Efficacy of various combinations of irrigants and medicaments on Candida albicans: An in vitro study

Mukund Singh, Vikrant O. Kasat

Departments of Conservative Dentistry and *Oral Medicine and Radiology, Rural Dental College, Loni, Maharashtra, India

Corresponding author (email: <dr.mukundvs@gmail.com>)
Dr. Mukund Singh, Department of Conservative Dentistry, Rural Dental College, Loni - 413 736, Maharashtra, India.

Abstract

**Objective:** To evaluate the efficacy of 3% sodium hypochlorite (NaOCl), 17% ethylenediaminetetraacetic acid (EDTA), calcium hydroxide \([\text{Ca (OH)}_2]\), and 2% chlorhexidine in various combinations on *Candida albicans* in root canals for 48 h and 10 days. **Materials and Methods:** In this *in vitro* study, 95 extracted teeth were instrumented. MTCC 183 strain of *C. albicans* was introduced into the root canals and after 7 days, the average value of colony forming units (CFUs)/ml was determined in all the roots. Then the teeth were randomly divided into three groups: Group I \((n = 15)\) was the control group and only saline was added to it. In group II \((n = 40)\), 3% NaOCl was used as the irrigant and in group III \((n = 40)\), 3% NaOCl and 17% EDTA were used as irrigants. Groups II and III were further divided into two subgroups (A and B) having 20 teeth each. After irrigation, \(\text{Ca (OH)}_2\) was placed in subgroup A and 2% chlorhexidine digluconate (CHX) solution in subgroup B of both groups as intracanal medicament. After 48 h, the CFUs/ml was determined in 45 teeth (5 from the control group and 10 from each subgroup). After 10 days, the CFUs/ml was determined in the remaining 50 teeth (10 from the control group and 10 from each subgroup). For statistical analysis, Statistical Package for Social Sciences (SPSS version 15, Chicago IL, USA) was used. **Results:** A decrease in CFUs/ml of *C. albicans* was noted in all subgroups at 48 h, but the combination of NaOCl, EDTA, and CHX (group IIIB) was the most effective showing value of \(0.095 \times 10^5\) CFUs/ml. On the 10th day, the CFUs/ml of *C. albicans* was further decreased in all subgroups; but again, subgroup IIIB was the most effective showing a value of \(0.025 \times 10^5\) CFUs/ml. There was statistically significant difference in CFUs/ml of *C. albicans* at 48 h and on 10th day in all study groups. **Conclusion:** The antimicrobial activity of irrigant and intracanal medicament has a positive role in controlling the growth of *C. albicans*.

**Key words:** Calcium hydroxide, Candida albicans, chlorhexidine, ethylenediaminetetraacetic acid, sodium hypochlorite

INTRODUCTION

Endodontic infections are polymicrobial in nature with preponderance toward anaerobic species, and several studies have found fungi, apart from bacteria and viruses, in endodontic infections.\(^1\) The most common organisms isolated from the root canals with post-treatment apical periodontitis are *Candida albicans* and *Enterococcus faecalis*.\(^2-4\) The goal of endodontic treatment essentially is debridement, i.e. to disrupt and remove the microbial ecosystem associated with the disease process and to neutralize any antigen that may be left in the canal after the elimination of the microorganisms. Therefore, the infected root canal is subjected to combined chemo-mechanical treatment involving instrumentation and copious irrigation with disinfectants. Still some bacteria and their products could persist in the dentinal tubules, in the root canal system, or in the smear layer produced by instrumentation. To inactivate this inflammatory burden, lasting antibacterial intracanal medication can be utilized between endodontic appointments until the final sealing of the canal.

