Comparative Study of Antifungal Efficacy of Various Endodontic Irrigants with and without Clotrimazole in Extracted Teeth Inoculated with *Candida albicans*

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**ABSTRACT**

Aim and objective: To assess the application of clotrimazole (1%) as a complementary antifungal agent along with sodium hypochlorite (5.25%), chlorhexidine gluconate (2%), and doxycycline hydrochloride (5%) against *Candida albicans*.

Materials and methods: Seventy freshly extracted single-rooted premolars with matured apices were collected, stored, and handled according to the Occupational Safety and Health Administration (OSHA) and the Center for Disease Control and Prevention (CDC) guidelines and recommendations. These were divided into three groups (two tests and one control group) depending on irrigants used. The efficacy of each irrigant group was compared. The observations were statistically analyzed by the multiple intergroup comparisons using ANOVA and Scheffe multiple comparisons (p < 0.001).

Results: The sodium hypochlorite (group IA—mean 129.6) has shown a statistically significant decrease in colony-forming units (CFUs) (p < 0.01) on comparison with chlorhexidine ([IB] mean 190.2). A similar result was obtained in comparison with the sodium hypochlorite group (IA) and doxycycline HCl group ([IC] mean 318.4) and also between the sodium hypochlorite group (IA) and the control group ([III] mean 554.2). The intragroup comparison of group II, group IIA (mean 63.3), and group IIB (mean 73.8) showed no statistically significant difference. Group III (mean 554.2) was the least effective of all the subgroups.

Conclusion: Sodium hypochlorite showed better antifungal efficacy than chlorhexidine and doxycycline when used alone. The addition of clotrimazole increased the efficiency of doxycycline also, but it was less compared to sodium hypochlorite and chlorhexidine. Within the limitations of this study, the inclusion of 1% clotrimazole increased the antifungal efficacy of all the three irrigants.

Clinical significance: Our study compared the efficacy of the various endodontic irrigants and also determined their efficiency with the addition of the antifungal agent. Clotrimazole (1%) addition in irrigating solutions showed better results and promoted faster healing.

Keywords: Chlorhexidine, Clotrimazole, Doxycycline, Endodontic irrigants, Sodium hypochlorite.

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**INTRODUCTION**

Endodontic therapy aimed to eliminate the microorganisms from the root canal and to prevent reinfection. The continuing or persisting infection or the reinfection caused by the bacteria may sustain or induce the apical periodontitis. The bacteria can infect the dental pulp mainly by following routes, i.e., through the open carious lesions, gingival sulcus, bloodstream, dentinal tubules, and marginal leakage from the broken restorations or due to postendodontic faulty restoration. The endodontic treatment seals these routes to avoid the contamination and thereby ensure a successful treatment.¹

The endodontic infections are generally polymicrobial and contain anaerobic species.¹ The Kakehashi et al. study showed the bacteria as one of the causes for the pulpal disease.² But other investigations have also shown that fungi and viruses play a probable role in the causation of endodontic infections. Studies also revealed the failure of root canal treatment is related to actinomycoses, *Candida albicans*, and *Enterococcus faecalis*.¹ ³ Fungi are present in the oral cavity of 30–45% of healthy adults and 95% of human immunodeficiency virus patients.⁴ ⁶ It is confirmed that fungi are also present in caries, plaque, dentinal tubules, and infected root canals as well as in subgingival flora. *Candida* is found in approximately 1–17% of cases of infected root canals.⁶ ⁸ The study of Rocas et al. showed the presence of *C. albicans* and *E. faecalis* around 6% and 47%, respectively, in root canal treated teeth with posttreatment apical periodontitis. This finding also
established a correlation between secondary infection and the posttreatment apical periodontitis.

There has been little attention given to the antifungal activity of disinfectants for cleaning the canal. The cleaning prevents reinfection and it is the need of the hour to avoid root canal failures related to fungal activity. In this context, antifungal properties of various irrigating solutions and endodontic disinfectants have been explored including sodium hypochlorite (NaOCl), calcium hydroxide (CaOH), and chlorhexidine gluconate (CHX). 9–12 0.5–5.25% NaOCl is extensively used as a root canal irrigant in endodontics. Previous studies demonstrated substantial antifungal properties of ethylenediaminetetraacetic acid (EDTA). 13,14 However, contrary to the literature, no specific single antifungal agent is used at the moment for the irrigation of the infected root canals.

