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COMBINED EFFECT OF GENTAMYCIN, AMPICILLIN WITH N – ACETYLCYSTEINE AGAINST ENTEROCOCCUS FAECALIS BIOFILM : AN IN VITRO STUDY

Dentistry

Dr. Nayana Nair* Post Graduate, PMNM Dental College And Hospital, Bagalkot, Karnataka, 587103. *Corresponding Author

Dr. Prahlad A Saraf MDS, Professor, HOD, PMNM Dental College And Hospital, Bagalkot, Karnataka-587103.

Dr. Shrishail Totad MDS, Professor, PMNM Dental College And Hospital Bagalkot, Karnataka-587103.

Dr. Rashmi P. Yadahalli MDS, Reader, PMNM Dental College And Hospital, Bagalkot, Karnataka-587103.

Dr. Sneha Vanaki MDS, Reader, PMNM Dental College And Hospital, Bagalkot, Karnataka-587103.

Dr. Pradeep Chigadani MDS, Sr.Lecturer, PMNM Dental College And Hospital, Bagalkot, Karnataka-587103.

ABSTRACT

Biofilm-related infections pose a major problem in the society from both an economical and health perspective. Persisting biofilms inside the root canal system after root canal therapy may result in re-infections and persistent apical periodontitis. Enterococcus Faecalis (E.Faecalis) has been found in 38% of the failed root canal-treated teeth.

Aim: Evaluation of in vitro antibacterial efficacy of Gentamycin (GMC), Ampicillin (AMP) and their combination with N-acetylcysteine (NAC) in root canals infected with E.Faecalis.

Methods And Materials: 60 extracted single rooted human teeth were prepared and randomly divided into six groups: Calcium hydroxide (CH), Gentamycin (GMC), Ampicillin (AMP), Gentamycin and N-acetylcysteine (GMC+NAC), Ampicillin and N-acetylcysteine (AMP+NAC) and Normal Saline as a positive control. According to the name of the groups, intracanal medicaments were placed into the canals and the teeth were restored with a temporary filling. After one week, intracanal medicament was removed and the final count of bacteria was measured. Antibacterial effect of medicament was assessed by measuring the percentage reduction in the colony counts and Scanning Electron Microscopy (SEM). The Mann-Whitney U test and the Kruskal-Wallis test were used to compare the overall antibacterial efficacy of the intracanal medicaments at significance level of 0.05.

Result & Conclusion: Comparatively the combination of Ampicillin and NAC has a higher disruptive effect on biofilms when compared to calcium hydroxide.

KEYWORDS

Ampicillin, Calcium Hydroxide, E. Faecalis, Gentamicin, N Acetylcysteine

INTRODUCTION

Biofilm-related infections pose a major problem in the society from both an economical and health perspective. Persisting biofilms inside the root canal system after root canal therapy may result in re-infections and persistent apical periodontitis. Enterococcus Faecalis (E.Faecalis) has been found in 38% of the failed root canal-treated teeth. E. faecalis is an anaerobic gram-positive coccus that normally commences in the human oral cavity, gastrointestinal tract, and vagina because it has demonstrated good adaptation to such environments with rich nutrient and low oxygen levels and complex ecology.[1] Among all cases with primary endodontic infection, E. faecalis was more likely to be associated with asymptomatic cases than with symptomatic ones.[2,3,4] Failure to effectively eliminate them could lead to persistent inflammation and impairment of healing.[5] Although proper canal instrumentation and adequate irrigation with sodium hypochlorite can decrease the number of bacteria, it cannot remove E. faecalis from the root canal entirely.[5,6]

Intracanal medicaments have been thought as an important step in killing the bacteria in root canals; however, in modern endodontics, shaping and cleaning has been emphasized greater importance than intracanal medicaments as a means of disinfecting root canals. Furthermore, biocompatibility and stability are essential properties for intracanal medicaments.[7] To curtail bacterial regrowth and possibly even improve bacterial suppression, an intracanal medication can be advantageous and successfully used to eliminate the bacterial flora. Interappointment antimicrobial medication acts by inhibiting proliferation of bacteria and further eliminates surviving bacteria, as well as minimizes ingress of pathogens through a leaking restoration.[8]

Triple antibiotic paste (TAP), containing ciprofloxacin (CIP), metronidazole, and minocycline, has proved to be effective for disinfection of the infected necrotic immature teeth.[9] Calcium hydroxide (CH) is widely used as an intracanal medicament to prevent microbial re-growth.[10] Evidence shows that *E. faecalis* may benefit from a CH challenge and grow in mixed biofilms.[11] Different chemotherapeutic agents were investigated aiming at effective intracanal medicaments.

