of *E.faecalis* growth.

Table 2: Optical Density And Time Kill Study.

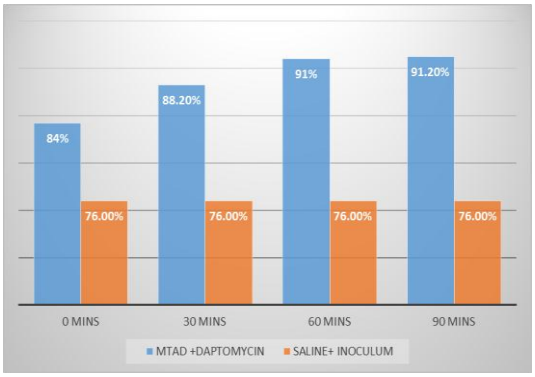
Groups	0 HRS	30MINS	60MINS	90MINS
Saline	100%	100%	100%	100%
Saline+Inoculum	76%	76%	76%	76%
Mtad	81.2%	87%	89.3%	90.5%
Mtad+ Nisin	88.6%	98%	98.30%	98.90%
Mtad+ Dap	84.2%	88.20%	91%	91.20%



Graph 2: Showing OD of MTAD in comparison with Saline+ inoculum.



Graph 3: Showing OD of MTADN in comparison with Saline+ inoculum.



Graph 4: Showing OD of MTAD in comparison with Saline+ inoculum.

DISCUSSION

It is well established that pulpal and periapical diseases as well as failure of endodontic therapy are due to the presence of microbes in the root canal system. Eliminating microbes from the infected root canals and prevention of re-infection is one of the fundamental aims of endodontic therapy[48] Although the root canal flora is dominated by obligate anaerobic bacteria, some facultative strains like *Enterococci*, *Staphylococci* and fungi such as *candida* species etc. have been involved in persistent infections, influencing the prognosis of the root canal therapy.[49]

Of the *Enterococcus* species,*E. faecalisis* the most frequently isolated or detected species from oral infections, including marginal periodontitis, infected root canals, and periradicularabscesses.[9] *Enterococcus faecalis* is an unrelenting organism that, despite making up a small fraction of the flora in untreated canals, plays a major role in the etiology of persistent periradicular lesions after orthograde endodontic therapy. It has been hailed as the "star survivor" and is commonly found in a lofty percentage of root canal failures.[50] It thrives in treated root canals as a single organism or as a major portion of flora.[51]

E. faecalis is a Gram-positive, Catalase negative, fermentative, non-sporing facultative, coccus.[50] Its cells are ovoid, ranging from 0.5 to 1u m in diameter. These organisms occur singly, in pairs, or in short chains. Most strains are non hemolytic and non-motile. Surface colonies on blood agar are creamy white, circular, smooth, and entire.[52] It has the ability to survive in various adverse circumstances. These comprise the capability to survive in hyper-osmotic conditions, at temperatures ranging from 10 c to60 c and at a pH of over 9.6. Growth on bile-esculin is a useful trait to identify Enterococci.

Studies have shown that these bacteria attain an increased resistance or phenotypic tolerance to numerous disinfectants or physical agents. *Enterococci* possess a number of virulence factors that permit adherence to host cells and extracellular matrix, facilitate tissue invasion, effect immunomodulation and cause toxin-mediated damage. These factors include: 1. aggregation 2. enterococcal surface proteins 3. Gelatinase 4. Cytolysintoxin 5.Extracellular superoxide production, 6. capsular polysaccharides and 7. antibiotic resistance determinant.[48]

The survival of *E. faecalis* within infected root canals is aided by dentinal fluid. Dentinal fluid also aids in their invasion into dentinal tubules and helps these microbes to endure long-term starvation. Therefore, even in a well-debrided and obturated root canal, *E. faecalis* may grow by utilizing local sources of energy and nutrients.

An assortment of antimicrobial solutions have been tested for their ability to eradicate *E. faecalis* from the intricacies of the root canal system. These encompass both inter appointment dressings, such as calcium hydroxide, camphorated paramonochlorophenol etc, as well as irrigants such as NaOCl, Chlorhexidine gluconate, chlorhexidine acetate and iodine compounds and newer irrigants like MTAD ,MTAD with Nisin and MTAD with Daptomycin.

