

A New Irrigant against *E. faecalis* in Root Canal Disinfection

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Non surgical endodontic therapy uses biomechanical cleansing and shaping of the canal, which may be combined with the application of antimicrobial irrigants and intracanal medications, to eliminate these bacteria. The study evaluated the antimicrobial efficacy of Octenidine Dihydrochloride, a newer irrigant with Sodium Hypochlorite, a gold standard against *Enterococcus faecalis* in root canal disinfection. 30 single rooted intact pre-molar teeth were collected. The teeth were decoronated to measure 7mm long segments. The root apexes were enlarged to size 40 using K files. The root apex was sealed using light cure composite resin and surface was coated with nail varnish. Following sterilization of the blocks, they were infected with pure cultures of *E. faecalis*, which was grown in Muller-Hinton agar for a period of 7 days. The specimens were divided into 3 distinctive groups containing 10 teeth each: Group I with Octenidine, (0.1%), Group II with NaOCl (5%), Group III with no irrigation (control). Each block was carefully removed and was irrigated with 3ml of the irrigant for 1 min. After irrigation, the dentine debris were harvested using Gates Glidden drills No.2 and No.3. The antibacterial assessment was carried out by finding out the MIC and MBC values by serial dilution method, and the proportions of viable bacteria under epifluorescence microscope using Syto9 dye. Statistical analysis comparing the inter-group was done using MANN-WHITNEY U test and KRUSKAL-WALLIS test. From the results it was observed that the antimicrobial efficacy of Octenidine was found to be higher than 5% Sodium hypochlorite against *E. faecalis* and in creating a "bacteria-free" root canal system.

Key words: *E. faecalis*, octenidin, syto-9, irrigant.

It is firmly established that bacteria are the prime etiological factor in the development and progression of dental caries and pulp and periapical disease¹. Non surgical endodontic therapy uses biomechanical cleansing and shaping of the canal, which may be combined with the application of antimicrobial irrigants and intracanal medications, to eliminate these bacteria².

In the past few years, *Enterococcus faecalis* has been mentioned with increased

frequency with regard to teeth with post-treatment disease (PTD), where it has also been detected as monocultures. The prominence of *E. faecalis* in root-filled teeth with apical periodontitis has made it a focus of attention as an etiological factor of PTD³.

Sodium hypochlorite has long been the irrigant of choice because of its antimicrobial activity and tissue dissolving ability, but it has been found to cause severe inflammatory reactions when placed in contact with vital tissues⁴.

Octenisept (Schülke & Mayr, Nordersedt, Germany) (N,N=-(1,10-decanediyl)-

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1(4H)-pyridinyl-4-ylidene)bis-[1-octanamine] dihydrochlorine), a new bispyridine antimicrobial compound effective against Gram-positive and Gram-negative bacteria, fungi and several viral species is an antiseptic for skin burns, wound disinfection and mouth rinse consisting of octenidine hydrochloride and phenoxyethanol⁵. These properties have led to the suggestion that this may be a potent root canal irrigant.

Identification of the root canal isolates from previous studies has traditionally been performed using standard microbiological and biochemical techniques. These methods have shown that the polymicrobial infections are mainly caused by obligate and facultative anaerobes. However, correlation of the microbiological findings from these studies is affected by certain limitations of the culture techniques, leading to the underestimation of bacterial diversity within the root canal system⁶.

SYTO 9, a nucleic acid stain have been used in diverse applications from staining DNA spotted on microarrays to staining live and fixed cells. The SYTO dyes can be used to stain RNA and DNA in both live and dead eukaryotic cells, as well as in Gram positive and Gram negative bacteria. The SYTO dyes are compatible with a variety of fluorescence-based instruments that use either laser excitation or a conventional broadband illumination source (e.g., mercury- and xenon-arc lamps)⁷.

The aim of this study was to evaluate and compare the antimicrobial effect of Octenidine and sodium hypochlorite as a root canal irrigant in the elimination of *E. faecalis*.

MATERIALS AND METHODS

Preparation of tooth specimens:

Thirty single-rooted human intact mandibular premolar teeth were collected. Digital images of the teeth were captured to eliminate teeth other than single root with single canal. Calculus and tissue remnants were removed using ultra sonic scalers and the teeth were incubated in 0.2% sodium azide solution for 14 days. The teeth were de-coronated with a diamond disc under water cooling and shortened, resulting in 7 mm of remaining root length. The specimens were

kept in tap water during all procedures to avoid dehydration. The root canals were enlarged to a size 40 with k files under constant irrigation with a total of 10 ml NaOCl (1%). It was then rinsed with 10 ml citric acid (10%) for 5 min to remove smear layer and, finally with 20 ml of physiological saline. The root surface was coated with nail varnish, and apex was sealed using light cure composite resin.