Clotrimazole is an imidazole and commonly used for treating the systemic mycoses as an antifungal agent in both medical as well as dental practice. Ketoconazole, econazole, and miconazole are the other antifungal agents that come under the same group. These antifungal agents have a wide range of activity against Candida, Dermatophytes, Streptococcus faecalis, and Staphylococcus aureus. 15 These are widely used in diseases like oral, otomycosis, cutaneous, as well as vaginal candidiasis. 15 Thereby, the spectrum of antimicrobial activity of endodontic irrigants should also include the antifungal medications to assist in the successful management of secondary or persistent endodontic infections caused by the fungi. 16,17 So, the present study aims to examine the efficiency of 1% clotrimazole as a complementary antifungal agent along with 5.25% NaOCl, 2% chlorhexidine gluconate, and 5% doxycycline hydrochloride against C. albicans.

**Materials and Methods**

The experiment was performed in the Department of Conservative Dentistry and Endodontics, Government Dental College, Thiruvananthapuram, and the DDRC Microbiology Centre, Thiruvananthapuram. Seventy single-rooted human permanent premolar teeth with matured apices extracted due to orthodontic treatment and periodontal disease were collected, stored, as well as handled according to the Occupational Safety and Health Administration (OSHA) and the Center for Disease Control and Prevention (CDC) guidelines and recommendations. The age range of the patients included in the study for the extracted teeth collection ranged from 17 to 60 years. The other inclusion criteria comprised of a fully matured (closed apex), noncarious single-rooted tooth with intact radicular portion without any resorption, i.e., internal, external, or apical.

The standard strain of C. albicans (MTCC3108) used in the study was obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh. The commercially available NaOCl (5.25%—DENTPRO), distilled water, EDTA (17%), sterile saline, chlorhexidine gluconate (2%—DENTOCHLOR), 5% doxycycline hydrochloride, clotrimazole (1%—Candid Mouth Paint, Glenmark, India) were used in the experiment. All irrigating solutions were stored in an autoclavable polypropylene container, in a cool, dry, and sterile field. Sabouraud dextrose agar—HIMEDIA was used as a microbiological media. The statistical analysis was done using the SPSS software by using ANOVA and Scheffe multiple comparisons (p < 0.001).

**Preparation and Mounting of the Samples**

The extracted teeth were prepared by overnight immersion in the NaOCl (to remove the surface and soft tissue debris), followed by removal of the coronal part of the tooth at the cementoenamel junction (CEJ) to obtain approximately 13 mm length of the root (Fig. 1). Further, the tooth apices were sealed with the glass ionomer cement and the remaining root surface was coated with a double layer of nail varnish to isolate the internal root canal environment. The tooth roots were cleaned and shaped to the ISO size of 50 K file by using hand files. The copious irrigation with NaOCl (5.25%) and EDTA (17%) was done for 5 minutes to remove the smear layer. Finally, each canal was flushed with NaOCl, dried up with the absorbent paper points, and stored in the distilled water until use. So, prepared root samples were mounted in four sterilized micro-tip polypropylene boxes (Tarsons, Kolkata) and sterilized using an autoclave at 121°C for 20 minutes at 1 psi pressure.

Further following steps were performed to complete the experiment:

First step—to assure the negativity of the specimen, all samples were injected and aspirated with 1 mL of sterile saline solution using an insulin syringe in the root canal. The aspirate was placed onto a Sabouraud 4% dextrose agar plate and incubated aerobically at 37°C for 2 days. The samples were stored under sterile conditions during the waiting period for culture results. After culture negativity, step 2 was followed.

Second step—the candidal suspension was inoculated in the root canal, keeping the teeth mounted in an upright position in the sterilized micro-tip boxes, and the samples were incubated in a BOD incubator for 96 hours.

Third step—for identification of the Candida growth, a small sample was taken from the canal of every tooth at 48 hours and was kept on a 4% Sabouraud dextrose agar plate to verify the growth of C. albicans. All the cultures were positive, confirming the growth of C. albicans within the root canals. After 96 hours, teeth were removed and the canal was cleaned with the help of sterile paper points and was randomly divided into groups I and II as test groups and group III as control based on the irrigants used. Groups I and II further divided into three subgroups as A, B, and C consisting of 10 teeth each. Group III served as a control group comprising of 10 teeth (Fig. 2).

**Group I (Only Irrigant Group)**

IA: Irrigated with 2 mL of 5.25% NaOCl.
IB: Irrigated with 2 mL of 2% CHX.
IC: Irrigated with 2 mL of 5% doxycycline hydrochloride.

**Group II (Irrigant with an Antifungal Group)**

IIA: Irrigated with 2 mL of 5.25% NaOCl and with 1% clotrimazole.
IIb: Irrigated with 2 mL of 2% CHX and with 1% clotrimazole.

Figs 1A and B: Samples: (A) Decoronated samples with nail varnish coating; (B) Teeth mounted on the microtip box
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IIC: Irrigated with 2 mL of 5% doxycycline hydrochloride and with 1% clotrimazole.