Among these *E. faecalis* appears to be extremely resistant to the medicaments used during treatment and is known to resist the antibacterial effects of calcium hydroxide which is generally a highly effective antimicrobial agent. This is because *E. faecalis* can endure a high alkalinity up to around pH 11.5. Certain mechanisms have been postulated which enable *E. faecalis* to survive the high pH of calcium hydroxide. These include a stress induced protein synthesis and the presence of a potassium/proton antiport system in maintaining cytoplasmicpH in an alkaline environment. The natural buffering effect of dentine, affords further protection since pH levels in dentine do not reach higher than 9.7-10.8. Thus, the highly complex nature of this organism poses a great challenge to endodontists.

Whilst the ability of *E. faecalis* to resist the antimicrobial effect of calcium hydroxide remains a significant clinical challenge in endodontic retreatment, other treatment regimens aimed at eradicating *E. faecalis* during each of the phases of endodontic therapy are being continuously evaluated.

Among various treatment steps involved with infection control, the chemo mechanical preparation assumes a pivotal role in root canal disinfection. In addition to the mechanical effects exerted by instruments and the flow and backflow of the irrigant solution during preparation, the use of an antimicrobial substance for irrigation has been shown to be necessary to enhance bacterial elimination.

A large number of substances have been used as root canal irrigants, including acids (citric and phosphoric), chelating agents (EDTA), proteolytic enzymes, detergents (cetrimide), alkaline solutions (sodium hypochlorite, urea), oxidative agents (hydrogen peroxide

and Gky-oxide), MTAD, peptides like Nisin and Daptomycin.

An ideal root canal irrigant should a) Have a broad antimicrobial spectrum and high efficacy against anaerobic and facultative microorganisms organized in biofilms b) Dissolve necrotic pulpal tissue remnants c) Inactivate endotoxins d) Prevent the formation of a smear layer during instrumentation or dissolve the latter once it has formed .Furthermore, as endodontic irrigants come in contact with vital tissues, they should be systemically nontoxic, noncaustic to periodontal tissues and have a little potential to cause an anaphylactic reaction.

One of the most routinely used root canal irrigant is sodium hypochlorite, because of its potent advantages. However the use of sodium hypochlorite has various inherent disadvantages, principally due to its toxicity- it injures all living tissues except keratinized epithelia. At very low concentrations, contact withvital tissues induce an inflammatory reaction. Accidental extrusion beyond the apex causes excruciating pain, immediate swelling, and profuse bleeding and also pharyngeal edema andoesophageal burns when swallowed unintentionally. Due to these biocompatibility issues other irrigants have come into the endodontic scenario such as MTAD, Nisn and Daptomycin.

Bio Pure (DENTSPLY, Tulsa Dental, Tulsa, Ok, USA) otherwise known as MTAD was introduced by Torabinejad and Johnson in 2003. This solution contains Doxycycline hyclate (at a concentration of 3%), citric acid (concentration 4.25%) and a detergent, Polysorbate 80 (concentration 0.5%, also known as tween 80). Several studies have evaluated the effectiveness of MTAD for disinfection of root canals. Torabinejad et al have shown MTAD is able to remove the smear layer and is effective against *E. faecalis.*

Doxycycline, an important component of MTAD belongs to the tetracycline family of antibiotics. Tetracyclines have been used to remove the smear layer from instrumented root canal walls, for irrigation of retrograde cavities during periapical surgical procedures, and as intracanal medicaments. Tetracyclines readily attach to the dentine and are subsequently released without losing their antibacterial activity. This property creates a reservoir of active antibacterial agent, which is then realesed from the dentine surface in a slow and sustained manner Thus, the presence of doxycycline may be accountable for substantivity of MTAD Doxycycline has also been shown to possess anticollagenase activity.

Root canal instrumentation produces a layer of organic and inorganic material called the smear layer that may also contain bacteria and their by-products. Removal of the smear layer remains controversial, however it can prevent the penetration of intracanal medicaments in to dentinal tubules and influence the adaptation of filling material to canal walls. Thus demineralizing agents such as ethylenedimine terraaceticid (EDTA) and citric acid have been recommended as adjuvants in root canal therapy. Loel 1975 and Tidmarsh 1978 demonstrated the effectiveness of citric acid as a root canal irrigant. It has been shown to be more effective than NaOCI Layer (Baumgartner et al 1984). Although citric acid appears to be slightly more potent at similar concentration than EDTA, both agents show high efficiency in removing the smear layer. In addition to their cleaning ability, chelators such as EDTA and citric acid may detach biofilms adhering to root canal walls. Thus, the presence of citric acid in MTAD accounts for removal of the smear layer.