Sterilization of tooth specimens

The tooth specimens were sterilized in an autoclave at 121°C for 15 min under 15lbs of pressure. The teeth were then transferred to individual sterile Eppendorf tubes and 1ml of Mueller Hinton broth was added to the tubes using a sterile pipette. The EP tubes were sealed with paraffin strips. The EP tubes containing the tooth samples and Mueller Hinton broth were re-sterilized in the autoclave.

Preparation of Media plate

The prepared sterilized Mueller Hinton agar was poured into sterilized petridish to a depth of 5mm, under laminar flow. For every 100ml of the medium, 5 plates were poured. The poured plates were allowed to solidify and refrigerated. For every batch of prepared plates, one plate was kept for sterility check for 24 hrs in the incubator at 37°C.

Preparation of Broth Tubes:

The prepared sterilized Muller-Hinton broth was dispersed in quantity of 20 ml in sterile screw capped test tubes. One tube was kept for sterility checking at 37 °C for 48hrs and one tube was used as control to check the performance to support the growth of *E. faecalis*.

Inoculation of the dentin blocks with *E. faecalis*

Isolated 48 hour colonies of *E. faecalis* (ATCC 29212) grown on sheep blood agar were suspended in 5ml of Muller Hinton broth and was adjusted resulting in a total density of approximately 2.4×10^5 cfu ml⁻¹ (McFarland's Standard 0.5). 50ml of the inoculum was transferred to individual Eppendorf tubes containing 1 ml of Muller Hinton broth and dentin block. The blocks were incubated at 37°C for 7 days and every second day the blocks were transferred to fresh tubes containing 1 ml of broth contaminated with 50ml of *E. faecalis*. Broth purity was checked by sub culturing 5ml on sheep blood agar.

Antibacterial assessment**Dentine sampling**

Dentin blocks were incubated at 37°C in Mueller- Hinton broth for 7 days. The blocks were divided into 3 groups containing 10 teeth each.

Each block was carefully removed from the broth and held with artery forceps. The block was irrigated with 3ml of each irrigant for 1 min, except for the ‘control group’, which was not subjected to any irrigation. After irrigation, the dentin block was dried with sterile paper points. Sampling was carried out by preparing the root canal circumferentially with sterile Gates Glidden drills No.2 & No.3. The fine dentine chips obtained were collected in an Eppendorf tube containing 1 ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

Microbiological analysis:**Proportion of viable bacteria (PVB) using epifluorescence microscope**

0.5 ml of the undiluted sample from each specimen was vortexed for 5 min, washed once with sterile saline, harvested by centrifugation and resuspended with 100µl of staining solution (syto 9 and propidium iodide) using live/dead backlight bacterial viability kit. After incubation in a dark chamber for 15 min, the sample was centrifuged, and the supernatant was discarded. The pellet was resuspended in 10µl of distilled water. It was then immediately analysed under epifluorescence microscope which labels viable bacteria by green fluorescence (excitation FITC 450-490nm) and propidium iodide which marks dead bacteria by red fluorescence (excitation Rhodamine 540nm).

Visual fields at a magnification of 1000X were recorded. PVB was calculated as the number of bacterial cells associated with green fluorescence divided by the total number of bacteria emitting either green/ red fluorescence.

Minimum Inhibitory Concentration (MIC) & Maximum Bactericidal Concentration(MBC)

Minimum inhibitory concentration of the irrigant was determined by working out the initial concentration. A further dilution of 1/10 was made using saline. A serial dilution was made by mixing 0.5ml of diluted irrigant with 0.5ml Mueller-Hinton broth. Further doubling dilution were made by transferring 0.5ml into subsequent tubes and a range was obtained. control tube was prepared by adding 0.5ml of Muller-Hinton broth. To all the tubes 0.05 ml of *E.feacalis* which was grown at 37°C for 48 hrs and opacity adjusted to McFarland standard of 0.5 was added. The tubes were incubated at 37°C for 18 hrs. Minimum inhibitory concentration was determined by as the lowest concentration of irrigant showing inhibition of growth by absence of turbidity.

After the MIC readings were recorded, 5µl of suspension was subcultured from all tubes including control on Mueller-Hinton blood agar plates and incubated at 37°C for 48 hrs. MBC was determined as the lowest concentration of the antimicrobial agent in µg/ml showing no growth.

