Effect of intracanal medicament gel materials separate and in combination in the elimination of Enterococcus faecalis biofilm

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Received 7 September 2014; revised 29 October 2014; accepted 5 November 2014
Available online 16 December 2014

Abstract

Objective: This study was to evaluate the antimicrobial efficacy of prepared medicament gels on induced Enterococcus faecalis biofilm using the fluorescent microscope.

Methods: The rheological determination was done for the prepared medicaments, namely: ECHXT (mixture of EDTA + CHX + Triss buffer), ENaOCl (2.5% NaOCl/18.6% EDTA gel) and ECHX (18.6% EDTA/17% CHX gel). Antimicrobial efficacy of each medicament gel was evaluated by bacterial viability method as examined using the fluorescent microscope. Fifty-five distal roots of extracted lower molars were contaminated with E. faecalis and incubated for 14 days at 37°C. Scanning Electron Microscope was used to confirm the biofilm formation in five samples. After mechanical preparation, Forty five roots were divided into three equal groups according to the used medicament gel, and incubated for 1, 24 h and 1-week.

Results: In fluid samples, there was no statistically significant difference between the three medicaments after recommended time interval. While in dentin shavings samples; ENaOCl and ECHX groups showed the statistically significant lowest mean viability after 24 h. The statistically significant highest mean viability percentage was with the fluid samples when compared with the dentin shaving.

Conclusion: Variation in the physical nature of irrigant improved its ability to penetrate the dentinal tubules, while minimizing the time of application.

Keywords: Chlorohexidine; Enterococcus faecalis; Intracanal medicament; NaOCl

1. Introduction

Bacteria and their by-products play an essential role in the initiation and perpetuation of pulpal and periapical diseases [1]. Enterococcus faecalis occurs primarily in retreatment scenarios; it survives in root canals as a single organism without the support of other bacteria [2]. Various substances have been used during and immediately after root canal preparation to remove debris and necrotic pulp tissue and to help eliminate microorganisms that cannot be reached by mechanical instrumentation [3].
Sodium hypochlorite is to date, the most commonly used root canal irrigant. However, no general agreement exists regarding its optimal concentration, which ranged from 0.5% to 5.25%. The gel form overcomes the side effects of NaOCl solution when introduced beyond the tooth apex [4]. Chlorhexidine in a higher concentration has antimicrobial activity against a wide range of Gram positive and Gram-negative organism as well as yeast, facultative anaerobes and aerobes [5]. While in a lower concentration, it has a bacteriostatic effect [6]. Chlorhexidine when used as an intracanal medication in a gel form demonstrated a good performance [7]. EDTA as a chelating agent when used in 18.6% had a better cleaning effect [8].

The aim of the present study was to evaluate in-vitro the antibacterial effect of prepared intracanal medicament gel materials against induced E. faecalis biofilm through the presence of viable microorganisms using the fluorescent microscope.

2. Materials and methods

2.1. Part I: preparation of the medicament gel

Ethical committee, Faculty of Oral and Dental Medicine Cairo University, approved the present study. Intracanal medicament gel materials were prepared; namely ECHXT (0.01% EDTA1 + 0.01% CHX2 + 6% Triss buffer3 + 12% HPMC 15 cps polymer4), ECHX (17% CHX with12% HPMC 15 cps polymer and 18.6% EDTA with 2% Sodium alginate polymer5), ENaOCl (2.5% NaOCl6 with 15% Guar gum polymer7 and 18.6% EDTA with 2% Sodium alginate polymer) and rheological determination was done. Using a cone and plate Brookfield viscometer for each of the freshly prepared medicament gel material and the stored for 2 h.

2.2. Part II: application of the medicament gel

Fifty-five distal roots of extracted lower molars were standardized to 12 ± 1 mm length and up to #20 K-file8 apex's width. A cervical seat was created of 1 mm dimensions at the coronal third of the root. The root surfaces were coated with varnish and root apices were sealed with cyanoacrylate. Each root was placed separately inside Eppendorf tube and autoclaved for 15 min at 15Psi and 121 °C. To confirm the procedure of sterilization; random sample (N = 5) from sterilized teeth were incubated in brain heart infusion broth for 48 h at 37 °C. All the next steps were performed under aseptic conditions inside class laminar flow cabinet with HEPA filter.9

2.2.1. Induced biofilm in initially prepared root canals

The canals were contaminated with 1 McFarland bacterial suspensions of E. faecalis (ATCC 29212) using sterile insulin syringe (gauge 27) and incubated for 14 days at 37 °C. Refreshing broth was added every 48 h throughout the incubation period. Randomly five roots were selected to confirm the formation of E. faecalis biofilm by SEM10. The roots were grooved vertically using a diamond disc without touching the canal. Then they were longitudinally split into two halves.