Nisin, an antimicrobial peptide produced by *Lactococcus lactis,* has been extensively used as a preservative in dairy products the peptide is composed of 34 amino acid residues. Nisin inhibits the proliferation of most gram-positive bacteria and is heat-stable, odourless, colourless, tasteless and active at low ph. Nisin also has a strong bactericidal effect and microorganisms generate little resistance to Nisin Furthermore Nisin has low toxicity. These advantages of Nisin suggest it has potential as a intracanal irrigant. Nisin attaches to the plasma membranethrough a specific docking molecule, creating a preformingunit comprised of several Nisin molecules. These interactions lead to the formation of transient pores in the cell membrane, which dissipate the cell's ion gradients and cause the leakage of cellular components, ultimately leading to cell death.

Daptomycin is a novel lipopeptide antibiotic with bactericidal activity against a wide range of clinically important Gram-positive bacteria, including vancomycin-resistant enterococci (VRE). Daptomycin is believed to act by Ca^{2+}-dependent insertion of its acyl tail into the Gram-positive cell membrane, which is followed by the development of potassium efflux channels, depolarization of the membrane and cell death. For synergy screening, Daptomycin was tested with the following antibiotics by Etest: ampicillin, oxacillin, piperacillin, ceftriaxone, cefepime, imipenem, tetracycline, chloramphenicol, clindamycin, linezolid, synercid, and rifampicin.

This study was undertaken to evaluate the antimicrobial activity of MTAD and MTAD with Nisin and Daptomycin.

Different approaches have been used to test the effectiveness of antimicrobial agents in the laboratory. These include : incubation of broth cultures of selected bacteria with the antimicrobial agent (O'Hara et al 1993, D'Arcangelo et al. 1999), growth of selected bacteria as 'lawns ' on agar surfaces and use of the disc diffusion method (Siqueira et al.1998) Use of calorimeter or spectrophotometer for checking of optical density seen with reduction of bacterial count.

In the present study the antimicrobial efficacy of different irrigants was evaluated by incubation of broth cultures of selected bacteria with the antimicrobial agent using zone inhibition method and optical density seen with reduction of bacterial count. According to present study results MTAD with combination of Nisin has shown greater zone of inhibition of 12mm.

(Zhongchum tong, Lin zou et al 2011) reported there was no significant difference between the viable bacterial counts in root canals with and without MTAD applied as a final rinse after root canal preparation, indicating that *E. faecalis* was strongly resistant to MTAD.

It can be explained by saying; It is difficult to completely eradicate *E. faecalis* from root canals with MTAD, in part because doxycycline, the primary antibacterial agent in MTAD, is bacteriostatic rather than bactericidal. For endodontic treatments, a bactericidal agent is needed to achieve a greater killing effect. Nisin, an antibacterial peptide, has strong bactericidal activity and is associated with little drug resistance, although there are no reports on its ability to eradicate the smear layer. In contrast, MTAD can effectively remove the smear layer. A combination of Nisin and MTAD could compensate for the weaknesses of each drug and kill and eradicate E. faecalis from the root canal. Furthermore, MTAD provides an acid environment that is favourable for Nisin because Nisin has higher antibacterial activity and stability at low ph.

In MTAD, polysorbate 80 is a detergent rather than an antibacterialagent. Citric acid has weak antibacterial activity at a concentration of 4.25%, and only when the concentration is greater than 10% can citricacid exert effects on E. faecalis. Therefore, the greater antibacterialactivity of MTADN can be attributed mainly to the synergetic action ofDoxycycline and Nisin. Doxycyclineis a semisynthetic tetracycline antibiotic that exerts its bacteriostaticaction by penetrating the bacterial cell. The antibiotic can bindto the 30S ribosomal mRNA complex at the donor site to blockaminoacyl-tRNA in microorganisms and inhibit protein synthesis atthe ribosomal level. Nisin exerts its antibacterial activitythrough pore formation on the cell membrane and disruption of cellwell synthesis, resulting in the rapid efflux of small requiredcytoplasmic compounds.

