

ANTIMICROBIAL EVALUATION OF SODIUM HYPOCHLORITE AND OZONATED WATER ON *E. FAECALIS* BIOFILM

Original Article

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ABSTRACT

This study aim to evaluate antimicrobial efficacy of sodium hypochlorite (NaOCl) and ozonated water against *Enterococci faecalis* biofilm. The bacterial biofilm was exposed to 2.62%, 1.31% NaOCl and 0.1ppm ozonated water over a range of time periods. The presence of viable cells was determined by enumeration of colony forming units (CFU). All experiments were repeated four times (n=4). The effectiveness of the agents was compared using non-parametric Kruskal-Wallis test. The result revealed that 2.62% of NaOCl can completely kill *E. faecalis* biofilm in 15 minutes whereas 1.31% NaOCl required a longer time to produce such effect. 0.1ppm ozonated, however, did not exhibit any antimicrobial effect within the period of time tested. From this study, it can be concluded that 0.1ppm ozonated water was not comparable with 2.62% and 1.31% NaOCl in antimicrobial efficacy against *E. faecalis* biofilm.

Key words: sodium hypochlorite, ozonated water, biofilm, antimicrobial efficacy

INTRODUCTION

Pulp and periradicular diseases are of microbial origin (1). *E. faecalis* is commonly isolated in root canals of failing treatment (2, 3). It is among the few facultative bacteria associated with persistent apical periodontitis (2). This bacteria species has the ability to invade dentinal tubules (4) and one of the hardest microorganisms to be eliminated once it has established itself in root canals (3). In addition, it exhibits a high level of resistance to a wide range of antimicrobial agents (5).

Bacteria in planktonic suspension and biofilm form have shown different response to antimicrobial agent tested on them. Of particular concern is that our ability to eradicate biofilm from infected hosts is substantially lower than that for dispensed, planktonic bacteria (6). A clinically valid and simple assay for microbial susceptibility testing could be based on a biofilm model. Generation of such biofilm in root canals of extracted teeth provides a more realistic scenario (7, 8). However, the result may be confounded by the variation in root canal

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anatomy between teeth. In addition, the collection and preparation of the teeth prior to experimentation is laborious and very labour intensive. There are several biofilm model that have been used in laboratory such as the closed growth model using solid media and membrane filter, continuous growth model using tubular model and others. In endodontic research, the most reliable and allows a more accurate assessment of antimicrobial efficacy thus far is the membrane filter based model (9, 10).

NaOCl is by far the most commonly used irrigant in endodontic therapy. It provides gross debridement, lubrication, destruction of microbes and dissolution of tissues. However, this irrigant lead to problems including toxicity, odour and discoloration of operatory items. Thus, an equally effective, but safer irrigant is desirable.

Ozone is a very powerful bactericide that can kill microorganisms effectively. It is an unstable gas, capable of oxidizing any biological entity. It was reported that ozone at low concentration, 0.1 ppm, is sufficient to inactivate bacterial cells including their spores (11). It is present naturally in air and can be easily produced by ozone generator. When introduced in water, ozone dissolves rapidly and dissociates rather quickly. The concentration of ozone in ozonated water can be measured using a dissolved ozone meter.

Although ozonated water is a powerful antimicrobial agent against bacteria, fungi, protozoa, and viruses, less attention has been paid to the antibacterial activity of ozonated water in bacterial biofilm (12) and hence in root canal infections. Therefore, it is the purpose of the present study to evaluate the antimicrobial effectiveness of ozonated water and NaOCl against *E. faecalis* biofilm.

MATERIALS & METHODS

Bacterial strain

E. faecalis strain was supplied as cultured in brain heart infusion broth plus 10% glycerol, BHIG (BHIG - Merck, Poole, UK) and were kept frozen (-70°C). This procedure ensured mid-log-stationary-phase cells in all of the experiments (13). Throughout this study the pure culture were maintained and sub-cultured weekly and the stock were re-frozen in BHIG broth for future use.

Test solutions

In this study, NaOCl (Clorox Malaysia Sdn. Bhd.) and ozonated water were used as the test solutions. Concentration of Clorox® (Clorox Malaysia Sdn. Bhd.) was initially determined using standard thiosulphate titration method. Subsequently, the pure solution (4.77%) was diluted to achieve the desired NaOCl concentration of 2.62%, 1.31%, 0.65%, 0.32%, 0.16%, 0.08%, 0.04% and 0.02%. Fresh solution was prepared prior to each experimental testing.

