The Antimicrobial Efficacy of Ozonated Water, Chlorhexidine and Sodium Hypochlorite against Single Species Biofilms of Enterococcus faecalis and Candida albicans

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ABSTRACT

The aim of the study was to investigate the antimicrobial efficacy of 5.25% sodium hypochlorite (NaOCl), 2% chlorhexidine (CHX) and 24mg/L ozonated water against single species biofilm of Enterococcus faecalis and Candida albicans. Cellulose nitrate membrane filters were inoculated with the tested microorganisms' suspensions and incubated at 37°C for 48 hours. The generated biofilms were transferred into tubes containing the selected antimicrobial solution or saline for 20 minutes. Then, the antimicrobial activity of the agents was neutralized and semiquantitative culture was performed for calculation of the number of colony forming units (CFU) per ml. The tests were carried out in triplicates. Furthermore, scanning electron microscopic (SEM) examination was done on four extra generated single species biofilms per each microorganism; to examine the established biofilms and the antimicrobial effect of the test solutions. It was found that NaOCl was capable of producing no growth. CHX resulted in residual growth of both tested microorganisms with no statistical significant difference compared to NaOCl. Ozonated water was ineffective with a significant difference when compared with NaOCl or CHX. SEM examination of the untreated biofilms revealed a well developed dense layered aggregation of the microorganisms. After treatment with the test antimicrobials, SEM showed that NaOCl succeeded in complete degradation of the biofilm. This effect was less with CHX and even lesser with ozonated water. Thus, the antimicrobial effectiveness of 5.25% NaOCl was reinforced, whereas that of ozonated water was questionable.

Keywords: Biofilm, Enterococcus faecalis, Candida albicans, sodium hypochlorite, chlorhexidine, ozonated water.

INTRODUCTION

Microorganisms and their byproducts are the main cause of pulp and periapical infection. Their control and elimination during endodontic therapy provides a favourable environment for periapical lesion healing¹. Most endodontic infections have polymicrobial etiology; Enterococcus faecalis and Candida albicans are considered the most resistant species and possible causes of root canal treatment failures². In infected root canal systems, bacteria grow mostly in sessile biofilm aggregates; in which they are embedded in an extracellular matrix. This matrix is crucial for the initial attachment as well as for holding the biofilm microorganisms together³.

The elimination of microorganisms and their byproducts from the root canal system is enhanced by the use of antimicrobial irrigant solutions during instrumentation⁴. Different irrigating solutions with antimicrobial properties and tissue biocompatibility are being studied and applied clinically in endodontic therapy. Sodium hypochlorite (NaOCl) is the most widely used irrigant because of its antimicrobial and organic tissue dissolving ability. However, there are clinical concerns regarding its toxicity to the periapical tissues, possibility to damage permanent tooth follicles in root canal treatment of deciduous teeth⁵, and the potential to produce allergic reactions⁶. It also weakens dentine by reducing its flexural strength and resilience, rendering the tooth more susceptible to deformation and possibly fractures⁴. Chlorhexidine has been suggested as an alternative to NaOCl. It has a potent broad spectrum antimicrobial activity with the advantages of holding substantivity, and low grade of toxicity. However, it lacks tissue dissolving ability⁵,⁶. Considering the disadvantages of the available antimicrobial irrigants, the search for new alternatives is necessary. Ozonated water is a new option that is being studied. Ozonated water has shown an antimicrobial effect in periodontology and...
restorative dentistry. In Endodontics, its role as an effective antimicrobial has been evaluated only in few reports. 

Considering the clinical endodontic relevance of root canal biofilms and the role of different irrigating antimicrobial solutions in their destruction, the aim of the present study was to investigate the efficacy of sodium hypochlorite, chlorhexidine and ozonated water against single species biofilm of Enterococcus faecalis and Candida albicans generated on a biofilm model.