Nisin, a cationic peptide, can stably bind to cell membranes by adhering to anionic lipid II, whichis a principal component of the membranes of gram-positive bacteria. By using lipid II as a ‘‘docking molecule’’ to form pores on the surface of cell membranes in a targeted manner, nanomolar concentrations of Nisin are able to effectively kill bacteria. When doxycycline is applied in combination with Nisin, the pores formed by Nisin on the surface of the cell membrane can facilitate the penetration of doxycycline molecules into the microorganism, and thus more intracellular protein synthesis can be inhibited by the doxycycline molecules. SEM revealed that MTADN caused the most seriousdamage to *E. faecalis*, indicating that it is difficult for *E. faecalis* toresist this double attack.

(Zhongchun Tong, Rong et al. 2012) conducted a similar study to check for synergistic effects of MTAD with Nisn. He concluded as MTAD in combination with Nisin improved antibacterial efficacy against pathogens, especially for some gram-positive bacteria associated with persistent intracanal infection. Therefore, the combination had the potential to be used as effective intracanal irrigation.

(Zhongchun Tong, Junqi Ling et al 2014) reported a study where MTAD was used in combination with Nisin at sub inhibitory concentration against *E. faecalis* growth and expression of pathogenic genes. The study reported similar results where MTAD with Nisin combination showed complete elimination of *E. faecalis. E. faecalis* showed the least resistance to alkaline environments after treatment with MTADN. This result indicated that Nisin, in combination with doxycycline, could help calcium hydroxide intracanal dressing to better inhibit the pathogenic bacteria *E. faecalis*. Calcium hydroxide dissociates into calcium and hydroxyl ions on contact with aqueous fluids, and hydroxyl ions cause the lethal effect on bacterial cell by damage to the bacterial cytoplasmic membrane, denaturing of proteins, and damage to DNA. In MTADN and MTAN, the antibacterial peptide Nisin exerts its antibacterial activity by forming pores in cell membranes, thus disrupting cell well synthesis and causing a rapid efflux of essential cytoplasmic small molecules. The pores made by Nisin facilitate the penetration of hydroxyl ions into bacteria, resulting in rapid death.

In the present study optical density of the various irrigants where compared with Saline and Saline inoculated with *E. faecalis, which* was recorded using spectrophotometer. Saline and Saline inoculated with *E. faecalis* were used as control groups whose optical density values remained constant. Saline OD value was 100% due to complete absence of bacteria and Saline inoculated with *E. faecalis* was recorded to be 76% due to the presence of bacteria, which was taken as a comparative reading after being added by three different irrigants used in the present study.

According to the results of the present study the optical density value of MTAD with Nisin was greater when compared to MTAD with Daptomycin and MTAD alone ,which is better explained by *(Zhongchun Tong, Junqi Ling et al 2014)* the synergistic action of doxycycline and Nisin.

In the present study the OD value of MTAD with Daptomycin was superior than MTAD alone. No study used MTAD in combination with Daptomycin for killing *E. faecalis*. The zone of inhibition was recorded at 12mm for MTADd and 10mm for MTAD. A plausible

explanation for the synergy between Daptomycin and MTAD would be that, Daptomycin binds and opens channels that alone are insufficient to produce killing but can allow specific entry of Doxycycline to carry out the further lysis of the cell.

(Kenneth H, Herbert Houck et al 2004) conducted a study where synergy between Daptomycin and 18 other antibiotics against 19 strains of high-level vancomycin-resistant enterococci was tested, which included tetracycline groups. He concluded there was no significant synergy between Daptomycin and any other antibiotic by this screening method. If confirmed by further studies, Daptomycin with either rifampicin or ampicillin may be useful in the management of infections caused by VRE.

Time-killing curves were used to evaluate the antibacterial activities of MTAD, MTAD with Nisin and Daptomycin, where the solutions were tested of OD at different time intervals to check for the reduction of bacterial count, by using saline and saline with inoculum as control group.

