Comparative Evaluation of Antifungal Activity of Octenidine: An *In Vitro* Confocal Laser Study

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ABSTRACT

Aim: The aim of this study was to evaluate the antifungal efficacy of a novel endodontic irrigant octenidine against conventional irrigants sodium hypochlorite and EDTA on *Candida albicans* growth in the young and old population by calculating the number of colonies formed and by qualitative identification of dead/viable fungi by Confocal laser scanning microscopy (CLSM) method.

Materials and methods: The total number of samples used in the study was eighty samples after decoronation of the crown portion the middle third of root canal. Each group was subdivided into four subgroups with various irrigation protocols: (A) 17% EDTA + 5.25% NaOCl, (B) 100% Octenisept, (C) 17% EDTA + 5.25% NaOCl + 1% clotrimazole, and (D) phosphate buffer saline. After completion of irrigation ATCC samples (90028) of *C. albicans* were inoculated with 5 mL of peptone water each and incubated at 37°C for 72 hours to attain the turbidity corresponding to 0.5 McFarland standards CFU. Eight samples were analyzed for the formation of candidal colonies, and two samples for the assessment of viability of *Candida* by confocal laser scanning microscope in each subgroup.

Results: Comparison of antifungal efficacy of endodontic irrigants employed in the young and old populations revealed a significant reduction in the mean values of CFU and the mean values of percentage of non-viable microorganism by CLSM method. A positive relationship was revealed in the younger population which had a better antifungal efficacy than the older population in all the irrigant subgroups evaluated in the study.

Conclusion: All the endodontic irrigants employed in our study had a good antifungal efficacy against *Candida albicans*. Octenisept had a maximum antifungal efficacy, while phosphate saline showed the least efficacy in both age groups, which was quantitatively evaluated by CFU method, and the same was confirmed through qualitative evaluation by CLSM method.

Clinical significance: *Candida albicans* plays a major role in the establishment and pathogenesis of failed root canal treatment. Age-related changes alter the adhesion potential of dentin, in turn influence the outcome of endodontic therapy. Octenidine, a novel antifungal agent, can be substituted over the conventionally used EDTA and NaOCl with less adverse effects.

Keywords: Antifungal activity, *Candida albicans*, Confocal laser scanning microscope, Endodontic irrigants.

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INTRODUCTION

Microbial agents play a primary role in the establishment of pulp and periradicular diseases.¹ The incidence of endodontic failure possesses an enigma to the endodontist, predominantly due to the diversity of the microbial species involved in the secondary infections.² The mechanical instrumentation helps to debulk the microbial load and to combat the microbial challenge created by the residual microbes in the failed root canal cases, which has tended us to use antifungal agents as endodontic irrigants.³ *Candida albicans* is a dentinophilic yeast which is reported in 10–14 percentages of secondary infections. The unique features are its adaptive capacity toward survival in the absence of nutrients and in wide range of pH.⁴ This along with penetration into the dentinal tubule and capacity to form hyphae and blastospores helps in the progression of the infection.⁵

Sodium hypochlorite has been predominantly used because of its antimicrobial, sporicidal, fungicidal, and tissue-dissolving properties which act on the organic components of the dentin substrate.⁶ Its usage has to be confined within the root canal system and can cause hypochlorite accident if it is used without adequate precautions.⁷ EDTA was first introduced to the endodontic literature by Nygaard-Ostby. The mechanism of action is predominantly on the inorganic component of dentin, and it reacts with calcium ions in dentin and forms soluble calcium chelate, which reduces the availability of calcium ions essential for the pathogenesis of candidal infections.⁸

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Clotrimazole is an antifungal agent with a broad spectrum of action. Chandra et al. has used clotrimazole as an additional

antifungal agent and found that it exerts a synergistic action along with routine irrigation protocols employed for disinfection against *Candida*.⁹ Octenisept has been employed as an antiseptic for skin burns and wound disinfection. Octenidine hydrochloride has been suggested as a potential endodontic irrigant based on its antimicrobial effects and low cytotoxicity. It has a broad spectrum of activity against fungi and viruses, which has not been explored. This has tended us to select it as the primary endodontic irrigant.¹⁰

Age-related changes alter the adhesion capability of dentin which influences the outcome of endodontic therapy. *Candida albicans* has preponderance to get attached to the radicular dentin and exerts a collagenolytic action which affects the prognosis of treatment.¹¹

The usage of the novel irrigant octenidine in the extreme age groups has not been evaluated in the literature previously.