MATERIAL AND METHODS

I. Test organisms and preparation of standardized suspensions:
The study design was formed of two groups based on the microbial species used. Two strains were used; Enterococcus faecalis and Candida albicans isolated from infected root canals and identified by the conventional methods. Tubes containing 5ml. brain heart infusion (BHI) broth (oxoid, UK) were inoculated with colonies of either E. faecalis or C. albicans obtained from 48 hours culture at 37°C on blood agar plates. The resulting suspension was then adjusted to match the 0.5 McFarland turbidity standard with an approximate concentration of 1.5X10^8/ml. and 5x10^5/ml. for E. faecalis and C. albicans, respectively.

II. Antimicrobial agents and their neutralizers:
Three experimental subgroups were formed according to the test antimicrobial solutions: (1) Sodium hypochlorite 5.25% (NaOCl) (Clorox, Nobelwax Factories for chemicals, Egypt), (2) Chlorhexidine 2% (CHX) (Kahira Pharmaceutical and Chemical Industrial Company, Egypt), (3) Ozonated water was generated at the National Research Centre (NRC)- Department of Chemical Engineering - Giza. It was prepared by bubbling ozone through sterile deionized water using a closed-ended silicon tube attached to the ozone delivery nozzle. The oxygen flow-rate was kept constant and monitored into the ozone generator. Surplus ozone was captured by 2% (w/v) KI solution. The experiments were carried out at ambient temperature. The concentration of ozonated water at the time of application to the constructed biofilms was calculated to be 24mg/L, based on the time dependent biodegradation of ozone in water at 4°C (Figure 1). 0.9% sterile saline (Egypt Otsuka Pharmaceutical company, SAE) was used as a positive growth control. To halt the activity of the tested antimicrobials, a neutralizer 0.1% sodium thiosulphate was used for both NaOCl and ozonated water, whereas 0.5% Tween 80 plus 0.07% lecithin was used for CHX.

III. Biofilm formation:
The method used herein was based on that described by Sena et al. Single species biofilm of E. faecalis and C. albicans was generated on sterile cellulose nitrate membrane filters (0.2 µm pore size, 13mm. diameter) (Whatman International Ltd., Maidstone, UK). This was done by aseptically placing the membrane filter on the surface of a blood agar plate and thoroughly covering the membrane with 50 µl. of either the tested E. faecalis or C. albicans prepared suspension. Four membranes were used for each agar plate. Then the agar plates were incubated at 37°C for 48 hours.

VI. Biofilm susceptibility testing:
The formed biofilms were treated with the different test antimicrobial solutions and their susceptibility was evaluated by both semiquantitative culture as well as direct visualization with the scanning electron microscope (SEM).

A. Biofilm treatment with tested antimicrobial solutions:
The biofilms of either E. faecalis or C. albicans were aseptically transferred to 10 ml. of phosphate buffered saline (PBS Dulbecco’s, GIBCO BRL, Paisely, Scotland) to remove loosely attached planktonic microorganisms. This was followed by transferring them to 5ml. of one of the tested antimicrobial solutions or saline. After a contact time of 20 minutes, the treated membrane filters were transferred to tubes containing 2 ml. freshly prepared BHI broth plus the specific neutralizer.

B. Detection of the antimicrobials’ effect by:
   i. Semiquantitative culture:
The BHI broth plus the specific neutralizer containing the treated biofilm was vortexed for 30 seconds to re-suspend the present microorganisms. Ten fold serial dilutions
were made from the formed bacterial suspension; from which 50µl. was spread on blood agar plates. The inoculated plates were incubated at 37°C for 48 hours and the approximate number of colony forming units per ml. (CFU/ml.) was calculated. The tests were carried out in triplicates for each test solution and microorganism.

ii. Scanning electron microscopic examination:
To confirm the establishment of the biofilms and to examine the antimicrobial effect of the test solutions, four extra single-species biofilms per each of *E. faecalis* and *C. albicans*, were generated as previously described. After treatment with each of the three test solutions, the membranes were transferred to the freshly prepared broth plus neutralizer. The untreated and treated membranes were aseptically placed on blood agar plates. Then, they were transferred at 4°C to the Central Laboratory at the NRC for SEM examination.