Ozonated water was prepared using an ozone gas generator machine. The corona discharge ozone gas generator ORM AW600 (ORM beauty and health care SDN BHD) was used as the source of the ozone gas. The ozone gas produced from this ozonizer was introduced into 1 litre of de-ionized water for 20 minutes. The concentration of dissolved ozone in water was measured using an EcoZone™ Model EZ10W Portable Dissolved Ozone Meter. The concentration of ozonated water used for this study was 0.1 ppm. Ozonated water was freshly prepared and was used within 20 minutes of preparation.

Agar diffusion test – minimum inhibitory concentration (MIC) of test solutions

In the first part of this study, an agar diffusion test was carried out to determine the minimum concentration of test agents that produce zone of inhibition. Petri dishes containing Brain Heart Infusion (BHI) agar enriched with horse blood (BECTON DICKINSON, USA) were seeded with *E. faecalis* strain using sterile swabs that were brushed across the agar surfaces. 100µL of test solutions (NaOCl – 4.77%, 2.62%, 1.31%, 0.65%, 0.32%, 0.16%, 0.08%, 0.04% and 0.02%; ozonated water at 0.1 ppm) were pipetted onto an individual paper disc (13mm in diameter) until all of the solution was absorbed. NaOCl at 4.77% acts as a positive control and distilled water as a negative control. The paper disks were then placed on top of the agar surface and incubated in aerobic condition at 37°C for 24 hours. All experiment was performed in triplicate (n=3) for each of the test solutions. Following incubation, zone of inhibition were measured using a clear ruler. The minimum concentrations that produce zone of inhibition were recorded and act as

baseline concentration for later part of the experiment.

Minimum biofilm eradication concentration, MBEC

The next part of the experiment involves determination of antimicrobial effect of test solutions against *E. faecalis* biofilm using a membrane filter model. *E. faecalis* was revived by inoculating on BHI agar (BECTON DICKINSON, USA) and incubated overnight in an aerobic incubator (Mettler, Germany) at 37°C. The purity of the isolate was confirmed based on colony morphology and gram staining. A bacterial suspension was prepared by suspending a loop-full of *E. faecalis* colony from the culture into 10mL of 0.85% sterile saline. The cell suspension was adjusted spectrophotometrically to match the turbidity of a McFarland 0.5 BaSO₄ standard which was approximately 1.5 X 10⁸ colony forming units/mL. An aliquot of 50µL of the suspension was seeded onto 13.0 mm diameter (0.22µm pore diameter) cellulose acetate membrane filters (Sartorius Malaysia Sdn. Bhd.) which were then placed on the surface of BHI agar enriched with horse blood. Six membranes were used for each plate. The plate was then incubated in an aerobic incubator at 37°C for 48 hours. Following incubation, non-adherent bacteria on the membrane filters was washed away by transferring the membrane filters aseptically with sterile tweezers from BHI agar plate into a universal bottle containing 10mL of sterile phosphate buffered saline (PBS) solution. Each membrane filter was then transferred to a sterile universal bottle containing 5mL of each concentration of test solution accordingly and kept for 1 minute. The antimicrobial activity of the test solution was then ceased by transferring the membrane into a universal bottle containing 5mL of 1.93% sodium thiosulphate for neutralization of 0.5% NaOCl and 3.86% sodium thiosulphate for neutralization of more than 1% NaOCl and 0.1ppm ozonated water accordingly. The membrane was then transferred to a 5mL of BHI broth (BECTON DICKINSON, USA) and sonicated for 5 minutes in the ultrasonic bath (Segar Media Sdn. Bhd.). This helps in re-suspending the sessile bacteria from the membrane and produced a 'neat' bacterial suspension. Aliquot of 20µL of the bacterial suspension was then mixed into 180µL of BHI broth in microtiter plate (NUNC, Denmark). 50µL of the broth was then plated on BHI agar plate. Value of MBEC was then determined.

CFU/biofilm at different exposure time

In this part of the experiment, 5mL of test solution (2.62% NaOCl and 0.1ppm ozonated water – the highest concentration at which bacteria can regrow) were prepared. Following incubation, non-adherent bacteria on the membrane filter was washed away by removing the membrane filter

aseptically with sterile tweezers from BHI agar plate and transferred to a universal bottle containing 10mL of sterile PBS solution. Each inoculated membrane filter was then transferred to each sterile universal bottle containing 5mL of 2.62% NaOCl (5 bottles) or 0.1ppm ozonated water (5 bottles) accordingly.