Each half of root was immersed in 2.5% Glutaraldehyde, pH 7.4, at 4 °C for 24 h for fixation, washed with Phosphate buffer solution (PBS) for 15 min, and post-fixation for 12 h at 4 °C to 6 °C in 1% (wt/vol). Osmium tetroxide. PBS was used as a final wash. Dehydration was performed with an ascending acetone series (30%, 60% and 100%) for 10 min each. Each sample was mounted and coated with a 200 Å layer of gold palladium. Canal observations were performed by using SEM. Representative samples were taken at 5000-X magnification for each third.

2.2.2. Mechanical preparation of the root canal

The root canals were prepared using K3 Ni—Ti rotary system11 using crown-down technique. The sequence used was as follows; #40, #35, #30, #25, #20 and #15. The K3/0.6 taper sequence used in the apical third were from #25 up to #40. During instrumentation, the canals were irrigated with 2-ml of sterile distilled water.

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1 Ethylenediaminetetraacetic acid, Oxford, Mumbai.
2 Chlorohexidine HCL, The Arabic drug company, Cairo A.R.E.
3 Oxford, Mumbai.
4 Hydroxyl propyl methyl cellulose polymer, Cairo Drug Company, Egypt.
5 Oxford, Mumbai, India.
6 Commercial Clorox, The Egyptian company for house cleanser, Cairo, Egypt.
7 Loba Chemie, Colaba, Mumbai, India.
8 Mani, Tochigi, Japan.
9 Nuair, U.S.A.
10 FEI, Holland.
11 SybronEndo, Orange,CA.
2.2.3. Application of dressing gel

After mechanical preparations; dryness of the canals was done using sterile paper points\textsuperscript{12}; dressing of root canals were done as follows (Fig. 1): ECHXT: \((n = 5)\) used the ready mixed medicament gel (mixture of EDTA + CHX + Triss buffer), standard volume of medicament gel (0.1 ml) was injected under stable pressure into the root canal using a 5 ml/CC plastic syringe\textsuperscript{13} with an etch tip\textsuperscript{14} of 23 gauges. The needle was placed as far as possible into the canal without binding, and, gradually withdrawn while it was within the medicament gel inside the canal to avoid air bubble formation.

ENaOCl: \((n = 5)\) used separate medicament gel 2.5% NaOCl/18.6% EDTA gel. While ECHX \((n = 5)\) used separate medicament gel 18.6% EDTA and 17% CHX gel. Standard volume of each medicament gel (0.1 ml) was applied as in ECHXT. In ENaOCl and ECHX, EDTA was applied in the canal space for 5 min. After that, the canal orifice was sealed with a sterile cotton pellet and a temporary filling material\textsuperscript{15}. Then, each root was kept in the Eppendorff tube and incubated at 37 °C for 1 and 24 h and 1-week.

2.3. Evaluation of biofilm disruption

After each recommended period; the temporary filling and cotton were removed, the tooth was placed in sterile Eppendorff tube, and 1-ml of sterile distilled water was used to wash canal contents. Evaluation of viability of microorganisms was done as follows:

1. **Fluid sample collection**: First 1-ml of the wash of sterile distilled water was collected under aseptic condition into sterilized Eppendorff tube for each root to be examined under the fluorescent microscope.

2. **Dentin chip shavings sample collection**: Final washing using agitation with the needle utilizing 5-ml of sterile distilled water. For each dressing gel material was neutralized by 6-ml of specified neutralizer, namely NaOCl neutralizer (1.80 gm Sodium thiosulfate\textsuperscript{16} in 20 ml distilled water), CHX neutralizer (1.50 gm Tween 80\textsuperscript{17} + 0.21 gm Lecithin\textsuperscript{18} in 20 ml distilled water), EDTA neutralizer (4.70 gm Sodium thiosulfate + 38.7 mg Tween 80 + 1.20 gm Lecithin + 30.00 gm Calcium chloride\textsuperscript{19} + 18.75 gm Sodium bisulphate\textsuperscript{20} in 20 ml distilled water). A final flush with 6-ml sterile distilled water was performed. Sterile K\textsubscript{3} rotary files #45 (0.6) and #50 (0.6) were used to instrument the root canal to two sizes larger than the original master apical file reaching the full working length. Dentin chip shavings were collected from the canal and the rotary files, aseptically. The dentin chips suspension was prepared by adding 100 μL sterile distilled water to the dentin chips.