N-acetylcysteine (NAC) is a mucolytic agent that has been reported to inhibit biofilm formation.[12] NAC can detach bacteria by decreasing the production of "Extracellular Polysaccharides" (EPS).[13]

Ampicillin is categorized as a broad-spectrum beta-lactam antibiotic that has bactericidal activity.[14] The drug antibacterial activity mostly covers the Gram-positive bacilli, but it acts less effectively than amoxicillin.[15]

Gentamicin and other aminoglycosides have increased activity when they are combined with beta-lactams, resulting in increased bacterial aminoglycoside uptake. The proposed mechanism of synergism is damage to the cell membrane by the beta-lactam, followed by improved diffusion of gentamicin across the outer bacterial membrane. A second type of synergism, pharmacodynamic synergism, occurs when high serum concentrations of aminoglycosides cause efficient bacterial killing, resulting in reduced bacterial concentrations, which are more effectively eliminated by beta-lactams, as they work more efficiently against lower bacterial concentrations.[16]

Hence this study is aimed to evaluate the in vitro antibacterial efficacy of Gentamycin (GMC), Ampicillin (AMP) and their combination with N-acetylcysteine (NAC) in root canals infected with E.Faecalis.

METHODOLOGY

Inclusion Criteria

- Single-rooted teeth

Exclusion Criteria

- Cracks
- Resorption
- Rootcanal calcification

Preparation Of Specimen

Sixty single-rooted human teeth were selected for the study fulfilling the criteria. The external surfaces of teeth were cleaned with periodontal curettes. Then, they were immersed in 0.5% NaOCl solution overnight for disinfection. Clinical crowns were cut at cementoenamel junction to obtain a standard root length of 13-15 mm. A #10 K-file (Dentsply-Maillefer, Ballaigues, Switzerland) was inserted into the root canals until its tip was visible at the apical foramen. Next, 1 mm was subtracted from this length to determine the working length. The root canals were prepared using the crown-down technique with Pro-Taper rotary files (Dentsply-Maillefer, Ballaigues, Switzerland) to F3 as the size for master apical file.

The canals were irrigated with 1 mL of freshly prepared 5.25% NaOCl solution. After instrumentation, the canals were irrigated with 17% EDTA followed by 5.25% NaOCl to remove the smear layer. A final rinse was carried out using 5 mL of sterile water. The teeth were dried with sterile paper points. To prevent bacterial leakage, the external surfaces and apices of samples were covered with nail polish and resin, respectively. The specimens were then autoclave-sterilized in phosphate-buffered saline (PBS) for 30 min at 121°C.

Biofilm formation

E. faecalis (ATCC 29212) was grown overnight in brain-heart infusion (BHI) broth medium to get turbidity of 0.5 McFarland standard (1.5 × 108 cells/mL). Then, 2 mL of sterile PBS were removed and replaced with 2 mL of fresh bacterial inoculum in BHI broth. The flasks containing teeth were kept at 37°C for 30 days in an aerobic incubator. The fresh medium was introduced into the canals every 2 days to confirm the growth of bacteria. The teeth would be excluded, if any contaminants were observed.

After 30 days, the specimens were irrigated with 5 mL of sterile saline and dried with sterile gauze. The specimens were then randomly divided into 6 groups (n=10), according to the intracanal medicaments, as it follows:

- Group 1 :- Calcium Hydroxide (CH)
- Group 2 :- Ampicillin
- Group 3 :- Ampicillin + NAC
- Group 4 :- Gentamycin
- Group 5 :- Gentamycin + NAC
- Group 6 :- Saline (positive control)

Table No.1 The Minimum Inhibitory Concentration (MIC) Of Medicaments (µg/mL)

MEDICAMENT	MINIMAL INHIBITORY CONCENTRATION (MIC)
CALCIUM HYDROXIDE	16
AMPICILLIN	1
N ACETYL CYSTEINE	2.5
GENTAMICIN	0.016

Preparation And Application Of The Medicaments

The minimum inhibitory concentrations (MIC) of CH, AMP, GEN and NAC were determined. Each medication was prepared in concentrations of MICs at 1000 times (Table 1). In CH group, CH powder was prepared in paste by mixing CH powder and distilled water with a powder to liquid ratio of 1:2. The paste form of CH was placed into the canal using a #40 file. To make a sticky paste each concentration of the antibiotics were mixed with a sufficient amount of starch and then placed into the canal using a #40 file. The roots in group 6 were filled with saline as the positive control group.

After placing the desired material into the root canal, the coronal part of the respective tooth was sealed with Coltosol for one week. Then, the temporary filling was removed with a fissure bur . Subsequently, the medicaments were taken out by rinsing the canals with 20 mL of sterile saline.

