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Comparative evaluation of antimicrobial efficacy of triple antibiotic paste and amoxicillin clavulanate paste as an intracanal medicament against *Enterococcus faecalis*: An *in vitro* study

Dhandayuthapani Sasikala, Parisa Norouzi Baghkomeh, Jamaluddin Mohammed Farzan

Department of Pediatric and Preventive Dentistry, Faculty of Dentistry, Meenakshi Ammal Dental College and Hospital, Meenakshi Academy of Higher Education and Research, Chennai, Tamil Nadu, India

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Address for correspondence:
Dr. Jamaluddin Mohammed Farzan,
Department of Pediatric and Preventive Dentistry, Faculty of Dentistry, Meenakshi Ammal Dental College and Hospital, Meenakshi Academy of Higher Education and Research, Chennai - 600 095, Tamil Nadu, India.
E-mail: profhod.pedo@madch.edu.in

ABSTRACT

Background: Triple antibiotic paste (TAP) is the commonly used intracanal medicament against *Enterococcus faecalis*. Amoxicillin clavulanate paste (ACP) is recommended as a "fall-back" antibiotic when traditional dental antibiotics fail. Literature comparing the antimicrobial efficacy of TAP and ACP in eradicating *E. faecalis* from the root canal system is sparse; hence, this *in vitro* study was conducted to evaluate and compare the antimicrobial efficacy of TAP and ACP as an intracanal medicament for endodontic treatment of single-rooted permanent teeth against *E. faecalis*.

Materials and Methods: This *in vitro*, experimental study evaluated 60 root samples obtained from extracted single-rooted human permanent teeth. The canal diameter was enlarged and subsequently infected with *E. faecalis* for 21 days. Four groups of the contaminated samples were treated with TAP, ACP, calcium hydroxide (positive control), and saline (negative control), respectively. Dentinal shavings were collected at the end of the 1st, 7th, and 10th day and inoculated in agar plates. The number of colony-forming units was determined, and the data were statistically analyzed using the Kolmogorov–Smirnov and Shapiro–Wilks test. $P < 0.05$ was considered statistically significant.

Results: The mean number of *E. faecalis* colony counts across all 3 test days demonstrated that TAP exhibited the highest inhibition of bacterial growth, followed by ACP which is not statistically significant ($P = 1.00$).

Conclusion: Considering the limitations of this *in vitro* study, the findings suggest that ACP could be an effective alternative intracanal medicament to TAP for endodontic therapy.

Key Words: Amoxicillin-potassium clavulanate combination, antibacterial agent, calcium hydroxide, ciprofloxacin, *Enterococcus faecalis*, metronidazole, minocycline

INTRODUCTION

Pulp necrosis and periradicular lesions are triggered by the bacteria and their products.[1] To facilitate the healing of periapical tissues, the objective of endodontic treatment is to completely remove the pulp and eradicate all bacteria from the root canal system.[2] The favorable outcome of endodontic therapy is influenced by mechanical preparation, irrigation, microbial control, and the system for filling the root canals.[2] *Enterococcus faecalis* is frequently present in root canal-treated teeth with

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secondary endodontic infection and persistent lesions which are difficult to get rid of during retreatment procedures.[3-7] They form dense colonies on canal walls which are difficult to eradicate from the root canal system[8] and can survive using the fluid present in the periodontal ligament and create biofilms to defend themselves from the resistance shown by the host and disinfecting solutions.[9] They appear to be resistant to numerous antimicrobial agents.[10] The capacity of *E. faecalis* to acquire antibiotic resistance, particularly to erythromycin and azithromycin, as well as its capacity to enter dentinal tubules and adhere to collagen, add to those processes.[9] Hence, the use of an intracanal medicament with an efficient antibacterial action is advised.[6]

E. faecalis has been tested with several antimicrobial agents. Triple antibiotic paste (TAP) (metronidazole, ciprofloxacin, and minocycline) has the ability to destroy *E. faecalis* in the deeper layers of the root canal dentine.[11] However, there are some concerns regarding the use of TAP, which is discoloration of treated teeth, significantly decreases dentin flexural strength, microhardness, and toxic to dental papilla stem cells, and inhibits their attachment and proliferation.[12] The three antibiotics used in TAP are not available commercially as a single drug combination. To address the limitations of the absence of a combined drug formulation and the issue of discoloration in current treatment options, one possible alternative that has been suggested is the use of amoxicillin clavulanate paste (ACP).[13] Amoxicillin is a selective inhibitor of bacterial cell wall synthesis, and clavulanic acid is a β-Lactamase inhibitor.[14] ACP is a combination drug that has a broad spectrum of activity and bactericidal properties. Hence, this antibiotic combination is recommended as a "fall-back" antibiotic when traditional dental antibiotics fail to treat the infection.[1] The advantage of ACP includes its availability commercially as a single drug combination. The use of a single antibiotic paste is preferable to antibiotic combinations, which can raise the risk of bacterial resistance even when used for short periods of time.[15]