RESULTS

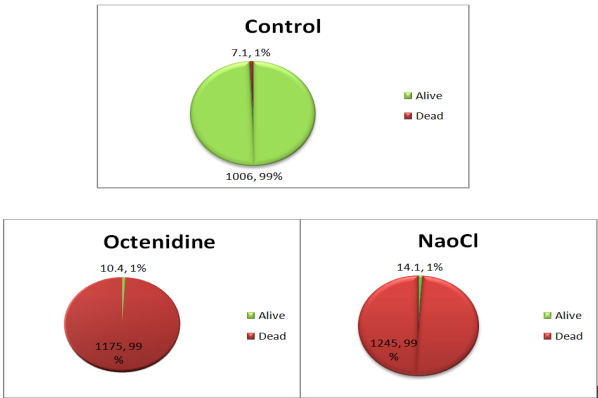
The results of the present study were subjected to statistical analysis to interpret the significant differences among the intracanal irrigants.

Table 1. MIC values of Octenidine hydrochloride

Tube	1	2	3	4	5
Potency(mg/ml)	2.5	1.25	0.62	0.31	0.15
	-	-	-	+	+

Table 2. MIC values of Sodium hypochlorite

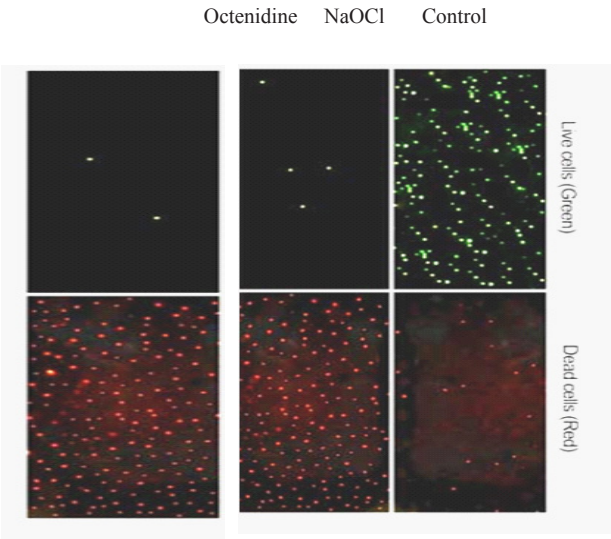
Tube	1	2	3	4	5
Potency (g/ml)	1000	500	250	125	62.5
	-	-	-	+	+



Graphs 1. Showing mean SYTO9 values for control group, Octenidine group and Sodium hypochlorite group



MIC and MBC of Octenidine and Sodium hypochlorite



Results of SYTO9 staining after irrigation

The data analysis can be summarized as follows:

1. The inhibition of growth in all the groups was statistically significant in comparison to control group (no irrigation)
2. Group 1 (Octenidine) was the more effective than Group 2 (Sodium hypochlorite) against *E. faecalis*.

DISCUSSION

The complex anatomy of teeth and root canals creates an environment that is a challenge to instrument and clean. In addition, the complex chemical environment of the root canal prevents antimicrobial irrigating solutions and medicaments from exerting their full potential against all microorganisms found in endodontic infections. While the knowledge of persistent bacteria, disinfecting agents, and the chemical milieu of the necrotic root canal has greatly increased, there is no doubt that more innovative basic and clinical research is needed to optimize the use of existing methods and materials, and to find new techniques and materials, or combination of materials, to achieve the goal of predictable, complete disinfection of the root canal system in apical periodontitis⁸.

Infection of the root canal is not a random event. The type and mix of the microbial flora develop in response to the surrounding environment. Factors that influence whether species die or survive are the particular ecological niche, nutrition, anaerobiosis, pH and competition or cooperation with other microorganisms. Species that establish a persistent root canal infection are selected by the phenotypic traits that they share in common and that are suited to the modified environment. Some of these shared characteristics include the capacity to penetrate and invade dentine, a growth pattern of chains or cohesive filaments, resistance to antimicrobials used in endodontic treatment, as well as an ability to grow in monoinfections, to survive periods of starvation and to evade the host response. Microorganisms that establish in the untreated root canal would experience an environment of nutritional diversity that changes with time. In contrast, the well-filled root canal offers the microbial flora little more than shelter from the host and microbial competitors, but in a small, dry, nutritionally limited space. In all cases, it is the environment that selects

for microorganisms that possess traits suited to establishing and sustaining the disease process⁹.

An endodontic irrigant should ideally exhibit powerful antimicrobial activity, dissolve organic tissue remnants, disinfect the root canal space, flush out debris from the instrumented root canals, provide lubrication, and have no cytotoxic effects on the periradicular tissues. However, the efficacy of these procedures also depends upon the vulnerability of the involved species¹⁰.

Studies that have recovered microbes from filled root canals with persistent periapical disease have shown a high proportion of enterococci, ranging from 29% to 77%. This contrasts with a rather low proportion of enterococci, around 5% or less, recovered from untreated infected root canals and raises the question of how and when enterococci establish in the root canal¹¹.