In groups I and II, all the samples were irrigated with respective irrigants for 1 minute and then flushed with 5 mL of distilled water to prevent the effect of irrigant. Following this, 2 mL of 1% clotrimazole was irrigated in all the samples of group II with the contact time of 1 minute, then flushed with 5 mL of distilled water to prevent the effect of irrigant.

**Group III (Control Group)**

The control group was irrigated with distilled water, and further flushing was performed using 15 mL of saline. The aliquots were removed from the fluid by using a 1 μm inoculation loop and were placed on the Sabouraud 4% dextrose agar plates incubated at 91% humidity and 36°C for 48 hours. After the incubation period, the colony-forming units (CFUs) of candida were taken as a measure of the antifungal activity of irrigants.

**Results**

The results obtained were statistically analyzed by the multiple intergroup comparisons using the SPSS software by using ANOVA and Scheffe multiple comparisons (p < 0.001). Sodium hypochlorite (group IA—mean 129.6) has shown a statistically significant decrease in CFU (p < 0.01) on comparison with chlorhexidine ([IB] mean 190.2). A similar result was obtained in comparison with the NaOCl group (IA) and the doxycycline HCl group ([IC] mean 318.4) and also between the NaOCl group (IA) and control Group ([III] mean 554.2).

The chlorhexidine group (IB) showed a statistically significant decrease in the CFU (p < 0.01) in comparison with the doxycycline HCl (IC) group; moreover, a similar result was obtained on comparison with the chlorhexidine group (IB) and the control group (III). Doxycycline HCl group (IC) showed a statistically significant decrease in CFU (p < 0.01) in comparison with the control group (Table 1 and Fig. 3). One-way ANOVA showed a statistically significant difference among group II subgroups and control (Table 2 and Fig. 4). No statistically significant results were obtained on comparison of NaOCl with clotrimazole group ([IIA] mean 63.3) and chlorhexidine with clotrimazole group ([IIB] mean 73.8).

However, NaOCl with the clotrimazole group (IIA) showed a statistically significant decrease in CFU (p < 0.01) in comparison with the hypochlorite and clotrimazole group (IIIA) with doxycycline HCl and clotrimazole group ([IIC] mean 250.9). A similar result was obtained when NaOCl with clotrimazole group (IIA) was compared with the control group (III). On comparison of doxycycline HCl and clotrimazole group (IIC) with the control group (III), doxycycline HCl and clotrimazole group (IIC) showed a statistically significant decrease in CFUs (p < 0.01) (Table 3 and Fig. 5).

**Table 1: Comparison of CFU within the group I and control**

| Group          | Mean | SD  | N  | F    | p Value |
|----------------|------|-----|----|------|---------|
| NaOCl (IA)     | 129.6| 8.2 | 10 | 595.03** | 0.000   |
| CHX (IB)       | 190.2| 12.5| 10 |      |         |
| Doxycycline HCl (IC) | 318.4| 15.8| 10 |      |         |
| Control        | 554.2| 43.6| 10 |      |         |

SD—standard deviation; ** significant 0.01 level; F—ANOVA
In an intragroup comparison of group I, group IA (mean 129.6) showed the highest antifungal efficacy followed by group IB (mean 190.2) and group IC (mean 318.4) (Table 1 and Fig. 3). In the intragroup comparison of group II, group IIA (mean 63.3) and group IIB (mean 73.8) showed no statistically significant difference. However, groups IIA and IIB comparison with the group IIC (mean 250.9), both IIA and IIB showed a statistically significant decrease in CFUs ($p < 0.01$) (Table 2 and Fig. 4). In intergroup comparison, all the group II subgroup irrigants (irrigant with an antifungal group), IIA, IIB, IIC, showed less number of CFUs than their respective group I irrigant (irrigant alone group) IA, IB, IC (Table 3 and Fig. 5). Group III (mean 554.2) was least effective than all the subgroups. Overall, in the present study, clotrimazole as a supplementary irrigant with other irrigants showed a significant decrease in CFUs than the irrigants alone.

**DISCUSSION**

Oral microbiota constitutes a small part of fungi, and *Candida* species are the most predominant. Some studies have shown the occurrence of fungi in endodontic infections and their role in the etiopathogenesis of periapical diseases. According to Sen et al. study, the root canal exhibits an ecologic environment that generally favors the growth of anaerobes. But, *Candida*, being an aerobic microorganism, can survive the harsh environment because of its unique “dentinophilic” character. It can also accommodate a broad range of pH levels. *Candida* shows pleomorphism. It can grow in various morphologic forms like blastospores, germ tubes, true hyphae, pseudohyphae, and chlamydocolones.

