

# Comparative evaluation of antimicrobial efficacy of triple antibiotic paste, calcium hydroxide, and a proton pump inhibitor against resistant root canal pathogens

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## ABSTRACT

**Objective:** The objective of this study is to compare the antimicrobial efficacy of triple antibiotic paste (TAP) and a proton pump inhibitor (PPI) (omeprazole) in combination with calcium hydroxide (CH) against *Enterococcus faecalis* and *Candida albicans*. **Materials and Methods:** *E. faecalis* and *C. albicans* were subcultured and inoculated at 37° overnight and were treated with different dilutions of TAP, 25 µg/ml (Group 1), CH (Group 2, control), CH 16 mg/ml + omeprazole 2 mg/ml (Group 3a) (CH 16 mg/ml + omeprazole 4 mg/ml (Group 3b) for 24, 48, and 72 h in sterile uncoated 96-well microtiter plates. Minimum concentration at which the medicaments produced least optical density was determined using ELISA reader (ELx 808 BioTek Inc., USA) device set at optical density of 630 nm. Results were analyzed statistically by one-way analysis of variance followed by Tukey's multiple comparison tests. The significance level was set at 0.05. **Results:** Mean concentration (irrespective of time) for TAP at which mean minimum optical density was recorded at 1.25 µg/ml (1:20 dilution) and 25 µg/ml (0 dilution) against *E. faecalis* and *C. albicans*, respectively. Least optical density for CH plus PPI group was obtained 1.6 µg/ml (1:10 dilution) and 16 µg/ml (0 dilution) for *E. faecalis* and *C. albicans*, respectively. However, CH alone showed a weaker antimicrobial action against either of the strains even at full concentration. **Conclusions:** PPI enhanced the antibacterial efficacy of CH against *E. faecalis* and *C. albicans*. However, TAP showed the best antibacterial property followed by CH plus PPIs against both the selected strains.

**Key words:** *Candida albicans*, *Enterococcus faecalis*, intracanal medicament

## INTRODUCTION

Bacterial microflora of primary endodontic infections differs vastly from secondary or persistent periradicular lesions. The most frequent survivors present in a very high proportion of root canal failure cases are *Enterococcus faecalis*, Gram-positive,

and facultative anaerobe with the prevalence of 24%–77%.<sup>[1,2]</sup> It has an inherent ability to invade deep inside dentinal tubules, survive periods of starvation, and withstand extremes of pH of medicaments such

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as calcium hydroxide (CH).<sup>[3,4]</sup> Interestingly, this resistance to withstand the alkaline stress induced by CH is also shared with *Candida albicans*; another resistant pathogen in teeth with periradicular lesions with the prevalence of 6%–18%.<sup>[5,6]</sup>

Both bacteria and fungi express a proton pump in their plasma membrane for energy metabolism and maintenance of constant cytoplasmic pH<sup>[7,8]</sup> that enables *E. faecalis* and *C. albicans* to maintain the homeostasis of the cytoplasm and survive the high alkaline pH of CH.<sup>[9,10]</sup> Moreover, dentin neutralizes the high pH of CH by exerting a buffering effect.<sup>[11]</sup>

Triple antibiotic paste (TAP) comprising ciprofloxacin, metronidazole, and minocycline has the potential to eradicate microbes residing deep inside the dentinal tubules.<sup>[12]</sup> However, its use TAP can cause discoloration of teeth due to minocycline present in it.<sup>[13]</sup> Another primary concern is resistance of bacteria to antibiotics and cytotoxic effects of TAP to stem cells and periradicular tissues when used at higher concentrations.<sup>[14,15]</sup>

Recently, proton pump inhibitors (PPIs) were proposed as an adjuvant to intracanal medicaments. These cause an irreversible inhibition of the H<sup>+</sup>/K<sup>+</sup> ATPase in the parietal cells of the stomach leading to reduced gastric acid secretion, and thus are a drug of choice for gastric/duodenal ulcers and gastroesophageal reflux diseases. They increase the efficacy of antibiotics by decreasing the intragastric acidity.<sup>[16]</sup> PPIs have also been found to exert antioxidant properties by directly affecting the neutrophils, monocytes, endothelial, and epithelial cells.<sup>[17]</sup>

Although the role of proton pump in the survival of resistant endodontic pathogens is known, a limited research is carried out to evaluate the antibacterial efficacy of PPIs. The aim of the present study was thus to evaluate the effect of the addition of omeprazole on antimicrobial efficacy of CH against *E. faecalis* and *C. albicans* in comparison to that of TAP.