Nosrat *et al*.[13] suggested the use of ACP as an intracanal medication, but there are no *in vitro* antimicrobial as well as clinical studies in the literature comparing the antimicrobial efficacy of TAP and ACP as an intracanal medicament. Hence, this *in vitro* study aimed to evaluate and compare the antimicrobial efficacy of TAP and ACP as an intracanal medicament for the endodontic treatment of single-rooted permanent teeth against *E. faecalis*. The objectives of the study were to evaluate the antimicrobial efficacy of TAP and ACP by measuring the colony-forming units on the 1st, 7th, and 10th day, followed by comparing the estimated antimicrobial efficacy using the statistical analysis to find if there is any statistically significant difference in the obtained values.

MATERIALS AND METHODS

This *in vitro*, experimental study was conducted at the Central Research Laboratory of the Institution. Sixty freshly extracted single-rooted human permanent teeth with complete root apices extracted for orthodontic reasons and periodontal diseases were collected after obtaining prior IRB approval (MADC/IEC-I/054/2021). These teeth were stored in 10% formalin until further use. Teeth with caries and restorations, those which were root canal treated, and those with multiple root canals and open apices were excluded from the study. The collected teeth were cleaned with saline, and then ultrasonic scaling was performed to remove debris from the teeth. An *in vitro* model was used in this study according to the modified Haapasalo and Orstavik method.[16] Vernier caliper was used to measure 6 mm from coronal and apical portions of all the tooth samples, and two markings were made at this 6 mm using a diamond disc. The separated 6 mm from the coronal and apical portions were discarded. The internal diameter of the root canals of these root samples was enlarged using Gates Glidden drill (MANI, Delhi, India) size 1, size 2, and size 3 in sequence for standardization.[17] The root samples were immersed in 17% ethylenediaminetetraacetic acid (EDTA) (Manual Anabond, DESMEAR, Airen Surgident, Raipur, Chhattisgarh, India) for 5 min, followed by 3% sodium hypochlorite (NaOCl) (Hyposol, PREVEST DenPro, Jammu and Kashmir, India) for 5 min, to remove debris from inside the prepared root canals. The root samples were then cleaned in an ultrasonic cleaner (RO ultrasonic cleaner, 600 mL, Uttar Pradesh, India) for 5 min for the removal of any remaining 17% EDTA and 3% NaOCl. This was followed by autoclaving at 121ºC for 30 min, and the sterile root samples were then immersed in 1 mL of Tryptic Soy Broth (TSB) in Eppendorf tubes. The inoculated broth containing 50 µL of the inoculum of *E. faecalis* and 1 ml of fresh TSB was taken in another set of 60

Eppendorf tubes. All the prepared root samples were then transferred to this prepared inoculated broth individually into the respective Eppendorf tubes to contaminate the radical dentinal tubules and produce biofilms of *E. faecalis* and were then incubated for 24 h at 37°C. A fresh inoculated broth was prepared every 48 h, and these inoculated tooth samples were transferred to it for 21 days. The inoculated broth was changed a total of 11 times. After the 3-week period of contamination, the inoculated root samples were removed from the inoculated broth and were irrigated with 5 ml of normal saline in a sterile syringe and then dried using paper points.

The powders of metronidazole (Medopharm Pvt. Ltd., India), ciprofloxacin (Medopharm Pvt. Ltd., India), minocycline (TCI Chemicals, India), amoxicillin (Medopharm Pvt. Ltd., India), potassium clavulanate (Medopharm Pvt. Ltd., India), and calcium hydroxide (Vinzai Chemical Industries Pvt. Ltd., India) in their purest forms were procured from pharmaceutical companies. A solution of metronidazole, ciprofloxacin, minocycline, amoxicillin, and potassium clavulanate was prepared by dissolving 10 mg of powder in 100 µL of distilled water separately. The solutions of metronidazole, ciprofloxacin, and minocycline were mixed together to form the TAP solution (1:1:1 ratio). The solutions of amoxicillin and potassium clavulanate were mixed together to form the ACP solution (1:1 ratio). A 1 mg/mL sol–gel of the TAP and ACP was prepared from the respective solutions by adding 1 mL of propylene glycol. A paste of calcium hydroxide was prepared by mixing the powder of calcium hydroxide with propylene glycol in a ratio of 1:1 in a glass slab.