At 0 min (i.e. no contact with the test solution), the individual inoculated membrane filters was removed aseptically with sterile tweezers from BHI agar plate, transferred to a sterile universal bottle containing 5mL of PBS and subsequently to 5mL of BHI broth and sonicated for 5 minutes to re-suspend the bacteria. This produced a "neat" bacterial suspension. Plating was carried out by aliquot 50 μ L of the broth to BHI agar plate. The plate was then incubated for 24 hours in an aerobic incubator at 37°C. The number of *E. faecalis* colonies on the agar surface was then manually calculated. The number of *E. faecalis* colony forming unit per biofilm (CFU/biofilm) was then enumerated. This was the negative control.

The biofilm was exposed to the test solution for 1, 2, 4, 8, 15, 30 and 60 minutes. Following this, the membrane filter from each test solution was transferred into a sterile universal bottle containing 5mL of 3.86% sodium thiosulphate to stop any further action of the test solution on the biofilm. Subsequently, the membrane filter was transferred into a sterile universal bottle containing 5mL of BHI broth. All universal bottles were sonicated for 5 minutes to re-suspend the bacteria from the biofilm. 10-fold serial dilution was performed in sterile microtitre plate by mixing 20 μ L of the bacterial suspension into 180 μ L of BHI broth. 50 μ L from each dilution was then plated on BHI agar plate. The plates were then incubated for 24 hours in an aerobic incubator at 37°C. The number of *E. faecalis* CFU/biofilm was then manually calculated.

The data collected was compared within and between groups whereby One-way ANOVA was used. Variances were compared using Levene's statistics. Since the homogeneity of variances assumption was not meet, the non-parametric Kruskal-Wallis test was performed. The level of significance was set at 5% ($p = 0.05$).

RESULTS

MIC of test solutions

Table 1 represents the mean diameter of zone of inhibition (mm) for various concentration of NaOCl and 0.1ppm of ozonated water. Distilled water (DW) was used as control. At concentration of 4.77%, NaOCl was capable in producing a mean diameter of 32 mm of zone of inhibition. At 0.02%, however, NaOCl did not produce any zone of inhibition. For ozonated water, the mean diameter of zone of inhibition produced was 14.0mm.

Determination of MBEC

Table 2 represents the mean value of CFU/biofilm using range of concentration of NaOCl and 0.1ppm of ozonated water against *E. faecalis* biofilm. DW was used as control. The result showed that *E. faecalis* biofilm can be totally eradicated (100% kill) by 4.77% of NaOCl. However at concentration of 1.31% and below, confluence growth were detected. Similarly, when tested with 0.1ppm ozonated water, confluence growth was observed.

CFU/biofilm at different exposure time

Table 3 represents mean CFU/biofilm with 2.62%, 1.31% NaOCl, 0.1ppm ozonated water and combination of 1.31% NaOCl and 0.1ppm ozonated water at 1, 2, 4, 8, 15, 30 and 60 minutes of exposure time. 2.62% NaOCl can only achieved 100% kill after 15 minutes of exposure time. However, with 1.31% NaOCl, reducing number (by 2 \log_{10}) of *E. faecalis* colony can only be seen after 30 minutes of exposure time and 100% kill was only achieved after 60 minutes of exposure time. In the case of 0.1ppm ozonated water, some reduction in CFU count was observed after 30 min exposure time. 100% kill was

Table 1. Agar diffusion test - Mean diameter of zone of inhibition (mm) of NaOCl and ozonated water (n=3)

Concentration	Mean diameter of zone of inhibition (mm)	
NaOCl (%)	4.77	32.0
(Available chlorine)	2.62	30.0
	1.31	23.1
	0.65	18.5
	0.32	16.0
	0.16	16.3
	0.08	15.0
	0.04	14.0
	0.02	NI
Ozonated water (ppm)	0.10	14.0
DW (C)		NI

DW(C) – Distilled water (control), NI – No inhibition

Table 2. MBEC of NaOCl & ozonated water (n=3)

Concentration	Mean CFU/biofilm	
NaOCl (%)	4.77	0
(available chlorine)	2.62	15.3
	1.31	CG
	0.65	CG
Ozonated water (ppm)	0.10	CG
DW (C)		CG

DW(C) – Distilled water, CG –Confluence growth

Table 3. Mean values and standard deviation (SD) of CFU counts (CFU/biofilm) following exposure to different test solutions at different exposure time (n=4)