Root Dentine Sampling And Scanning Electron Microscopy (SEM)

Each of the six groups of roots was divided into two equal subgroups: The first subgroup was used for counting "Colony Forming Units" (CFUs). Root dentine samples were taken from dentinal walls using #3, 4 and 5 sterile Gates-Glidden drills (Dentsply). Each drill removed the dentine layer from the inner surface of the canal and samples were transferred to a sterile Eppendorf tube containing 100 µL of fresh sterile broth. Each sample was streaked onto BHI agar plates, and the plates were incubated at 37°C for 48 h to observe any microbial growth. The colonies on the agar plates were counted with colony counter, represented in colony-forming units (CFU) per mL.

Another subgroup was used to examine the formed biofilms and their changes after the application of the medicaments on the root canal walls. The roots were prepared for SEM analysis and observed at 25 kV under scanning electron microscope.

Data Analysis

The CFU data was subjected to logarithmic transformation. To compare the efficacy of different medications for the amount of reduction in CFUs/mL and log CFUs/mL, non-parametric statistical analyses were performed using the two-tailed Mann-Whitney U test and the Kruskal-Wallis test. The level of significance was set at 0.05.

RESULTS

For disinfection of *E. faecalis* from the roots, all intracanal medicaments were significantly more effective than calcium hydroxide (P<0.05).

Table no. 2 Comparison of mean CFUs between different study groups using Kruskal Wallis Test

Groups	N	Mean	SD	Min	Max	P-Value
$Ca(OH)_2$	10	2.50	1.35	1	4	<0.001*
Ampicillin	10	0.10	0.32	0	1	
Ampicillin+NAC	10	0.00	0.00	0	0	
Gentamycin	10	0.10	0.32	0	1	
Gentamycin+NAC	10	0.40	0.97	0	3	
Saline	10	148.00	19.32	130	180	

• - Statistically Significant

Table no. 2 shows comparison of mean CFUs between different study groups using Kruskal Wallis Test. The test results demonstrate that the mean CFU for $Ca(OH)_2$group was 2.50 ± 1.35, Ampicillin group was 0.10 ± 0.32, Ampicillin + NAC group was 0.00 ± 0.00, Gentamycin group was 0.10 ± 0.32, Gentamycin + NAC group was 0.40 ± 0.97 and for saline group, it was 148.00 ± 19.32.

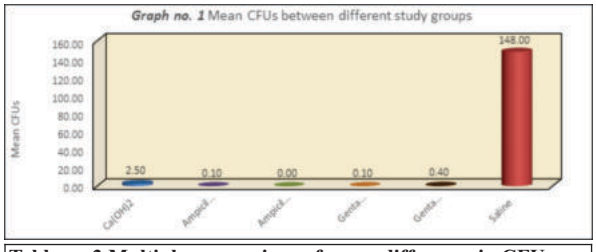
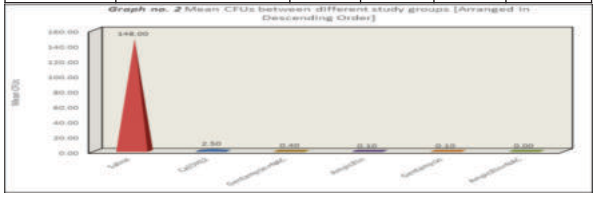
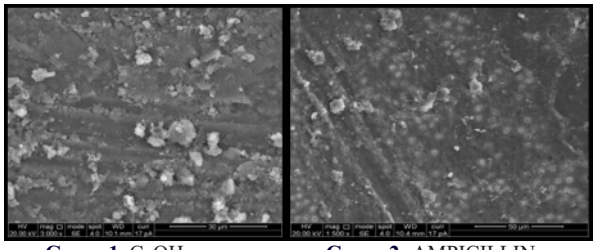


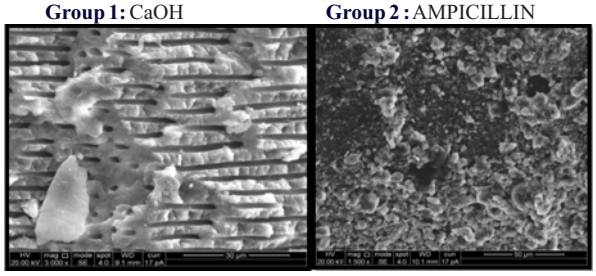
Table no.3 Multiple comparison of mean difference in CFUs b/w different study groups using Mann Post hoc Analysis