Sodium hypochlorite (NaOCl) is the most popular and widely used irrigant in endodontics, but it is not active against the smear layer. Studies have shown...
that 17% ethylenediaminetetraacetic acid (EDTA) effectively removes the smear layer and its antifungal activity has also been well demonstrated.\textsuperscript{15} Among various intracanal medicaments, calcium hydroxide \([\text{Ca} (\text{OH})_2]\) stands out because of its consistent antibacterial activity and minimal cytotoxicity, but its role against \(C.\ albicans\) is questionable as some have found it ineffective\textsuperscript{6,7} and some others have found it effective for the first 24 h.\textsuperscript{8} Also, 2% chlorhexidine digluconate (CHX), when used within the root canals as an intracanal medicament, has shown potent results against common endodontic pathogens including \(C.\ albicans\).\textsuperscript{4,6,9}

Since \(C.\ albicans\) are frequently recovered from the re-treatment cases, it shows that they are able to survive in environments with sparse nutritional supply.\textsuperscript{10} A combination of irrigant, chelating agent, and medicament may be useful to eliminate \(C.\ albicans\) from the root canals. Thus, the present \textit{in vitro} study was undertaken to check the efficacy of 3% NaOCl, 17% EDTA, 2% CHX, and \(\text{Ca} (\text{OH})_2\) in various combinations on \(C.\ albicans\) in the root canals for 48 h and 10 days.

**MATERIALS AND METHODS**

Ninety-five freshly extracted, caries-free human permanent single-rooted incisors, canines, and mandibular premolars were used in this study. Each tooth was radiographed to confirm the presence of a single and straight canal. The teeth were stored in 10% formalin for disinfection and fixation of organic tissue for a period of 24 h, followed by cleaning of the external debris and calculus with an ultrasonic scaler. They were stored in physiologic saline until use. The crowns were sectioned with a diamond disk such that the root length was standardized to 16 mm. The working length of each tooth was determined by using a size 10 K-file and teeth with apical diameter greater than size 10 K-file were discarded. After coronal preflaring with Gates Glidden drills, apical preparation was done using hand K-file till size 50. Canals were irrigated with 3 ml of 3% NaOCl solution during the procedure. After instrumentation, the smear layer was removed with a rinse of 1 ml of 17% EDTA for 1 min followed by rinsing with 3 ml of 3% NaOCl. Finally the canals were flushed with 5 ml of distilled water to remove any debris and residual irrigants. The external surfaces of the roots were coated with epoxy resin (Araldite, Huntsman, Switzerland) and the root apices were sealed with temporary cement (Cavit\textsuperscript{TM} G; 3M ESPE, Seefeld, Germany) [Figure 1a]. After setting of the epoxy resin, all the roots were sterilized by autoclaving at 121°C for 20 min [Figure 1b].

\(C.\ albicans\) broth (strain MTCC 183) was prepared and kept for overnight incubation. The broth was then matched with a spectrophotometer to 0.5 turbidity on the McFarland scale, which corresponds to \(1.5 \times 10^5\) microorganisms/ml. Ten microliters of the inoculum was injected into the prepared canals with the help of micropipette inside laminar air flow chamber. Teeth were sealed coronally with temporary cement (Cavit\textsuperscript{TM} G; 3M ESPE, Seefeld, Germany) after placing sterile cotton ball over access opening of the canals. Then, the teeth were placed on a gauze pad in sterile petri plate and incubated at 37 ± 1°C (in humid conditions) for 7 days. To facilitate the growth of \(C.\ albicans\), on the 4\textsuperscript{th} day, all teeth were reopened, 1 ml of nutrient broth was added into the canal, and the roots were again sealed with temporary cement (Cavit\textsuperscript{TM} G; 3M ESPE, Seefeld, Germany) for 7 days.

On the 7\textsuperscript{th} day, the teeth were reopened, microbiological sampling was carried out individually by placing sterile paper points into the root canals for 60 s, and then the teeth were again kept in the incubator after coronal sealing. The paper points were placed in a test tube containing 1 ml of sterile saline solution and incubated for 30 min at 37°C and then shaken in a vortex mixer for 60 s. After this, serial dilution of the microbiological sample was done in Eppendorf tubes and the serially diluted microorganisms were plated on to Sabouraud dextrose agar (SDA; HiMedia, Mumbai, India) and incubated at 37°C for 24 h to determine the colony forming units (CFUs)/ml. The CFUs/ml were counted with a 10 \times magnification and only the CFUs more than 30 units was taken into consideration. The average value of CFUs/ml of \(C.\ albicans\) was \(32 \times 10^9\) in all the roots.