The recommendations for dentin tubule disinfection model proposed by Haapasalo and Orstavik was modified in this study, the direct exposure method for testing antimicrobial agent was applied¹². Intact human mandibular premolars with complete root formation, extracted for orthodontic or periodontal reasons were selected for this study to simulate the clinical scenario. Radiovisiography was used to confirm single canal anatomy to facilitate standardization of specimens.

The dentine blocks were standardized to a measure of 7mm. This was in accordance with the study performed by L. Tandjung *et al.*¹³ Standardization of the root canal diameter of the dentin blocks were achieved using k-files to a size 40. The root surface was coated with nail varnish, which was allowed to dry at room temperature for 1 hour.

Ten test specimens were randomly assigned to one tube containing 20ml of Mueller-Hinton broth. In order to improve broth penetration into dentinal tubules, it was treated ultrasonically, for 30sec. Mueller-Hinton broth was used since it showed better infection of the dentinal tubules when compared to Brain heart infusion broth¹⁴. The dentin blocks were sterilized in an autoclave at 121°C for 15 min to remove presence of microorganisms within the canal system. For sterility check, the tubes were incubated at 37°C for 72 hrs.

Enterococcus faecalis (ATCC 29212) was used as the test organism as this Gram positive

facultative anaerobic bacterium is the most common isolate found in failed cases. Almyroudi *et al.*,¹⁵ found it easy to maintain and culture *E.faecalis* under laboratory conditions.

7 day infection period was used in this study. The broth was changed on alternate days to prevent saturation of broth with *E.faecalis* and to replenish the nutrient source for the organism. **Following infection of the dentin blocks antibacterial testing was performed**

The irrigants used in this study were, 0.1% octenidine, and 5% Sodium Hypochlorite. 3ml of each irrigant was taken based on the studies by Gomes *et al* and was irrigated for 1 min following the previous study done by Radcliff *et al*. The control was teeth which were exposed to *E.faecalis* but were not irrigated using any irrigants. This allowed inter-group comparison of the antibacterial activity of each group

Dentin debris was harvested first with Gates Glidden drill No.3 followed by No.4, this allowed sampling to a depth of 400µm from the canal lumen¹⁶. Similar sampling was done by Krithika Datta *et al.*,¹⁷. The debris was collected in Eppendorf tubes containing 1ml of phosphate buffered saline and 3 small glass beads. The suspension was homogenized by a vigorous vortexing for 5 min. The dentin chips were allowed to sediment for 5 min and the supernatant was used for microbiological analysis.

The results obtained from the present study revealed significant information on the newer intracanal irrigant used, against *E.faecalis*. Octenidine proved to be more effective against *E.faecalis*, than 5% Sodium hypochlorite.

The results are similar to a study by Tirali *et al*, in which Octenidine was found to be more effective than 5.25% NaOCl solution against *E.faecalis* as antimicrobial endodontic irrigants. There was a significant difference between the solutions in terms of producing negative cultures for the tested microorganism¹⁸.

The antibacterial potential of octenidine has been well documented and compared to some other disinfectants used in endodontics. The probable reason for the enhanced antimicrobial effect of Octenidine can be attributed to its cation-active structure that tends to bind readily to the negatively charged bacterial cell envelope,

automatically disrupting the vital functions of the cell membrane and killing the cell. Preliminary results point to a strong adherence particularly to lipid components (e.g. cardiolipin) prominent in bacterial cell membranes explaining the high antimicrobial efficacy without adversely affecting human epithelia or wound tissue¹⁹.

Furthermore, it has been shown that octenidine resists an organic challenge, i.e. maintains its antimicrobial efficacy in the presence of organic material comparably to chlorhexidine and iodine. This is of interest, as, in a root canal system both organic and inorganic inhibitory factors are present that may weaken the antimicrobial efficacy (Haapasalo *et al.*).(20)

From the results of this study, it can be concluded that the experimental irrigants used had good antibacterial efficacy in eliminating *E.faecalis*. Octenidine had the lowest MIC and MBC value suggesting it to be a potent irrigant.. However antimicrobial effect is not the only requirement of an endodontic irrigant. Many studies have proved the tissue dissolving property of NaOCl, which is a significant attribute that the other irrigants do not possess. Further studies are required to find out the tissue dissolving properties of Octenidine.

CONCLUSION

Within the parametric limitations of this in vitro study, it can be inferred that:

- 1) The MIC and MBC values of Octenidine hydrochloride were superior to Sodium hypochlorite, thereby revealing a good bactericidal property.
- 2) The special staining dye Syto9 showed significant reduction in the proportion of viable bacteria thereby proving the efficacy of the 2 dental irrigants. This method however, is technically very sensitive and not within reach of all laboratories.
- 3) Octenidine which is not yet evaluated widely as an irrigant showed the maximum efficacy, against *E.faecalis* thus proving to be a potent irrigant. Further in vivo studies are required to evaluate the tissue dissolving properties and also their safety in dental practice.

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