## MATERIALS AND METHODS

### Bacterial strains and media

*E. faecalis*, American type culture collection (ATCC 29212) and *C. albicans* (ATCC 90028) were subcultured on sheep blood agar and Sabouraud dextrose agar plates, respectively, and incubated aerobically at 37° overnight.

### Preparation of medicament working strength solutions

Ciprofloxacin, metronidazole, and minocycline for TAP, CH, and omeprazole were obtained in powder form (Sigma-Aldrich; Mumbai, Maharashtra, India). CH was dissolved in distilled water at room temperature to prepare a saturated solution, centrifuged at 3000 rpm for 15 min, and the aqueous supernatant layer was filter sterilized using a sterile 25-mm syringe filter. Working strength solution of each medicament group was prepared by diluting them with distilled water and final concentration tested for antibacterial efficacy was as follows:

- Group 1: (TAP)-25 µg/ml
- Group 2: (CH)-16 mg/ml
- Group 3a: (CH 16 mg + omeprazole 2 mg)/ml
- Group 3b: (CH 16 mg/ml + omeprazole 4 mg)/ml.

### Preparation of 0.5 McFarland

The density of each strain was adjusted equal to that of 0.5 McFarland standard ( $1.5 \times 10^8$ ) CFU/ml after inoculating in BHI-YE broth supplemented with 5 g yeast extract/L and 5% v/v Vitamin K<sup>+</sup> hemin. McFarland was used as a reference to adjust the turbidity of microbial suspension.

Two-fold microdilution assay was performed on 96-well sterile uncoated microtiter plates (tarsons) as per The Clinical and Laboratory Standards Institute guidelines.<sup>[18]</sup> Strains were treated with 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, and 1:2560 dilutions of each medicament group for 24, 48, and 72 h. Bacterial growth appeared as turbidity or as a deposit of cells at the bottom of a well. The turbidities of the bacterial cultures of each well were measured using a microplate reader device (ELx 808 Biotek Inc., USA) set at 630 nm. All the readings were taken in triplicate, and the minimum mean concentration of the drug which produced least optical density for that group was recorded. Eleventh well was negative control, in which only broth was added without bacterial inoculums and 12<sup>th</sup> well as a positive control, free of antibiotics.

### Statistical analysis

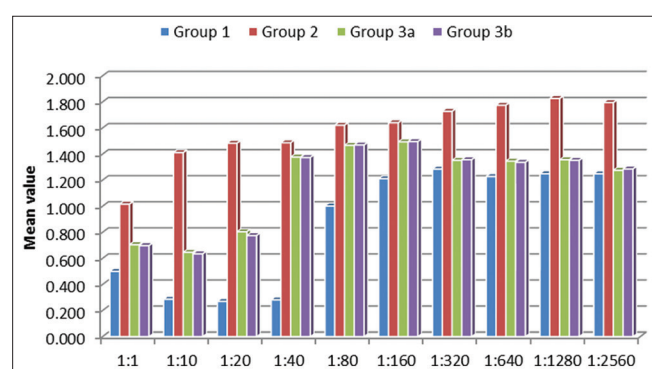
One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests was used to compare the optical density among the groups at different concentrations at 24, 48, and 72 h. The effect of time on the optical density of various groups was tested using two-way ANOVA and pairwise comparisons. The significance level was set at 0.05. All the analysis was carried out using SPSS version 16 (SPSS Inc., Chicago, IL, USA).

## RESULTS

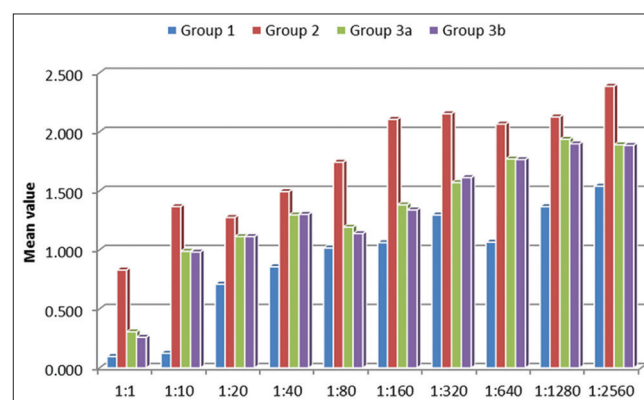
Mean concentration (in µg/ml) and dilution at which minimum optical density was recorded for each drug tested at various time intervals is depicted in Table 1.