After this, temporary filling was removed from all the 95 teeth kept in the incubator. The canals were dried using paper point for gross excess fluid removal.
to prevent the dilution of the irrigants before the experiment. Teeth were randomly divided into three groups. Group I \((n = 15)\) was taken as the control group and only saline was added to it. In group II \((n = 40)\), 1 ml of 3% NaOCl was used as the irrigant for 1 min, followed by normal saline. In group III \((n = 40)\), 3% NaOCl and 17% EDTA were used as irrigants followed by normal saline. Groups II and III were further divided into two subgroups (A and B) having 20 teeth each. After irrigation, Ca (OH)\(_2\) mixed with distilled water (3:1 ratio) was placed in subgroup A and 2% CHX solution in subgroup B of both groups as the intracanal medicament. After this, a sterile cotton ball was placed on root canal opening and teeth were sealed coronally with temporary cement (Cavit G; 3M ESPE, Germany). Then teeth were kept in the incubator at 37°C.

After 48 h, the coronal seal of 45 teeth (5 from the control group and 10 from each subgroup) was removed and the canals were irrigated with 1 ml of sterile saline. They were then instrumented with no. 50 H-file to create dentinal shavings and microbiological sampling was carried out by placing sterile paper points into the root canals for 60 s. The paper points were placed in test tubes containing 1 ml of sterile saline solution, incubated at 37°C for 30 min, and shaken vigorously for 60 s in a vortex mixer. Serial dilution and culturing was further carried out to determine CFUs/ml. On the 10th day of incubation, the coronal seal of remaining 50 teeth (10 from the control group and 10 from each subgroup) was opened, and subsequently, microbiological sampling and serial dilution followed by culturing were carried out as explained previously.

**Statistical analysis**

For statistical analysis, Statistical Package for Social Sciences (SPSS version 15; SPSS Inc., Chicago, IL, USA) was used. Significance level was set at \(P \leq 0.05\).

**RESULTS**

After 48 h, group I (control group, which was not treated with any medicament) had \(31 \times 10^5\) CFUs/ml of \(C.\) albicans. In the study groups (II and III), the application of irrigant and intracanal medicament resulted in decreased values of \(C.\) albicans. In subgroup IIA [NaOCl, Ca (OH)\(_2\)], the growth of \(C.\) albicans was reduced to \(1.98 \times 10^5\) CFUs/ml, while in subgroups IIB (NaOCl, CHX) and IIIA [NaOCl, EDTA, Ca (OH)\(_2\)], it was further reduced to \(0.29 \times 10^5\) and \(0.13 \times 10^5\) CFUs/ml, respectively. Subgroup IIIB (NaOCl, EDTA, CHX) was the most effective against \(C.\) albicans, showing a value of \(0.095 \times 10^5\) CFUs/ml at 48 h [Figure 2].

On the 10th day, all subgroups further reduced the growth of \(C.\) albicans [Figure 3], but again subgroup IIIB was the most effective against \(C.\) albicans when compared to any of the subgroups, showing a value of \(0.025 \times 10^5\) CFUs/ml. There was statistically significant difference in all the groups and subgroups between 48 h and 10 days [Table 1].

**DISCUSSION**

Microorganisms play a fundamental role in the etiology of pulp and periapical disease. While primary endodontic infections are polymicrobial in nature, the microorganisms involved in secondary infections are composed of one or few bacterial species. In 1997, Waltimo et al. studied the occurrence of microorganisms within 967 root canals of teeth with persistent periapical lesions and the most common species among the 48 strains of fungi isolated from 7% of the culture-positive samples was \(C.\) albicans.\(^4\) \(C.\) albicans has been considered a dentinophilic microorganism because of its invasive affinity to dentin and, hence, is most often found in endodontic infections.\(^{1,12}\) It has the ability to grow on the dentinal surfaces in the absence of oral fluids and penetrates into dentinal tubules to a variable extent by its different growth patterns like hyphae and blastospores.\(^{1,6}\) Hence, in this study, the antimicrobial activities of various irrigants and medicaments on \(C.\) albicans were tested in the extracted teeth in preference to agar diffusion test.