The inoculated root samples were randomly divided into four groups: TAP (Group A), ACP (Group B), calcium hydroxide as positive control (Group C), and normal saline as negative control (Group D). The intracanal medicaments were introduced into the root canal space of the inoculated root samples using a lentulo spiral for all the allotted groups. After the placement of the intracanal medicaments, both the orifices of the inoculated root samples were sealed using modeling wax and were stored in an incubator at 37°C and 100% humidity. The antimicrobial efficacy of the intracanal medicaments in the four groups was evaluated at 24 h, 7 days, and 10 days. On each of the evaluation days, five inoculated root samples with intracanal medicament from each group were randomly selected, and the wax sealing was removed. It was then irrigated with 10 mL of normal saline to remove the already placed intracanal medicaments from the prepared canals and dried using paper points. Using a size 4 GG drill, the dentin shavings from each of the 20 prepared and treated root canal walls were collected. The collected dentin shavings were transferred to five Eppendorf tubes containing 1 mL of TSB and were incubated for 24 h at 37°C. After the incubation period, the contents from each tube were serially diluted three times. Following this, 100 µL of this diluted dentin shaving sample from each group was taken and spread across a Tryptic Soy Agar (TSA) plate using an inoculation loop, and all 20 plates were incubated for 48 h at 37°C. After the incubation, the bacterial growth was assessed by counting the colony units formed. The spread plate method on the TSA medium showed *E. faecalis* colonies formed in the agar plates among various intracanal medicaments on the 1st day, 7th day, and 10th day.

Statistical analysis

The collected data obtained were analyzed using the SPSS (IBM SPSS Statistics for Windows, version 26.0, Armonk, NY, USA: IBM Corp. Released 2019) for normality using the Kolmogorov–Smirnov and Shapiro–Wilks test. As the data did not follow a normal distribution, nonparametric tests of significance were used for the comparison of the mean values. P <0.05 was considered statistically significant.

RESULTS

Tables 1 and 2 show that there is a statistically significant difference observed in the mean *E. faecalis* colony count within and between the groups at different time periods (P < 0.05). In addition, Group B exhibited the highest inhibition of bacterial growth, followed by Group A, as demonstrated by the mean number of *E. faecalis* colonies across all 3 test days. The results presented in Table 3 indicate a statistically significant difference in antimicrobial efficacy between Group A versus Group D (P = 0.0) and Group B versus Group D (P = 0.002) across all three time periods. However, no significant difference was observed in antimicrobial efficacy between Group A and Group B (P = 1.00). Table 4 shows that Group A, Group B, and Group C showed increased antimicrobial activity from the 1st to the 7th day to the 10th day which was statistically significant (P < 0.05).

DISCUSSION

In the present study, TAP and ACP showed an equal reduction in the mean colony-forming units when used as an intracanal medicament for endodontic treatment of single-rooted permanent teeth against *E. faecalis.*

One of the key strengths of this study was the utilization of ACP, which has been shown to have several beneficial properties in endodontic treatment. An advantage of using ACP is that it is readily available commercially as a single drug system. However, it is important to note that the use of a single antibiotic paste is often preferred over antibiotic combinations. This is because the use of multiple antibiotics can increase the risk of bacterial resistance, even when used for the short periods of time. Therefore, the use of a single antibiotic paste may be a more prudent approach to minimize the risk of developing bacterial resistance.[15] The limitation of this *in vitro* study was the potential for experimental artifacts or contamination, which could negatively impact the accuracy and reproducibility of results. Such studies are often conducted in highly controlled and artificial environments that may not fully reflect the complex and dynamic nature of biological systems *in vivo*. Furthermore, it should be noted that the characteristics of dentin may have been modified due to the storage of teeth in 10% formalin.