The membranes were fixed in 3% glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.2) for 1 hour, followed by exposure to osmium tetroxide for 48 hours. After rinsing the membranes in the buffer three times, they were dehydrated through graded ethanol series (from 10 – 100%) and critical point dried with liquid CO2. The membranes were then mounted on stubs with double-sided adhesive tapes, coated with gold and viewed with SEM (JOEL, JXA-840A-Electron probe microanalyzer-Japan). The topography of the membranes was examined at a magnification ranging from X2000 up to X5000.

Statistical Analysis:
Data were statistically described in terms of mean ± standard deviation (± SD). Comparison of quantitative variables between the study groups was done using Kruskal Wallis analysis of variance (ANOVA) test, with Mann Whitney U test for independent samples as post hoc multiple two-group comparisons. Within the group, comparison of quantitative variables was done using Wilcoxon signed rank test for paired (matched) samples. A probability value (p value) less than 0.05 was considered statistically significant. All statistical calculations were done using Microsoft Excel version 7 (Microsoft Corporation, NY, USA) and SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 13 for Microsoft Windows.
RESULTS

i. Semiquantitative culture:
The mean number of CFU of *E. faecalis* and *C. albicans* after the application of the tested antimicrobials and saline is shown in Table 1. After application of NaOCl, no growth occurred, while treatment with CHX resulted in residual growth. However, there was no statistical significance between NaOCl and CHX on the two test microorganisms. On the other hand, ozonated water was ineffective in reducing the bacterial growth of both tested microorganisms. It showed a significant difference when compared with either NaOCl or CHX, but did not show any statistical significance when compared with saline.

<table>
<thead>
<tr>
<th></th>
<th><em>E. faecalis</em> Mean (±SD)</th>
<th><em>C. albicans</em> Mean (±SD)</th>
<th><em>P</em> value</th>
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<tbody>
<tr>
<td>Saline</td>
<td>65.5 x 10^6 ± 48.4 x 10^6 (a)</td>
<td>1.7 x 10^6 ± 1.3 x 10^6 (a)</td>
<td>0.1</td>
</tr>
<tr>
<td>NaOCl</td>
<td>0 ± 0 (b)</td>
<td>0 ± 0 (b)</td>
<td></td>
</tr>
<tr>
<td>CHX</td>
<td>1060 ± 1500 (b)</td>
<td>46.6 ± 80.8 (b)</td>
<td>0.18</td>
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<tr>
<td>O3 water</td>
<td>96.6 x 10^6 ± 141.5 x 10^6 (a)</td>
<td>0.1 x 10^6 ± 1.04 x 10^6 (a)</td>
<td>0.10</td>
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<tr>
<td><em>P</em> value</td>
<td>0.028</td>
<td>0.024</td>
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*P* value <0.05, Different letters denote statistical significance.

ii. Scanning electron microscopic examination:
The formed biofilms as well as the effect of the three test antimicrobials on the established biofilm was examined by SEM. The untreated biofilms showed a well developed dense layered aggregation of the microorganisms (Figures 2a and 3a).

NaOCl succeeded in complete degradation of the biofilm (Figures 2b and 3b), CHX produced a less effect (Figures 2c and 3c) and a lesser effect was seen with ozonated water (Figures 2d and 3d).
Figure 2: SEM of: (a) untreated *E. faecalis* biofilm on cellulose nitrate membrane, and after treatment with (b) NaOCl; there is total biofilm degradation, (c) CHX; scattered cells are seen and (d) ozonated water; more scattered cells embedded in the extracellular matrix are detected (original magnification X5000).