**Figure 2:** SDA plate depicting growth of \(C.\) albicans at 48 h in (a) group IIA [NaOCl and Ca (OH)\(_2\)], (b) group IIB (NaOCl and CHX), (c) group IIIA (NaOCl, EDTA, and CHX), and (d) group IIIB [NaOCl, EDTA, and Ca (OH)\(_2\)].
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It is truly said that instruments shape and irrigants clean. Irrigation is important for root canal treatment as it assists in removing bacteria and debris within the intricacies of the root canal. Higher the concentration of NaOCl, better are the antibacterial and tissue dissolution properties, but at the same time, more are the chances of tissue reaction. Also, 5.25% NaOCl has an unpleasant odor when compared to a 3% solution. Hence, 3% NaOCl is clinically more acceptable and has been used in this study. Mechanical instrumentation produces a smear layer that adheres on the walls of the root canal. Manjunatha et al. have shown that hand instrumentation causes the least amount of smear layer as compared to rotary instrumentation. This smear layer is composed of both organic and inorganic particles. This deposit can be penetrated by bacteria and may offer protection to biofilms adhering to the root canal walls. Though NaOCl is the preferred irrigant in endodontic therapy, it cannot remove inorganic components and, therefore, cannot prevent the formation of a smear layer. One has to rely on demineralizing agents like EDTA or citric acid to remove the smear layer, which also softens the dentin and makes shaping procedures easier. EDTA reacts with calcium ions in dentin and forms soluble calcium chelates. It is highly effective in removing the smear layer from the root canal walls. EDTA is commercially available as a 17% solution as an endodontic irrigant. Hence, 17% EDTA was our choice as the irrigant in group III (NaOCl, EDTA, Ca (OH)₂/CHX).

Chemomechanical preparation is effective in reducing the number of microorganisms, but not in completely eliminating them. Microorganisms may remain viable even after root canal preparation, multiplying between appointments. Thus, intracanal medication may be a valuable adjunct to chemomechanical preparation in further disinfection of the root canal system. CHX is widely used for disinfection in dentistry because of its good antimicrobial activity. CHX has been shown to have long-term antimicrobial properties because of its unique ability to bind to hydroxyapatite. Unlike NaOCl, it lacks tissue dissolving properties. Vianna et al. proved CHX liquid to be a stronger disinfectant than CHX in gel form at various concentrations. In the same study, it was proved that 2% CHX liquid was more potent than 1% and 0.2% liquid against selected microorganisms. Hence, in this study, 2% CHX liquid was used.

Various effects take place if irrigants and medicaments are mixed together. NaOCl and EDTA are the two commonly used irrigating solutions and they have different characteristics and tasks. However, EDTA instantly reduces the amount of chlorine when mixed with NaOCl, resulting in the loss of NaOCl activity. Studies have shown that CHX and NaOCl are not soluble in each other, and a brownish orange precipitate (parachloroaniline) is formed when they are mixed and this may have mutagenic potential. Similarly, mixing CHX and EDTA immediately produces a white precipitate. Although the clear supernatant has not been thoroughly studied, it seems that the ability of EDTA to remove the smear layer is reduced. Hence, in this study, samples were irrigated with saline between each irrigant and before the placement of medicament to prevent any decrease of efficacy of the irrigants or medicaments used.