Test agent	Exposure time (min)						
	1	2	4	8	15	30	60
2.62% NaOCl (SD)	1.3 X 10 ⁴ (0.5 X 10 ⁴)	4.1 X 10 ³ (1.8 X 10 ³)	1.0 X 10 ⁴ (0.3 X 10 ⁴)	8.0 X 10 ³ (1.5 X 10 ³)	0	0	0
1.31% NaOCl (SD)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	2.4 X 10 ³ (0.5 X 10 ³)	0
0.1ppm OW (SD)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	1.7 X 10 ⁵ (0.2 X 10 ⁵)	1.4 X 10 ⁵ (0.4 X 10 ⁵)
1.31% NaOCl + 0.1ppm OW (SD)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	5.9 X 10 ³ (1.5 X 10 ³)	0	0
DW(SD)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)	(6.0 X 10 ⁵)* (-)

DW – Distilled water, OW – Ozonated water, () * means Confluence growth (CG) = 6.0 X 10⁵

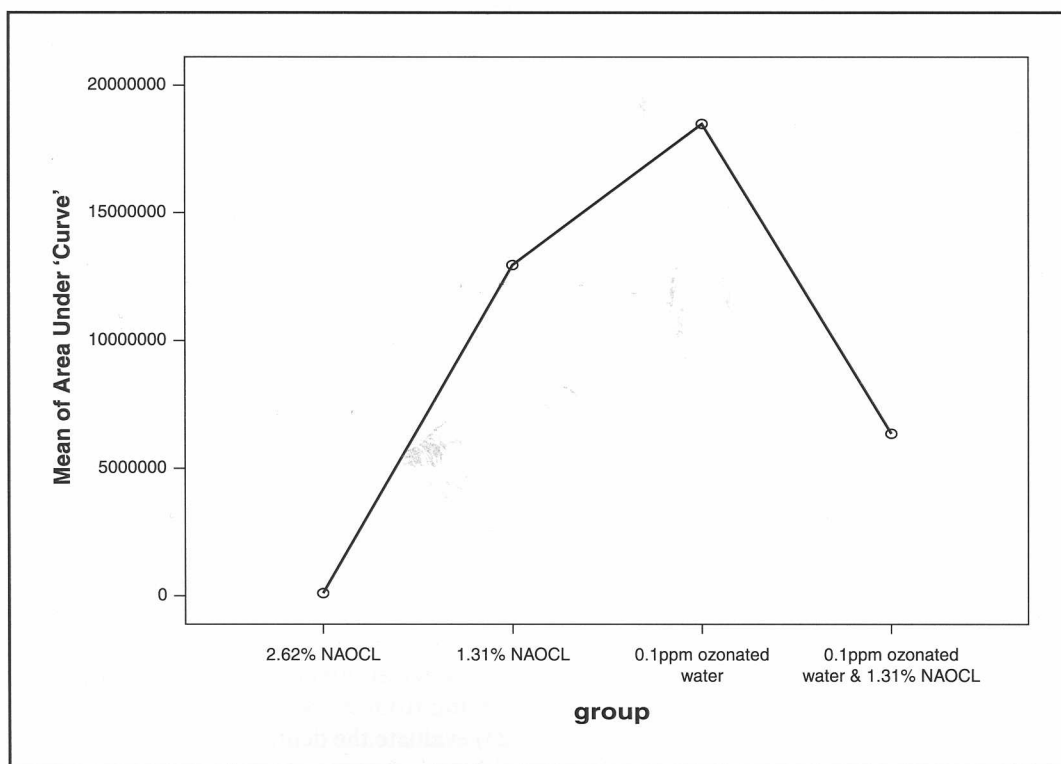


Figure 1: Mean plot of Area under 'curve' for various test agents for a period of time tested (n=4).

however not achievable even after 60 minutes of exposure time.