The result of our study was in contrast with the previous *in vitro* study by Kaur *et al.*[1] who investigated *in vitro* the efficacy of ACP with metronidazole, TAP, amoxicillin-cloxacillin with metronidazole, and amoxicillin with metronidazole combinations as intracanal medicaments against *E. faecalis* for 24, 48, and 72 h using agar well-diffusion method and found that a combination of ACP and metronidazole showed improved antibacterial activity for up to 48 h, whereas TAP showed sustained antibacterial activity for up to 72 h. However, our study did not find a significant difference between the antibacterial efficacy of TAP and ACP against *E. faecalis.* This finding could potentially be attributed to the inclusion of metronidazole in the ACP formulation.

Several studies have investigated the antimicrobial susceptibility of Enterococci and *E. faecalis* to various antibiotics using *in vitro* and *ex vivo* methods. Rams *et al.*[18] found that these organisms were resistant to therapeutic doses of penicillin G, tetracycline,

Table 1: Friedman test for intragroup comparison of *Enterococcus faecalis* colony count (10^3 CFU/mL) on the 1st, 7th and 10th

Groups	Mean±SD (10^3 CFU/mL)			*P* (intragroup comparison)
	1st day	7th day	10th day	
Group A	4.36±0.78	0.52±0.13	0.08±0.08	<0.001*
Group B	4.06±0.7	0.52±0.22	0.04±0.05	<0.001*
Group C	6.28±0.72	3.42±0.72	0.3±0.1	<0.001*
Group D	8.08±2.43	5.82±2.30	0.9±0.27	<0.001*

*Statistically significant. SD: Standard deviation; CFU: Colony-forming unit

Table 2: Kruskal–Wallis test for intergroup comparison of *Enterococcus faecalis* colony count (10^3 CFU/mL) on the 1st, 7th, and 10th

Groups	Mean±SD (10^3 CFU/mL)				*P* (intergroup comparison)
	Group A	Group B	Group C	Group D	
1st day	4.36±0.78	4.06±0.7	6.28±0.72	8.08±2.43	0.004*
7th day	0.52±0.13	0.52±0.22	3.42±0.72	5.82±2.30	0.002*
10th day	0.08±0.08	0.04±0.05	0.3±0.1	0.9±0.27	0.001*

*Statistically significant. SD: Standard deviation; CFU: Colony-forming unit

Table 3: *Post hoc* analysis of intergroup comparisons of colony-forming unit at different time periods

Groups	*P*		
	1st day	7th day	10th day
Group A versus Group B	1.00	1.00	1.00
Group A versus Group C	0.194	0.169	0.49
Group A versus Group D	0.05*	0.01*	0.01*
Group B versus Group A	1.00	1.00	1.00
Group B versus Group C	0.07	0.147	0.182
Group B versus Group D	0.003*	0.01*	0.002*

*Statistically significant

Table 4: *Post hoc* analysis of intragroup comparisons of colony-forming unit at different time periods

Days	Group A		Group B		Group C		Group D	
	MD±SD (10^3 CFU/mL)	*P*	MD±SD (10^3 CFU/mL)	*P*	MD±SD (10^3 CFU/mL)	*P*	MD±SD (10^3 CFU/mL)	*P*
1st versus 7th day	3.84±0.7	<0.001*	3.54±0.74	<0.001*	2.86±0.27	<0.001*	2.26±0.71	0.002*
7th versus 10th day	0.44±0.15	0.003*	0.48±0.22	0.009*	3.12±0.65	<0.001*	4.92±2.05	0.006*
1st versus 10th day	4.28±0.8	<0.001*	4.02±0.73	<0.001*	5.98±0.64	<0.001*	7.18±2.16	0.002*

*Statistically significant. SD: Standard deviation; CFU: Colony-forming unit; MD: Mean difference

clindamycin, and metronidazole but were sensitive to ciprofloxacin and amoxicillin–potassium clavulanate (Augmentin). Pinheiro *et al*.[19] tested the susceptibility of *E. faecalis* to multiple antibiotics, including amoxicillin, amoxicillin–clavulanic acid, vancomycin, and ciprofloxacin, among others and found that *E. faecalis* isolates were completely susceptible to amoxicillin–clavulanic acid. Therefore, in the present study, ACP was selected as the antibiotic of choice for use as an intracanal medicament against *E. faecalis.*

AlSaeed *et al.*[20] used the Epsilometer test method to determine the antimicrobial efficacy of TAP, ACP, and tigecycline using a slow-release hydrogel scaffold as a vehicle against several bacteria, including *Fusobacterium nucleatum, Porphyromonas gingivalis, Staphylococcus intermedius, and E. faecalis.* They found that all the tested antibiotics showed equal and effective bacterial growth inhibition at a concentration of 1 mg/mL. Therefore, in the present study, both TAP and ACP were utilized at a concentration of 1 mg/mL.