Table 1: Comparison of CFUs at different study time periods between the study groups by ANOVA

| Treatment group | CFUs in x10⁵ | P value | 48 h vs. 10 days |
|-----------------|---------------|---------|-----------------|
| Pre-treatment   | 48 h          | 10 days | 48 h vs. 10 days |
| Group I - Control | 34.25±2.79   | 31.32±1.57 | 39.09±5.18 | 0.077+ |
| Group IIA - NaOCl+Ca (OH)₂ | 32.9±2.67   | 1.98±0.62  | 0.309±0.07 | <0.001** |
| Group IIB - NaOCl+CHX | 31.76±3.36  | 0.29±0.11  | 0.051±0.23 | <0.001** |
| Group IIIA - (NaOCl+EDTA) and Ca (OH)₂ | 34.35±3.71  | 0.13±0.05  | 0.049±0.008 | <0.001** |
| Group IIIB - (NaOCl+EDTA) and CHX | 32.64±4.38  | 0.09±0.04  | 0.025±0.009 | 0.001** |
| P value         | 0.198         | <0.001**  | <0.001**       |

ANOVA=Analysis of variance; CFUs=Colony forming units; suggestive significance (0.05<p<0.10). **Strongly significant (p≤0.01)

Figure 3: SDA plate depicting the growth of C. albicans at 10 days in (a) group IIA, (b) group IIB, (c) group IIIA, and (d) group IIIB
In this study, after instrumentation, microorganisms were inoculated within the canal and allowed to grow for a period of 7 days, followed by placement of irrigant/intracanal medicaments. Hence, this study was done to evaluate the effectiveness of the irrigant/medicaments only and not to find the cumulative effect of instrumentation and irrigation/medicaments. Bacteriological samplings were taken at 48 h and at the end of 10 days. These two time periods were selected so as to evaluate the short- and long-term effects of the medicaments. In this study, the bacteriological sampling was accomplished with a sterile paper point that absorbed the root canal contents. The advantage of using paper points is that it can be used in vitro and in vivo, while its limitation is that the microorganisms in the root canal can be sampled, but those in the dentinal tubules are sampled to a limited extent. Hence, the canals were instrumented with no. 50 H-file to create dentinal shavings and then sampling was done. Further, serial dilution was performed in the control and study groups at 48 h and on the 10th day. Serial dilution is a standard microbiological method that allows us to precisely count the CFUs/ml.

The results showed greater reduction of CFUs/ml from 48 h to 10 days in all groups. In groups IIB (NaOCl, CHX) and IIIB (NaOCl, EDTA, CHX), the effect can be attributed to the substantivity shown by CHX. CHX has an affinity to dental hard tissues (hydroxyapatite), and once bound to a surface, it shows prolonged antimicrobial activity by release of biologically active component over a period of time. This phenomenon is called “substantivity.” This is in contrast to the effect of other disinfectants, which rapidly lose their potency and have no residual antimicrobial effects.[18] A study by Rosenthal et al. has shown that the gradual release of this bound CHX could maintain the bacteriostatic effect in the root canals over a prolonged period of time which may be up to 12 weeks.[19] Groups IIA [NaOCl, Ca (OH)2] and IIIA [NaOCl, EDTA, Ca (OH)2] also showed reduction of CFUs/ml from 48 h to 10 days.

When comparing groups IIA and IIB, the study proved that the combination of NaOCl and CHX showed more effectiveness against C. albicans than the combination of NaOCl with Ca (OH)2. This is because the role of CHX is greater than that of Ca (OH)2 in reducing the growth of C. albicans. Combination of NaOCl, EDTA, and Ca (OH)2 (group IIIA) showed less CFUs after 48 h, when compared with group IIA [NaOCl, Ca (OH)2]. The reason can be attributed to the action of EDTA. Furthermore, NaOCl, EDTA, and CHX (group IIIB) showed least CFUs/ml when compared with all the other subgroups. This may be the result of the combined effect of EDTA and CHX.

One limitation of this in vitro study is that a single microorganism was used to infect the root canal in contrast to clinical situations with polymicrobial endodontic infections. Thus, the irrigant and medicament combination that is effective in vitro may not necessarily be effective against the same microbe in vivo.

CONCLUSION

Within the limitation of this in vitro study, it can be said that the combined effect of both irrigants and medicaments has a positive role in controlling the growth of C. albicans, which may contribute to a more favourable outcome of the root canal treatment.

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