ANOVA revealed a significant difference in the *E. faecalis* and *C. albicans* optical density at 24, 48, and 72 h among all the groups in different concentrations. When evaluated irrespective of time, mean minimum optical density for TAP was obtained at 1.25 µg/ml (1:20 dilution) and 25 µg/ml (0 dilution) against *E. faecalis* and *C. albicans*. Least optical density for CH plus PPI group was achieved 1.6 µg/ml (1:10 dilution) and 16 µg/ml (0 dilution) for *E. faecalis* and *C. albicans* [Table 2 and Figures 1, 2]. The pairwise comparison tests revealed that optical density for both strains was

significantly ( $P = 0.0001$ ) lower in Group 1 (TAP) than Group 2 (CH), Group 3a (CH + omeprazole 2 mg), and Group 3b (CH + omeprazole 4 mg) at all the concentrations but significantly higher ( $P = 0.0001$ ) in Group 2 (CH) compared to Group 3a (CH + omeprazole 2 mg) and Group 3b (CH + omeprazole 4 mg) at all the concentrations. There was no significant ( $P > 0.05$ ) difference in the optical density for both strains between Group 3a (CH + omeprazole 2 mg) and Group 3b (CH + omeprazole 4 mg) at all the concentrations. The two-way ANOVA revealed that there was a significant effect of time ( $P < 0.01$ ) at all the concentrations in the optical density for both the strains. The interaction of Group X Time was also found to be statistically significant ( $P < 0.05$ ) at all the concentrations in the optical density.



**Figure 1:** Mean  $\pm$  standard deviation of effect of dilutions on *Enterococcus faecalis* optical density among the groups in different concentrations irrespective of time



**Figure 2:** Mean  $\pm$  standard deviation of effect of dilutions on *Candida albicans* optical density among the groups in different concentrations irrespective of time

**Table 1: Mean concentration (µg/ml) and dilution at which minimum optical density was recorded for each drug tested at various time intervals**

	<i>E. faecalis</i>			<i>C. albicans</i>		
	24 h	48 h	72 h	24 h	48 h	72 h
TAP	1.25 µg/ml (1:20 dilution)	1.25 µg/ml (1:20 dilution)	1.25 µg/ml (1:20 dilution)	25 µg/ml (0 dilution)	2.5 µg/ml (1:10 dilution)	1.25 µg/ml (1:20 dilution)
CH	1.6 µg/ml (1:10 dilution)	1.6 µg/ml (1:10 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)	0.8 µg/ml (1:20 dilution)
CH + omeprazole 2 mg	0.8 µg/ml (1:20 dilution)	1.6 µg/ml (1:10 dilution)	1.6 µg/ml (1:10 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)
CH + omeprazole 4 mg	1.6 µg/ml (1:10 dilution)	1.6 µg/ml (1:10 dilution)	1.6 µg/ml (1:10 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)

*E. faecalis*: *Enterococcus faecalis*, *C. albicans*: *Candida albicans*, TAP: Triple antibiotic paste, CH: Calcium hydroxide

**Table 2: Overall mean concentration (µg/ml) and dilution at which minimum optical density was recorded for each drug tested irrespective of time**

Microbial strains	Drug tested			
	TAP	CH	CH + omeprazole 2 mg	CH + omeprazole 4 mg
<i>E. faecalis</i>	1.25 µg/ml (1:20 dilution)	16 µg/ml (0 dilution)	1.6 µg/ml (1:10 dilution)	1.6 µg/ml (1:10 dilution)
<i>C. albicans</i>	25 µg/ml (0 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)	16 µg/ml (0 dilution)

*E. faecalis*: *Enterococcus faecalis*, *C. albicans*: *Candida albicans*, TAP: Triple antibiotic paste, CH: Calcium hydroxide

## DISCUSSION

Microtiter plate method was adopted considering the usual contamination and difficulty in obtaining bacterial samples from the inherent complexities of root canal system. In addition, it is a quantitative and reproducible method that simulates the contact of the test microorganism with endodontic medicaments inside the root canal. Furthermore, the accuracy and feasibility of this approach substantially reduce the waiting time involved in standard laboratory antibacterial sensitivity assays.