Figure 3: SEM of: (a) untreated *C. albicans* biofilm on cellulose nitrate membrane, and after treatment with (b) NaOCl; there is total biofilm degradation, (c) CHX; two yeast cells can be seen (arrows) and (d) ozonated water; more yeast cells are shown, some of which are in a state of budding (original magnification X5000).
DISCUSSION

One of the primary objectives of endodontic therapy is the microbial reduction, which in turn promotes the normal healing process of the periodontal tissues. Mechanical instrumentation and irrigation are important agents in eliminating the microorganisms from the root canal system. Mechanical debridement alone, however, does not result in total or permanent reduction of bacteria. Therefore, the use of antimicrobial agents has been recommended as an adjunct to mechanical instrumentation to reduce the number of microorganisms.

A number of laboratory approaches have been used to test the effectiveness of antimicrobial agents. These include: inoculation of broth cultures of selected bacteria with the antimicrobial agent or growth of selected bacteria on agar surfaces and the use of the disc diffusion method. These tests use planktonic cultures for testing the antimicrobial efficacy of endodontic irrigants; whereas microorganisms in the actual clinical condition attach to the root canal walls forming biofilms. Accordingly, the use of planktonic cultures alone would not represent a clinically relevant test.

Organization of bacteria within biofilms confers a range of phenotypic properties that are not evident in their planktonic counterparts and confers a reduced susceptibility to antimicrobial agents. Although in vivo testing of antimicrobial agents is the most definitive method for establishing efficacy, yet the controlled experimental conditions in the in vitro testing is also useful when evaluating potency and spectrum of activity. The most realistic in vitro model would be to grow bacterial biofilms on the root canal surfaces of extracted teeth. Many studies have tried artificial infection of extracted teeth with selected bacteria and the in-use irrigation with the test antimicrobial agents was performed.

The membrane filter disc adopted in the present study was, however, preferred to the extracted tooth model. It has been used by several researchers to compare antimicrobial efficacy of the test agents on oral bacterial biofilm. It eliminates the variation of root canal anatomy, properties of dentin (collagen and organic content, degree of calcification, and content of dentinal tubules) and reduces the variations in quantity of growth and contact of the biofilm with the antimicrobial agent. Moreover, this model has the advantage of growing biofilm on standardized surfaces, thus allowing more accurate assessment of the efficacy of antimicrobial agents. It is therefore a useful and convenient method for preliminary testing of antimicrobial agents.

_E. faecalis_ and _C. albicans_ were selected in this study searching for an effective endodontic antimicrobial irrigant. _E. faecalis_, Gram-positive facultative anaerobic cocci, is commonly found in root canals of failing endodontically treated cases. It has the capability to form biofilm in root canals, penetrate the dentinal tubules, exhibit strong adhesion to collagen and display resistance to chemomechanical preparation and intracanal medication. It can also survive in a quiescent phase with low metabolic activity for a long period of time. _C. albicans_ is the fungal species most commonly isolated from root canals of teeth with pulp necrosis, especially those with persistent endodontic infections. It has also shown the ability to colonize dentinal walls, penetrate into tubules and form biofilm.

NaOCl, CHX and ozonated water were the antimicrobial agents selected in this study to test their efficacy. Many studies have evaluated the antimicrobial effects of NaOCl and CHX in endodontic treatment, thus they may be considered as a reference to which new irrigants can be evaluated. The use of ozone is justified as a new option of an irrigating agent with antimicrobial action. Ozone acts on bacterial cell walls and cytoplasmic membranes, as well as on fungi, protozoa and viruses. It forms oxidized radicals in the presence of water that penetrate and act on cell membranes, affecting the osmotic stability, promoting the oxidation of amino acids as well as nucleic acids, and causing cellular lysis. Ozonated water rather than gaseous ozone was chosen in this study as it would be more clinically applicable.

The effectiveness of the irrigant is dependent on the contact time, concentration, and the nature of the microorganism. In the present study, 20 minutes was the duration chosen for contact between the tested irrigant and the biofilm, guided by previous in vitro
studies on ozonated water with a contact time of 10 minutes\textsuperscript{7} and 20 minutes\textsuperscript{8}. The chosen contact time was also based on a pilot study initially performed using ozonated water against both species’ biofilms for a contact time of 10 minutes; which proved to be ineffective. Since the antimicrobial effectiveness of NaOCl and CHX was reported to be concentration dependent\textsuperscript{31}, concentrations of 5.25% NaOCl and 2% CHX were used in this study. The concentration of ozonated water however, depended on the maximum concentration that could be generated by the ozone generator.