2.3.1. Stains preparation and microscopic examination

Propodium iodide stain\textsuperscript{21} for non-viable bacteria and Tetrazolium blue stain\textsuperscript{22} for viable bacteria were prepared according to manufacture. After that; using pipette 20 μL of each stain were added to 40 μL of each sample; the first wash or the dentin chips suspension. Then 5 μL of the prepared bacterial sample was placed on a microscopic slide using a pipette and examined using a fluorescent microscope\textsuperscript{23} at 40-X/0.65 pH 2. Bacteria viability was expressed as the mean percentage of viable \textit{E. faecalis} over the total number of microorganisms by randomly counting of three fields\textsuperscript{9}.

2.4. Statistical analysis

Statistical analysis was performed with IBM SPSS Statistics Version 20 for Windows. Tabulated data were

\textsuperscript{12} Dentsply Mailflefer, Ballaigues, Switzerland.

\textsuperscript{13} HG Company, USA.

\textsuperscript{14} Super etch tip, SDH limited, Victoria, Australia.

\textsuperscript{15} Provis\textsuperscript{8}, Favodent, Karlsruhe, Germany.

\textsuperscript{16} Loba Chemie, Mumbai, India.

\textsuperscript{17} Oxford, Mumbai, India.

\textsuperscript{18} Fluka, U.S.A.

\textsuperscript{19} Oxford, Mumbai, India.

\textsuperscript{20} loba Chemie, Mumbai, India.

\textsuperscript{21} Sigma—Aldrich, Fluka, USA.

\textsuperscript{22} Riedel-de Haen, Germany.

\textsuperscript{23} Olympus, Japan.
presented as mean and standard deviation (SD) values. One-way Analysis of Variance (ANOVA) was used for comparisons between groups and between different periods. Tukey’s post-hoc test was used for pair-wise comparison between the groups when ANOVA test is significant. Student’s \( t \)-test was used to compare between viability % in dentin shavings and fluid sample. The significance level was set at \( P \leq 0.05 \).

3. Results

The viscosity of all the tested gels slightly decreased after storage for 2 h. The gels could be arranged in descending order according to their viscosity values as medicament gel NaOCl > medicament gel ECHXT > medicament gel CHX > medicament gel EDTA.

After 14 days the biofilm was formed along the whole root in the apical, middle and coronal thirds (Fig. 2).

Both fluid and dentin chips samples by the fluorescent microscopic examinations showed cocci in single or aggregates form with two colors; bluish-green which represent the viable bacteria, while the red color represent the non-viable bacteria. In the fluid samples, the bluish-green color was more predominant than the red color and it was reversed that is red color crowding in the dentin chips suspension samples (Figs. 3 and 4).

For the dentin chip shavings samples; the results showed that after 24 h, ECHXT group had the statistically significant highest mean viability percentage. While there was no statistically significant difference between ENaOCl group and ECHX group; both

| Groups                  | Time  | ECHXT | ENaOCl | ECHX  |
|-------------------------|-------|-------|--------|-------|
|                         | Mean  | SD    | Mean   | SD    | Mean   | SD    | P-value |
|                         |       |       |        |       |        |       |
|                         | 1 h   | 23.7  | 8.1    | 42.5  | 10.6   | 31.7  | 10.2   | 0.376 |
|                         | 24 h  | 46a   | 19.4   | 19b   | 4.5    | 15b   | 4.1    | 0.040* |
|                         | 1-week| 29    | 4.7    | 32    | 10.4   | 31    | 11.4   | 0.916 |
|                         | P-value| 0.271 | 0.188  | 0.278 |

*: Significant at \( P \leq 0.05 \), different letters are statistically significantly different according to Tukey’s test.

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Fig. 2. SEM photomicrographs showed the formed *E. faecalis* biofilm at 5000× magnification in the (a) apical, (b) middle, (c) coronal thirds.

Fig. 3. Selected fluorescent photomicrographs representing red non-viable bacteria in the dentin chip shavings samples.

Fig. 4. Selected fluorescent photomicrographs representing predominant bluish-green viable bacteria in fluid sample.
showed the statistically significant lowest mean viability percentage. By comparison between the periods for dentin shaving samples; the results showed no statistically significant difference between the three times 1, 24 h and 1-week with the three testing gels (Table 1). For the fluid samples there was no statistically significant difference between viability % when compared between samples of the three medicament gel and between the different periods (Table 2).