Moreover, it also exhibits a variety of virulence factors and can secrete “aspartyl protease,” which is a degenerative enzyme that can degrade the dentinal collagen. In HIV-infected patients, 95% of the patients may present with a history of oral candidiasis. The invasion of dental tissues, including root canals by the yeasts, is inevitable. Thereby, to perform a successful endodontic treatment, thorough debridement of the root canal system is crucial. Apart from adequate instrumentation of the root canals, irrigation with an appropriate agent has also been a norm of the debridement procedure. Thus, in the patients having local or systemic predisposing factors to oral candidiasis, specific antifungal agents may be recommended in their endodontic therapy.

The present study aimed to evaluate and compare the antifungal efficacy of root canal irrigants with and without clotrimazole. The efficiency of NaOCl and CHX on *C. albicans* has been well recorded in the literature. But very few studies have evaluated the efficacy of NaOCl and CHX along with an antifungal agent. The present study used and adopted an *in vitro* model from the study by Orstavik and Haapasalo. The bacterial growth can be restricted from the pulpal side when there is intact root cementum due to the limited nutritional availability. This indicates that the different environments (i.e., nutrient-rich or nutrient-deficient) can determine the variability of the microorganisms in dentinal tubules.

According to the results obtained in the present study, it was found that 5.25% NaOCl was more effective against *C. albicans* than 2% CHX and 5% doxycycline hydrochloride ($p < 0.01$). Besides, 5% of doxycycline hydrochloride irrigant was the least effective against *Candida*. Moreover, when clotrimazole was included with the experimental irrigants, there was a pronounced decrease in the CFU count. It has been suggested by various studies that chlorhexidine gluconate and sodium hypochlorite diffuse through the root canal,

**Table 2:** Comparison of CFU within group II and control

| Group                  | Mean | SD  | N  | F     | p Value |
|------------------------|------|-----|----|-------|---------|
| NaOCl + clotrimazole (IIA) | 63.3 | 10.1| 10 | 889.84*** | 0.000   |
| CHX + clotrimazole (IIB)   | 73.8 | 14.0| 10 |       |         |
| Doxycycline HCl + clotrimazole (IIC) | 250.9 | 12.7| 10 |       |         |
| Control (III)            | 554.2| 43.6| 10 |       |         |

SD—standard deviation; ** significant at 0.01 level; $F$—ANOVA
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**Table 3:** Comparison of CFU based on group and represents mean, standard deviation, and p value of all experimental groups and control

| Group                      | Mean  | SD    | N   | F    | p Value |
|----------------------------|-------|-------|-----|------|---------|
| (IA) NaOCl                 | 129.6 | 8.2   | 10  | 728.63** | 0.000   |
| (IB) CHX                   | 190.2 | 12.5  | 10  |      |         |
| (IC) Doxycycline HCl       | 318.4 | 15.8  | 10  |      |         |
| (IIA) NaOCl + clotrimazole | 63.3  | 10.1  | 10  |      |         |
| (IIB) CHX + clotrimazole   | 73.8  | 14.0  | 10  |      |         |
| (IIC) Doxycycline HCl + clotrimazole | 250.9 | 12.7  | 10  |      |         |
| (III) Control              | 554.2 | 43.6  | 10  |      |         |

SD—standard deviation; ** significant at 0.01 level; F—ANOVA

A limitation of the present study is that a single organism has been used to infect the root canal. Since the root canal system consists of various microorganisms, hence the irrigant used effectively *in vitro* against a single microbe may not necessarily be effective against the same microbe *in vivo*. However, further investigations are necessary to assess the efficacy of clotrimazole *in vivo*. Moreover, the reaction between the irrigants and antifungal agents also needs to be evaluated in the future.

**Conclusion**

Among the three irrigants, sodium hypochlorite showed better antifungal efficacy than chlorhexidine and doxycycline when used alone. But, the inclusion of clotrimazole as a final irrigant increased the antifungal efficacy of both sodium hypochlorite and chlorhexidine, resulting in no statistical difference between both the irrigants. The addition of clotrimazole increased the effectiveness of doxycycline also, but less when compared to sodium hypochlorite and chlorhexidine. Within the limitations of this study, the inclusion of 1% clotrimazole increased the antifungal efficacy of all the three irrigants.

**Author Contributions**

Sudhakar Srinivasan, Rahul VC Tiwari—drafted the research protocol and developed an experimental strategy. Gayathri Velusamy, Meer Ahamed Ibrahim Munshi, Karthikeyan Radhakrishnan—collected samples and performed the preparation of samples. Sudhakar Srinivasan, Rahul VC Tiwari—collected the results and interpreted. Gayathri Velusamy, Meer Ahamed Ibrahim Munshi, Karthikeyan Radhakrishnan—revised existing literature for writing introduction and discussion. Sudhakar Srinivasan, Rahul VC Tiwari—finalized the article draft. Gayathri Velusamy, Meer Ahamed Ibrahim Munshi, Karthikeyan Radhakrishnan—approved the draft with corrections. All authors approved final version of the article for submission.

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