Results of the present study indicated that the most effective root canal irrigant for disrupting biofilms and achieving a negative culture was 5.25% NaOCl, and that even though CHX effectively reduced the CFU, it did not achieve the 100% kill as achieved with NaOCl. This was in agreement with many studies; Giardino et al.\textsuperscript{31} showed that 5.25% NaOCl could disintegrate and remove the \textit{E. faecalis} biofilm generated on cellulose nitrate membrane filters at all tested times starting from 5 minutes up to 60 minutes. Dunavant et al.\textsuperscript{21} revealed that 6% NaOCl was able to eliminate the \textit{E. faecalis} biofilm after 1 and 5 minutes. Whereas, 2% CHX was less effective; achieving 60.5% kill. Moreover, Clegg et al.\textsuperscript{31} reported that 6% NaOCl applied for 15 minutes was capable of rendering bacteria non viable and physically removing the polymicrobial biofilm generated on hemisections of root apices. They also reported that 2% CHX applied for the same time duration resulted in negative cultures from the specimens, but failed to disrupt the biofilm as revealed by SEM examination. Abdullah et al.\textsuperscript{27} found \textit{E. faecalis} grown in biofilm to be more resistant to 0.2% CHX than to 3% NaOCl; where the latter achieved a 100% kill in 2 minutes’ time. Similarly, Spratt et al.\textsuperscript{21} showed that 2.25% NaOCl achieved a 100% kill of \textit{E. faecalis} grown on cellulose nitrate membrane filters after 15 minutes while 0.2% CHX was effective after 60 minutes. Furthermore, Sena et al.\textsuperscript{5}, revealed that 5.25% NaOCl, with and without mechanical agitation, eliminated single species biofilm of either \textit{E. faecalis} or \textit{C. albicans} in 30 seconds’ time. They also revealed that 2% CHX, with agitation, eradicated both organisms in 30 seconds’ time, but required longer contact time (5 minutes) to produce no growth of \textit{C. albicans} when the same concentration of CHX was used without agitation.

On the other hand, the results were not consistent with Önçag et al.\textsuperscript{6}, who tested CHX on root segments infected with \textit{E. faecalis}. They showed that CHX, whether alone in a concentration of 2% or in a concentration of 0.2% and combined with cetrimide, was more effective than NaOCl. The difference could be attributed to the inactivation of NaOCl by the root dentin or the inability of NaOCl to penetrate into accessory canals or dentinal tubules in their model.

5.25% NaOCl was equally effective on \textit{E. faecalis} and \textit{C. albicans} biofilms. The SEM observation confirmed the negative culture results, which was in accordance with Clegg et al.\textsuperscript{31}. Its anti-biofilm effectiveness might be attributed to its tissue dissolving capacity and therefore it may be less inhibited by the extracellular matrix of the biofilm\textsuperscript{23,27}. Consequently, the removal of the organic tissue eliminates the bacterial attachment to the surface and to other microorganisms\textsuperscript{3}.

The antimicrobial effectiveness of CHX in the present study might have been enhanced by increasing the contact time\textsuperscript{5}, agitation\textsuperscript{5,31} or the addition of agents to physically disrupt the biofilm as cetrimide\textsuperscript{6}.

Under the conditions of the present study, 24mg/L ozonated water was the least effective antimicrobial agent. In vitro reports on the antimicrobial effect of ozonated water are controversial. Several in vitro studies have shown that the antimicrobial activity of ozone depends on a number of variables such as: the used experimental model, the species of the microorganism, whether planktonic or in a biofilm, the ozone concentration, its flow rate, contact time, delivery system: gaseous ozone, or ozonated water, or ozone bubbled in the experimental model at constant flow rate, the depth of action, the use of sonication and the degradation of ozone in water by time\textsuperscript{4,7,8-10}.