Comparison results between dentin shavings and fluid showed that fluid had statistically significantly higher mean viability percentage than dentin shavings through all periods (Table 3).

4. Discussion

The aim of the present study was to evaluate the antibacterial effect of intracanal medicament gel against induced *E. faecalis* biofilm in-vitro. *E. faecalis* was chosen in this study for several reasons: It occurs primarily in retreatment scenarios; it has been extensively used in experimental work in endodontic, including antiseptic susceptibility [10]; and it is an easy-to-grow bacterium in the laboratory [11].

Rheological determination of the prepared medicament gel materials was done to study the behavior of the prepared medicament gel with time; proved the stability of the prepared gel. Such findings are in accordance with El-Hadidy et al. [12] study.

The materials used in this study were prepared: as medicament gel ECHXT. Combined use of EDTA gel as a chelating agent to increase the bactericidal activity of CHX, while a buffer enabled a low concentration of CHX to be effective against selected microorganisms was reported [13]. In the present study the benefit of the chelating agent and the medication was done in one mixture and one application step.

The 18.6% EDTA concentration chosen in this study was due to its better cleaning effect [8]. EDTA was applied for 5 min [14], before application of CHX gel and after NaOCl gel application. Chlorohexidine in 17% concentration has a bactericidal effect because of precipitation and/or coagulation of intracellular constituents [6].

The injection of the medicament gel was done using a syringe [15] while Lentulo spiral was used in other studies [16,17]. Application of medicament gel in ENaOCl group was NaOCl/first followed by EDTA [18]. While for ECHX group, it was EDTA first/followed by CHX.

The removal of medicament gel was done using standardized volume of sterile distilled water with agitation with the needle. While in other studies [19,20] copious sterile saline and the master apical file were used. The present study expressed the viability of bacteria as the mean percentage of viable *E. faecalis* over the total number of microorganisms by randomly counting of three fields [9]. Other study [21] used a confocal laser scanning microscopy to capture and analyze the photos by calculating the volume ratio between dead and live bacteria.

For the fluid samples when compared between the medicaments gels used there was no statistically significant difference between them after 1, 24 h and 1-week. Other studies; Dametto et al. [22] and Vianna et al. [23] contradict the present study. They showed that the 2% CHX (gel and liquid) antimicrobial ability was more effective than 5.25% NaOCl in keeping low CFUs of *E. faecalis* for 7 days after the biomechanical preparation. While Vianna et al. showed that the strongest irrigant was 5.25% NaOCl, followed by 2% CHX liquid, and 1% CHX liquid producing negative
cultures in 15 s; while 2% CHX gel required 1-min and the weakest one was 0.2% CHX gel (2 h). There was no statistical significant difference between the three periods within each dressing gel used. These could be due to the hardening of the medicament gel material.

For the dentin chips shavings sample it was found that after 24 h ENaOCl and ECHX groups showed the statistically significant lowest mean viability but ECHXT group showed the highest percentage these could be due to the low concentration of the antimicrobial agent (CHX). Such findings indicated that the ENaOCl and ECHX groups had the most effective antimicrobial action after 24 h. Ercan et al. [24] contradicts the present study; showed that CHX was significantly more effective than NaOCl 48-h after instrumentation.

The results of comparison between periods for the dentin shavings revealed no statistically significant difference between the three periods indicating that by increasing the time there was any improvement in the antimicrobial action of the medicament gel material it could be due to the hardening of the gel that might prevent the diffusion of the active ingredients.

The results of the comparison between the fluid and dentin chip shavings revealed the statistically significant highest mean viability percentage with the fluid samples which proved by the culturing techniques and CFUs counting used by other studies [16,17].

In conclusion, the results indicated that the used medicament gel materials have ability to penetrate the dentinal tubules to make its effect on reducing the bacterial biofilm in the root canal. Long acting effect or dentinal tubules to make its effect on reducing the medicament gel materials have ability to penetrate the dentinal tubules revealed the statistically significant lowest mean viability but after 24 h ENaOCl and ECHX groups showed the highest percentage these could be due to the low concentration of the antimicrobial agent (CHX). Such findings indicated that the ENaOCl and ECHX groups had the most effective antimicrobial action after 24 h. Ercan et al. [24] contradicts the present study; showed that CHX was significantly more effective than NaOCl 48-h after instrumentation.

Acknowledgment

The authors express their gratitude to Dr. Nadia Mohammed Madany for her input during the study, lecturer of Medical Microbiology & Immunology, Faculty of Medicine, Cairo University.

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