We in this study evaluated the antifungal efficacy of the novel endodontic irrigant octenidine against conventional irrigants sodium hypochlorite and EDTA on *Candida albicans* in the young and old populations by calculating the number of colonies formed and by qualitative identification of dead/viable fungi by CLSM method.

MATERIALS AND METHODS

The ethical clearance for the study was obtained from the IRB and ethics committee of APDCH. The total number of tooth samples included in the study was 80, of which forty tooth samples were collected from young population aged between 12 years and 25 years, which were extracted for orthodontic reasons, and the remaining forty tooth specimens [40] were collected from older population aged above 60 years, which were extracted for periodontal and prosthodontic reasons. The samples were obtained from the Oral Surgery Department, Adhiparasakthi Dental College and Hospital, Melmaruvathur.

Sample Preparation

The teeth were cleaned and subjected to thermocycling, which were then stored in artificial saliva procured from laboratory for a week to simulate the oral environment. Decoronation was done, and the remaining portion was subjected to pulp extirpation with broaches. A 25-mm, size 15 K-File was used to determine the working length, and the orifice was enlarged with Gates Glidden drill size 2 and the apical preparation was done until ISO size 50. The irrigants employed in the study were subdivided into four subgroups with 10 samples for each subgroup, and the following irrigation protocols were employed: [A] 2 mL of 17% EDTA for one min and 2 mL of 5.25% NaOCl for one min, [B] 2 mL of Octenisept for one min, and [C] 2 mL of 17% EDTA for one min and 2 mL of 5.25% NaOCl for one min. The middle third of the root canal was sectioned and employed for the study. Microbial aspects of the study were done at the Department of Microbiology, MAPIMS, Melmaruvathur.

The ingredients of the prepared Sabouraud dextrose broth include [1] special peptone 10 g/L, and [2] dextrose 40 g/L, the pH of which is adjusted to 5.6. A 6.5 g agar was weighed and dissolved in 100 mL of distilled water in a conical flask. The conical flask was plugged with cotton and sterilized in autoclave at 121°C for 15 minutes at 15 lbs pressure. The sterilized Sabouraud dextrose agar was poured into petri dishes to a depth of 5 mm under the laminar flow chamber. A quantity of 100 mL of medium was used to pour six culture plates. The poured plates were allowed to solidify and were refrigerated.

Inoculation of Samples

Dentin shavings were collected with Gates Glidden burs [0.9 mm], and the dislodgement of samples was done using sonics. The dentin shavings collected were inoculated with 0.3 mL of the adjusted *C. albicans* suspension. *Candida albicans* (ATCC 90028) was the test organism chosen in this investigative study and was procured from Dr. Lal Microbiological Culture Lab in Chennai. The inoculums with dentin scrapings were kept in the incubator for 72 hours. The replenishment of samples was done at regular periodicity of one day with fresh candidal suspension broth. After a two-day period, the aliquots were taken using a syringe and transferred using a one-micron inoculation loop on a 4% Sabouraud dextrose agar plate for verification of *Candida albicans* growth, and the mean and standard deviation were calculated for the number of colony-forming units.

Dyes and Kits

Samples preparation was done using the hard tissue microtome, SYTO9, and propidium iodide (PI) staining agents employed for CLSM evaluation.

Colony-forming Unit Evaluation

Among the ten dentin disks in every subgroup eight disks were quantitatively assessed for formation of colonies (CFU).

Confocal Laser Evaluation

Each cross section was examined under a confocal laser scanning microscope and was recorded at 63× magnification employing fluorescent mode with a laser illumination source. Two samples in each subgroup were assessed qualitatively to detect the viability, that is the presence of live or dead fungi in the dentinal tubules.

RESULTS

Comparison of the antifungal efficacy of the endodontic irrigants employed in the young and old populations revealed a significant reduction in the mean values of CFU. A positive relationship was revealed between the mean values of CFU, while more reduction was revealed in the younger population than in the older population in all irrigant subgroups evaluated in the study.

In this study one-way ANOVA followed by Tukey's HSD test showed statistically significant difference among various subgroups concerning the discrepancy in the colony-forming units in each group. Unpaired *t*-test showed a significant difference in the number of colony-forming units between the groups.