The results suggest that CH alone was not as effective against the tested strains compared to other groups. Visible bacterial growth was observed in all the wells at 24, 48, and 72 h and minimum optical density was obtained only at full concentration. CH owes its antimicrobial property mainly to its high pH (>11) but it is practically difficult to achieve this high pH throughout the length of the canal because of the buffering capacity of dentin, especially in the dentinal tubules.<sup>[19]</sup> Furthermore, the presence of proton pump in its cell membrane *E. faecalis* sustains the alkaline effect of CH dressing by allowing pumping of the protons into the cytoplasm and hence lowering the pH.<sup>[10,20]</sup> Tang *et al.* listed the ability of microorganisms to survive in dentinal tubule ramifications, fall in pH, and microleakage of the temporary filling as some of the limitations of CH as an intracanal medicament.<sup>[21]</sup>

TAP delivered statistically significant inhibition of *E. faecalis* and *C. albicans* at 24, 48, and 72 h. TAP achieved least optical density at 1:20 dilution (1.25 µg/ml) against *E. faecalis* at all time intervals; however, for *Candida*, maximum inhibition of growth was seen only at highest concentration, i.e., 25 µg/ml at 24 h, 1:10 dilution (2.5 µg/ml) at 48 h, and 1:20 dilution (1.25 µg/ml) at 72 h. Hoshino *et al.* concluded that a combination of ciprofloxacin, metronidazole, and minocycline at a concentration of 25 µg/ml each per milliliter of paste was able to sterilize infected root dentin *in vitro*.<sup>[12]</sup> Sato *et al.* reported 50 µg/ml of each antibiotic per milliliter was sufficient to sterilize infected root dentin *in situ*.<sup>[22]</sup> In this study, TAP was found to be effective even at high dilutions, indicating that low concentrations of antibiotics might be sufficient to obtain the required antibacterial effect.

The addition of omeprazole to CH showed synergism and achieved least optical density at 1:10 dilution (1.6 µg/ml) against *E. faecalis*; however, for *Candida*, it showed antifungal property only at maximum

concentration and variation in the concentration; whether 2 mg or 4 mg of omeprazole did not affect the result. This synergism may be due to the irreversible inhibition of proton pump in the cell membrane of the *E. faecalis* and *C. albicans*.<sup>[23]</sup>

Evans *et al.*<sup>[10]</sup> demonstrated that survival of *E. faecalis* in CH is unrelated to stress-induced protein synthesis, but more the result of a proton pump that pumps protons into the cell to acidify the cytoplasm. The present data are in accordance with a previous *in vivo* study which has revealed that, the combination of a PPI, omeprazole with CH when used as an intracanal medicament exhibited increased antimicrobial efficacy against *E. faecalis* and superior radiographic healing of periapical lesions with increase in reparative bone areas in male Wistar rats compared CH.<sup>[24]</sup> Apart from antibacterial properties, PPIs exert anti-inflammatory and pro-reparative effects which enhance the healing of the periapical area.<sup>[20]</sup>

Although the results of this study using PPIs as an adjuvant to intracanal medicament were promising, an *in vitro* agar diffusion assay demonstrated that the addition of PPI (Pantoprazole) to CH did not enhance the antimicrobial efficacy of CH when compared to chlorhexidine.<sup>[25]</sup> The variation in results may be due to the difference in the tested drugs and methodology used. The present experiment suggested that the PPIs were not very effective against *C. albicans* at the concentrations employed in the study. Omeprazole exhibits an “eagle” or ‘paradoxical effect,” i.e., its antifungal action is seen at a particular concentration but disappears at a higher or lower range.<sup>[26]</sup> One of the limitations of the present study was not using a biofilm model for testing *E. faecalis* which would have more closely simulated the clinical situation as it occurs mostly as extra- or intra-radicular plaque.

## CONCLUSIONS

Although in the present study, the best antibacterial property among the study groups was exhibited by TAP, followed by CH plus PPIs, the results also demonstrate the synergistic antimicrobial efficacy of CH and PPIs against *E. faecalis* and *C. albicans*. In addition, the results suggest that the TAP can be used at a lower concentration than it is being used to avoid the deleterious effects associated with higher concentrations.

However, in real-life situations, dentin exerts a buffering effect on endodontic disinfectants. Hence,



the antibacterial activity of PPIs along with CH as an intracanal medicament needs to be tested in the clinical scenario to establish it as a potential chemical adjunct to endodontic therapy.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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