Some studies reported the insufficient effectiveness of ozone in accordance with the present study. In their experiment to investigate the antibacterial efficacy of ozone using \textit{E. faecalis}, Hems et al.\textsuperscript{4}, concluded that 0.68mg/l ozonated water had an antibacterial effect against planktonic cells after 240 seconds. However, it was ineffective against cells in a biofilm for the same time
duration, unless the cells were displaced in the surrounding medium by agitation. Estrela et al. found that ozonated water circulating at a constant rate of 50ml/min for 20 minutes, was ineffective against E. faecalis in extracted human root canals.

On the other hand, other studies reported its antimicrobial effectiveness. Nagayoshi et al., created an in vitro model for the infection of bovine teeth root canals using E. faecalis. Contrary to the present study results, irrigation with 4 mg/L ozonated water at a constant flow rate of 30ml/min. for 10 minutes with sonication, resulted in significant reduction of the colony forming units. In their study, ozonated water had nearly the same antimicrobial activity as 2.5% NaOCl. Such a difference may be attributed to their use of sonication during irrigation. This justification is supported by further results within the same study; where irrigation with the same concentration of ozonated water but without sonication resulted in a number of colony forming units nearly the same as when irrigation was done using distilled water with sonication. In a study using a freshly prepared suspension of yeast cells, de Faria et al., observed that bubbling 3.3mg/l. ozonated water at a flow rate of 3L/min. resulted in a reduction of the C. albicans concentration, which was directly proportional to the contact time. They concluded that the complete fungicide effect was observed only after 5 minutes. Cardoso et al., inoculated single-rooted human teeth with C. albicans and E. faecalis, and then used 24mg/L ozonated water as an irrigant for 20 minutes in the form of 3ml irrigation between successive instrumentation from size 35 up to size 80. They detected significant reduction of both organisms at the immediate sampling but increased values were detected after 7 days; suggesting absence of a residual effect for ozonated water. They also revealed that ozonated water was more effective on C. albicans than on E. faecalis.

Ozone is relatively unstable in aqueous solution; undergoes auto decomposition with lack of residual power effects. Accordingly, the concentration of ozone changed by time, in contrast with NaOCl and CHX; whose concentrations remained high and constant throughout the experiment. The absence of an effect by ozonated water might be attributed to the depletion of ozone as it diffused into the biofilm by virtue of its organic composition. The biofilm’s extracellular matrix probably provided large suitable targets for ozone to react with, thus shielding the microorganisms from ozone.

In conclusion, the biofilm model was effective in determining the in vitro antimicrobial efficacy of different root canal irrigants. The effectiveness of 5.25% NaOCl was reinforced. It proved to be able to remove completely the biofilm organized on the surface of the membrane; CHX produced a valid reduction in the CFU, whereas ozonated water failed in this action.

Further studies are recommended to test for the effect of NaOCl and CHX using infected tooth models as well as against multispecies biofilms to mimic the clinical conditions. In addition, more tests should be directed towards the study of the effect of ozonated water applied at higher concentrations with constant flow. Attention should be directed towards overcoming its problem of instability; ozonation may be complemented with the addition of another reagent to take over the action of ozone. Moreover, it might be of help if ozonated water is generated on the site of application.

Although the present study tested irrigants without mechanical agitation, strategies to treat biofilms in root canals may benefit from disruption of the multicellular structure mechanically; by root canal instrumentation, ultrasonic vibration, and the use of surfactants with dissolution of the matrix polymers. Moreover, further search for new root canal irrigants having greater antimicrobial and biofilm potential as well as tissue biocompatibility is still recommended.
REFERENCES


الجراثيم

**ملاحظة: يوجد خطأ في النص العربي، حيث يظهر بعض الكلمات المتكررة أو غير واضحة.**