(I) Groups	(J) Groups	Mean Diff. (I-J)	95% CI for the Diff.		P-Value
			Lower	Upper	
$Ca(OH)_2$	Ampicillin	2.40	-8.06	12.86	<0.001*
	Ampicillin+NAC	2.50	-7.96	12.96	<0.001*
	Gentamycin	2.40	-8.06	12.86	<0.001*
	Gentamycin+NAC	2.10	-8.36	12.56	0.001*
	Saline	-145.50	-155.96	-135.04	<0.001*
Ampicillin	Ampicillin+NAC	0.10	-10.36	10.56	0.32
	Gentamycin	0.00	-10.46	10.46	1.00
	Gentamycin+NAC	-0.30	-10.76	10.16	0.50
	Saline	-147.90	-158.36	-137.44	<0.001*
Ampicillin+ NAC	Gentamycin	-0.10	-10.56	10.36	0.32
	Gentamycin+NAC	-0.40	-10.86	10.06	0.15
	Saline	-148.00	-158.46	-137.54	<0.001*
Gentamycin	Gentamycin+NAC	-0.30	-10.76	10.16	0.50
	Saline	-147.90	-158.36	-137.44	<0.001*
Gentamycin + NAC	Saline	-147.60	-158.06	-137.14	<0.001*

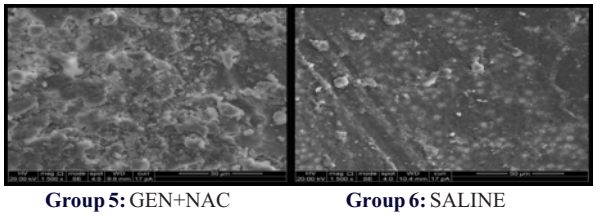




Group 1: CaOH **Group 2 :** AMPICILLIN



Group 3: AMP+NAC **Group 4:** GENTAMICIN



Group 5: GEN+NAC **Group 6:** SALINE

SEM IMAGES OF ALL GROUPS

DISCUSSION

CH is the most frequently used intra-canal medicament, some concerns exist about its limited efficacy against biofilm-producing bacteria, including *E*. faecalis. The antimicrobial activity of CH depends on the release of hydroxyl ions into an aqueous environment. Kayaoglu et al.concluded that an increase in the pH of CH up to 8.5 could lead to an increase in collagen-binding ability of *E.* faecalis producing *E.* faecalis from the infected roots. Incubation of root dentine with *E.* faecalis for 21 days led to a well-developed biofilm that was highly resistant to CH dressing. This might be due to the use of CH in paste form, which was shown to be incompetent in hydroxyl ion release and antimicrobial activity compared to its aqueous suspension.[10]

N-acetylcysteine (NAC) is a mucolytic agent that disrupts mature biofilm formation through decreasing bacterial adhesion on solid surfaces. NAC can detach bacteria by decreasing the production of "Extracellular Polysaccharides" (EPS). Quah et al showed that NAC inhibits growth and eradicates *E.* faecalis biofilm. However, antibacterial character of NAC was not affected in the presence of dentine.[17]

According to Samantha Yilling Quah, Siwen Wu concluded that NAC was most bactericidal at pH 11 with minimum inhibitory concentration and minimum bactericidal concentration of 1.56mg/mL and 12.5mg/mL.The antibacterial property of NAC was unaffected by the presence of dentin.[17,18]In addition, in an invitro study conducted by Camargo et al it was shown that an experimental resin-based epoxy based sealer bonded with NAC could indicate an enhanced antimicrobial effect on E-faecalis in comparison to unmodified epoxy sealers.[19]

Selection of antimicrobial agents was based on previous studies that tested the susceptibility/resistance patterns of E.faecalis. Penicillin and ampicillin are generally not selected because exclusive strains of E.faecalis express β - lactamase enzyme.

Ampicillin is the drug of choice for monotherapy of susceptible E.faecalis infection. For most isolates,the MIC of ampicillin is 2-to-4 fold that of penicillin. For rare strains that are resistant to ampicillin because of β-lactamase production, ampicillin plus sulbactam may be used. Ampicillin remains the treatment of choice for enterococcal infections that lack the other mechanisms for high-level resistance.The cornerstone for treating susceptible enterococcal infections is the β-lactam antibiotics.Among the β-lactams the compounds with the best invitro activity include the amoinopenicillin (ampicillin).[20]

Gentamicin and streptomicin are the two main aminoglycosides used in the clinical practice to achieve bactericidal therapy, and the clinical experience supports the use of combination of ampicillin and either of these two aminoglycosides as the first line of therapy for severe enterococcal infections that are susceptible for both classes of antibiotics.Gentamicin irreversibly binds to the 30S ribosomal subunits.The binding interferes with the formation of messenger RNA and subsequent formation of non-functional proteins and eventual death of susceptible bacteria.[21]

Results of this study indicate that all the chemotherapeutic agents used were significantly better than calcium hydroxide in the elimination of biofilm bacteria. Comparatively the combination of Ampicillin and NAC has a higher disruptive effect on biofilms when compared to calcium hydroxide.

CONCLUSION

AMP and NAC could be used as an alternative intracanal medicament for eradication of E. faecalis biofilm in endodontics.

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