The use of single-rooted human permanent teeth was preferred due to their ease of availability and their suitability for fulfilling the objectives of the study. In this study, the modified Haapasalo and Orstavik[16] model was used to assess the effectiveness of the intracanal medication. To assess the penetration of intracanal medicaments into the dentin from the entire root canal space, standard 6-mm markings were made from the coronal and apical portions of all tooth samples and then cut them using a diamond disk. The pulpal tissue remaining in the root canals was removed by treating the samples with NaOCl. The removal of hard-tissue debris was achieved by treating the samples with EDTA, which also enhanced the effectiveness of NaOCl.[16]

Estrela *et al.*[21] suggested that three factors should be considered when validating biofilm models, including the biological indicator, bacterial colonization structure, and the time required for biofilm development. The root canal models in this study fulfilled all of these objectives. The biological marker used in this study was *E. faecalis*, which is a common endodontic pathogen known for its multiple virulence characteristics.

The human root canal, with its own unique microenvironment, was utilized as the bacterial colonization structure in our study. The tooth substrate was selected as the surface for biofilm formation since the biofilm-forming capacity and structural organization of *E. faecalis* are known to be influenced by the chemical nature of the substrate. Therefore, using the tooth substrate, the biofilm model developed in this study mimics the natural conditions of an infected root canal more accurately.[7]

The infection period was set at 21 days to allow sufficient time for the biofilm to form and mature, which has been suggested as an appropriate duration for biofilm development in the previous studies.[7] This duration was chosen as it has been suggested that biofilms formed after 21 days are less susceptible to antibiotics.[7]

Propylene glycol exhibits hygroscopic properties, enabling the absorption of water and facilitating a sustained release of intracanal medicaments over extended periods of time.[22] Mandal *et al*.[6] conducted a study to assess the impact of various vehicles on the antimicrobial effectiveness of TAP in root canals infected with *E. faecalis*. Their findings indicated that the propylene glycol group combined with TAP demonstrated the highest efficacy in eliminating *E. faecalis*.[6] Hence, propylene glycol was chosen as the preferred vehicle for preparing the antibiotic paste.

TAP is a widely used intracanal medicament for treating persistent endodontic infections in permanent teeth, and therefore, it was selected as one of the medicaments in the present study. However, a major drawback of this paste is the observed discoloration, which can be attributed to the presence of minocycline in the formulation.[12] To address the limitations associated with the lack of a combined drug formulation and discoloration, ACP was utilized as an alternative in this study.

ACP has emerged as a potential alternative intracanal medicament for endodontic treatment due to its antimicrobial activity against commonly found bacterial strains in endodontic infections.[18] Its efficacy in controlling infections within the root canal system may contribute to successful endodontic treatment. Furthermore, the commercial availability of ACP makes it a convenient option. However, it is important to note that the clinical use of ACP as an intracanal medicament is an active area of research, and further studies are necessary to fully assess its effectiveness in clinical settings.

To fully understand the potential benefits and drawbacks of using TAP and ACP as intracanal medicaments in

endodontic treatment, further studies are necessary. These studies should assess the antimicrobial efficacy of both medicaments against other oral pathogens, as well as compare their clinical outcomes, patient-reported outcomes, and cost-effectiveness. Long-term follow-up studies would also be beneficial in assessing the durability and longevity of treatment outcomes. While TAP can be obtained separately, mixed, and stored for a limited period of time,[23] the storage of ACP does not have established evidence. Therefore, further research is needed to determine the shelf life of ACP and provide guidance on its storage recommendations. In addition, it is important to investigate potential adverse effects, stability, and the development of bacterial resistance with the use of ACP and other antibiotic-containing medicaments. Such research can help clinicians make informed decisions about the appropriate use of antibiotics in endodontic treatment and minimize the risk of developing antibiotic resistance.

CONCLUSION

Within the scope of the study's limitations, the results of this *in vitro* study indicated that Groups A and B demonstrated effective inhibition of bacterial growth on the 1st, 7th, and 10th day. However, there was no statistically significant difference between the two groups. Hence, ACP can be regarded as a viable alternative to TAP for endodontic treatment, demonstrating its effectiveness as an intracanal medicament. Further clinical studies with longer follow-up periods are needed to validate these findings.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, and financial or non-financial in this article.

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