According to the results in the present study Doxycycline and Nisin have showed the superior antimicrobial activity at 30mins of 98%, followed by MTADd of 88.2% and least being MTAD of 87%. Which showed a significant difference between the three groups. The OD value of MTAD with Nisin was greater with increase of time where it could eliminate complete bacteria of 98.9% at 90mins, when compared to MTADd and MTAD which was 91.2% and 90.5. MTAD alone showed a least killing of *E. faecalis at 30mins* and 90mins interval.

(Bradley M. Newberry, Shahrokh Shabahang. et al 2007) conducted a studyto determine theAntimicrobial effect of MTAD as a final irrigant on eight strains of *Enterococcus faecalis (E. faecalis)* and to measure the minimum inhibitory concentration (MIC) of MTAD. He concluded that this treatment regimen was effective in completely eliminating growth in seven of eight strains *of E. faecalis. Which can be explained by* that the superior bactericidal effect of MTAD caused by a carryover effect of the doxycycline in the MTAD preparation

(Trisha A. Krause . et al 2007) carried out a study to check for the Antimicrobial effect of MTAD, Sodium hypochlorite, Doxycycline and citric acid on E. faecalis using zone inhibition method. Zone inhibition of doxycycline and MTAD was superior than NaOcl.

(Joshua M. Davis, James Maki .et al 2007) conducted a study using Zone inhibition method to check for Antimicrobial Effects of Various Endodontic Medicaments on Enterococcus faecalis. The

Largest diameter of the zones of microbial inhibition was measured in millimetres and recorded. Where Bio Pure MTAD showed significantly more zones of microbial inhibition than 5.25% NaOCl, 2% CHX, and Dermacyn.

*(ShahrokhShabahang, Manouchehr Pouresmail. et al 2003) A*ntimicrobial efficacy of MTAD and Sodium hypochlorite. The efficacy of MTAD in disinfecting the internal and external surface of roots is a result of the presence of the antibacterial effect of doxycycline – its ability to remove organic and inorganic substances from the surface of roots, which is facilitated by the presence of citric acid and presence of detergent that aids its propensity to diffuse into the root canal and the dentinal tubes.

CONCLUSION

Within the limits of the present study, it can be concluded that

1. The antimicrobial efficacy of MTAD with Nisin (MTADN) is significantly greater when compared to MTADd and MTAD alone.
2. The inhibition zone size was seen greater with MTADN followed by MTADd and MTAD.
3. The optical density value of MTAD with Nisin was significantly greater when compared to MTADd and MTAD, which can be concluded that MTAD and Nisin have a greater synergistic effect.

SUMMARY

Successful endodontic therapy largely depends on the adequate cleaning and shaping of the root canal and elimination of the main etiological factors i.e. the microorganisms. For this purpose various irrigants and intracanal medicaments have been introduced. Among which to of the most common irrigants are sodium hypochlorite and chlorhexidine, but due few potent disadvantages andits inability to completely eliminate the bacteria lead to the development of few new irrigants like MTAD, Nisin and Daptomycin. This study was therefore undertaken to evaluate the antimicrobial efficacy of MTAD, MTADN AND MTADd. The present study was undertaken in Panineeya Institute of Dental Sciences, Hyderabad and NRI medical college Vijayawada.

Total four protocols were taken to calculate the antimicrobial efficacy of three different irrigants, which include Zone inhibition method, where blood agar medium was taken and divided in four different compartments and labelled as *E. faecalis,MTAD, MTADN and MTADd*. All the four compartments where streaked with E. faecalis and incubated. 6mm disc dipped in each irrigant to be tested is placed in their in their respective compartments and check for the amount of zone of inhibition.

Second and third method was check for reduction in bacterial count by checking the optical density at different time intervals underspectrophotometer. Five 10ml test tubes were taken each filled with Saline,

Salineinoculated, MTAD to inoculation, MTADN to inoculation and MTADd to inoculation and checked of OD at 0hr, 30mins, 60mins and 90mins interval. Morphological changes were observed under SEM.
Results of the present study reveal significant zone of inhibition and decrease in optical density for all the irrigants.Among the three irrigantsused, MTADN showed a large zone of inhibition of 20mm and reduction in OD of 98.9% at 90mins interval, which is significantly higher than MTADd and MTAD.

The present study concluded that MTAD with NIsin showed a superior antimicrobial efficacy and have a greater synergistic effect than MTADd and MTAD alone.

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