Comparison of Antifungal Efficacy by CFU Method

Octenidine subgroup had a highest reduction in CFU with mean values of 9.38 and 38.75 in the young and old populations, respectively, and was the most efficient one (Fig. 1 and Table 1). Among the other irrigants evaluated phosphate saline subgroup had the least reduction in CFU with mean values of 570.63 and 997.25 in the young and old populations, respectively, and hence was the least efficient one (Fig. 2 and Table 1).

Clotrimazole subgroup had a significantly better reduction with mean values of 14.88 and 51.13 in the young and old populations, respectively (Table 1), compared to sodium hypochlorite/EDTA subgroup with mean values of 62.25 and 185.75 in the young and old populations, respectively [(Table 1). There was a positive but nonsignificant relation between octenidine and clotrimazole

Figs 1A and B: CFU image of octenidine group

Figs 2A and B: CFU image of phosphate saline group

Table 1: Comparison of CFU between the groups

Groups	Young-I (mean $\pm$ SD)	Old-II (mean $\pm$ SD)
I 17% EDTA + 5.25% NaOCl	62.25 $\pm$ 8.73	185.75 $\pm$ 16.36
II Octenisept	9.38 $\pm$ 3.02	38.75 $\pm$ 6.23
III 17% EDTA + 5.25% NaOCl + 1% Clotrimazole	14.88 $\pm$ 4.49	51.13 $\pm$ 10.87
IV Phosphate buffer saline	570.63 $\pm$ 33.02	997.25 $\pm$ 74.66
p value	0.000	0.000

subgroups with *p* values of 0.919 and 0.918 in the young and old groups, respectively, suggesting both irrigant combinations were equally effective and are represented in Table 2.

Confocal Laser Results Analysis

The viability of microbes read as red and green voxels for dead and live organisms, respectively, and the mean values of percentage of non-viable microorganism were calculated employing special software.

Octenidine subgroup had the highest reduction with the presence of 17% and 15% of non-viable microorganism in the young and old populations, respectively, and was the most efficient one (Fig. 3 and Table 3). Among the other irrigants evaluated phosphate saline subgroup had the least reduction with the presence of 7% and 5% of non-viable microorganism in the young and old populations, respectively, and was the least efficient one (Fig. 4 and Table 3).

Clotrimazole subgroup had a significantly better reduction with the presence of 12% and 10% of non-viable microorganism in the young and old populations, respectively (Table 3), compared to sodium hypochlorite/EDTA subgroup with the presence of 8% and 6% of non-viable microorganism in the young and old populations, respectively (Table 3).

There was a positive but nonsignificant relation between octenidine and clotrimazole subgroups with *p* values of 0.719 and 0.710 in the young and old groups, respectively, suggesting both irrigant combinations were equally effective as represented in (Table 4).

DISCUSSION

Residual microbes with diverse endodontic flora lead to compromised outcome of root canal therapy and require

Table 2: Significance of fungal growth among the four irrigants

Groups	Young-I (<i>p</i> value)	Old-II (<i>p</i> value)
I vs II	0.000	0.000
I vs III	0.000	0.000
I vs IV	0.000	0.000
II vs III	0.919	0.917
II vs IV	0.000	0.000
III vs IV	0.000	0.000

employment of novel strategies in management. The unique features of the above study were to compare the antifungal potency of the newer endodontic irrigant Octenisept against *Candida* organism with exploration of its significance in different age groups. This study also employed a novel evaluation method to directly estimate the viability of organism.

This comparative study revealed a significant variation among the mean values of CFU and the percentage of non-viable microorganism remaining after the irrigation protocols of all the four tested irrigants. There was no statistically significant difference between octenidine and clotrimazole in both age groups, suggesting both irrigant combinations were equally effective. Clotrimazole greatly augmented the antifungal effect against *Candida albicans* in both age groups as it interferes with the synthesis of ergosterol causing disruption of the cell membrane. Baumgartner et al. revealed *E. faecalis* and *Candida albicans* as the most common organisms associated with root canal failure.¹²

Octenidine subgroup had the highest reduction in CFU with mean values of 9.38 in young population and 38.75 in old population. A higher amount of calcium present in the old age group contributes to the stabilization of bond established between the *Candida albicans* and dentin.¹³ The dimension of fungi was found to be smaller than the size of obliterated dentinal tubules associated with aging. The surface molecules with invasive affinity mediates the adherence of *Candida* to the collagen component of dentin.¹⁴

The sodium hypochlorite/EDTA subgroup with mean values of 62.25 and 185.75 in the young and old age groups, respectively, ranks third with the presence of 8% of non-viable microorganism in the young age group and 6% in the older age group. The high pH of sodium hypochlorite along with hydroxyl ions generation interferes with the integrity of cytoplasmic membrane causing irreversible damage to the cell wall.¹⁵ Ates et al. revealed that 17% EDTA had anti-colonization and anti-collagenolytic properties against *C. albicans*, which prevents its binding and reduces the

Figs 3A and B: CLSM image of octenidine group at 63× magnification

Figs 4A and B: CLSM image of phosphate saline group at 63× magnification

Table 3: Yeast viability of the two groups

Groups	Young-I (mean ± SD) (%)	Old-II (mean ± SD) (%)
I 17% EDTA + 5.25% NaOCl	8	6
II Octenisept	17	15
III 17% EDTA + 5.25% NaOCl + 1% Clotrimazole	12	10
IV Phosphate buffer saline	7	5
p value	0.000	0.000

Table 4: Significance of yeast viability among the four irrigants

Groups	Young-I (p value)	Old-II (p value)
I vs II	0.000	0.000
I vs III	0.000	0.000
I vs IV	0.000	0.000
II vs III	0.719	0.710
II vs IV	0.000	0.000
III vs IV	0.000	0.000

growth by removing calcium through chelation from the cell walls and aids in collapse of the cell wall.¹⁶

Techniques available for the quantitative evaluation of bacterial flora includes histologic sections, scanning electron microscope, and microbiological assays. Microbiological sampling techniques estimate the number of colony-forming units they do read on spatial distribution of bacteria in tubules. The histologic sections show the distribution of the bacteria in the infected dentin, but lack the details of viability.

In consideration of the above facts confocal laser scanning microscopy (CLSM) becomes important because it is a suitable method for the identification of live and dead bacteria inside the tubules to analyze the viability profile, architecture, and spatial distribution. We in this study employed the CLSM for the qualitative evaluation of bacterial flora, and according to Vitkov et al., SYTO9 probe [473 nm] was used for the evaluation of live bacteria and propidium iodide probe [559 nm] for the evaluation of dead bacteria as it penetrates the damaged plasma membrane. The contact time for dye was kept as 5 minutes at 1:1 ratio.

The stains were assessed for fluorescence, and the images were captured by special software at a resolution of 512 pixels² and with a zoom factor of 1.0, giving a final pixel resolution of 0.41 mm/pixel.¹⁷ The antifungal efficacy of octenidine might be related to the fact that it resists the challenges created by organic and inorganic inhibitory factors in the root canal system.¹⁷ It maintains its antimicrobial efficacy in the presence of organic material in the biologically complex environment and exerts a substantive effect on dentin.¹⁸ This was in accordance with Tirali et al. who demonstrated that 100% Octenisept was more effective than 5.25% NaOCl as an antimicrobial endodontic irrigant.^{19–22}

Tandjung et al. investigated the biocompatibility and safety aspects of Octenisept on *E. faecalis* and concluded that toxicity

parameters were all within the compliant limits. No carcinogenic or mutagenic effects have been reported, which has tended us to select it for our study.²³ Results obtained in the CFU method were reflected in the CLSM evaluation with the presence of more *C. albicans* in the older population as compared to the young population.

LIMITATIONS OF THE STUDY

The effectiveness of endodontic irrigants needs to be evaluated by clinical trials with protocols to assess the effect of inflammatory mediators on the establishment and progression of secondary infections. Furthermore, there is no direct method to assess the antifungal effect and we rely on the indirect methods to assess the clinical scenario.

CONCLUSION

Within the limitations of our study it can be concluded that all the endodontic irrigants employed in our study had a good antifungal efficacy against *Candida albicans*.

Octenisept had a maximum antifungal efficacy, while phosphate saline showed the least efficacy in both age groups, which was quantitatively evaluated by the CFU method, and the same was confirmed through the qualitative evaluation by CLSM method.

In future, studies need to be concentrated to find out the antimicrobial effectiveness in clinical trials as it has better validity and reliability.

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