Statistical analysis

Figure 1 showed mean plot for different group of test agents. Since the time tested is not consistent, the mean of Area under 'curve' was measured and represent the mean CFU for the period of time

tested i.e. 60 minutes. 0.1ppm ozonated water showed the highest mean CFU after 60 minutes exposure time

Table 4 showed result from non parametric test in comparison between groups of test agent. Since the p-value is less than 0.05, at least one pair is different. The result was further tested to see multiple comparisons using Dunnett T3, as in Table 5. This

Table 4. Results from nonparametric test

Group (test agents)	n	Mean Rank	p-value
2.62% NaOCl	4	2.50	0.003
1.31% NaOCl	4	10.50	
0.1ppm ozonated water	4	14.50	
0.1ppm ozonated water & 1.31% NaOCl	4	6.50	
Total	16		

Table 5. Multiple comparisons using Dunnett T3

Group	Group	Sig.
2.62% NaOCl	1.31% NaOCl	.000
	0.1ppm ozonated water	.000
	0.1ppm ozonated water & 1.31% NaOCl	.000
1.31% NaOCl	0.1ppm ozonated water	.006
	0.1ppm ozonated water & 1.31% NaOCl	.000
0.1ppm ozonated water	0.1ppm ozonated water & 1.31% NaOCl	.001

Dependent variable: Area under curve

table revealed that the p-values for all pair-wise comparisons are less than 0.05 therefore all groups are significantly different from each other in term of their antimicrobial effectiveness. The most significant different was 2.62% NaOCl in comparison to other test groups.

DISCUSSION

E. faecalis was chosen as it is considered as one of the most resistant species encountered in the root canal system (2, 14). In addition, some studies have shown that the bacteria are capable to survive as a mono-infection in roots canals (15, 16) and can invade dentinal tubules (17).

In the present study, NaOCl was used as one of the test agents due to its well known antimicrobial action. However, no general agreement exists regarding its optimal concentration, which range from 0.5% to 5.25%.

Ozone is a powerful oxidizing agent (11) and was stated to have an antimicrobial property (18). When it is dissolved in water, ozonated water becomes highly unstable and rapidly decomposed through a complex series of chain reactions (19). This resulted in the production of hydroxyl (OH⁻) radicals which were amongst the most reactive oxidizing agent.

Agar diffusion test is the widely accepted method in evaluating antibacterial activities of dental

materials and medicaments (20). This method allows direct comparison of the tested agent against the test microorganisms. Although determination of MIC is the standard procedure in evaluating antimicrobial effect of test agent, however, it did not take into account that target microorganisms may exist in the form of a biofilm. Therefore, in this study MIC of the test agents was first determined acting as a baseline concentration prior to MBEC. The MBEC was then performed to obtain the initial test concentration, thus allowing consideration of existence of microorganisms in the form of biofilm.

In this study, membrane filter model was used to form the biofilm. This technique offers a simple means of determining the susceptibility of oral bacterial biofilm to antimicrobial agents. In addition, it is reproducible and easy to be used. Nevertheless, it is perhaps more relevant to test effectiveness of test solution in a tooth model. However, considering the anatomical variety of the root canal system and individual growth responses within each tooth during the incubation periods, these factors might lead to biased in data and wide confidence intervals. Thus, it will require a larger sample size in order to increase confidence in the estimates. Moreover, collection, selection and preparation of tooth sample would be quite laborious.

In this study the inoculated membrane filter was incubated for 48 hr. Gulabivala *et al.* (21) in their pilot study had established that the 48-hr time-frame was sufficient to develop single species biofilm.

The volume of all test solutions were standardized to 5mL each and no attempt was made to test on different volume applied to antimicrobial effect since clinically it is the replenish activity that plays an important role in eliminating the microorganism and debris rather than extended volume.

Previous studies have shown that NaOCl was a very effective agent in disinfecting the root canal. Its optimal concentration is still debatable. Siqueira *et al.* (22) reported that from clinical and laboratory studies, there is no significant difference in antibacterial effect between NaOCl concentration ranging from 0.5% to 5.0%. However, Berber *et al.* (23) evaluate the dentinal tubules following irrigation with various concentration of NaOCl. In this study, 0.50%, 2.50% and 5.25% NaOCl were tested associated with hand and rotary instrumentation techniques against *E. faecalis* within root canals and dentinal tubules. 5.25% was shown to be the most effective irrigant solution tested when dentinal tubules were analysed, followed by 2.50% regardless of the canal preparation technique used. However, no differences among concentrations in cleaning the canals were found. In the present study, 4.77% NaOCl was shown to be an effective antimicrobial action after 1 min exposure whereas 2.62% NaOCl

require 15 min to be fully effective. In addition, with reduced concentration, 1.31%, an additional time was needed to produce such similar effect. This result is in line with Radcliffe *et al.* (24) in which their finding indicated a significant interaction between tested time and concentration of NaOCl. They found that when higher concentration was employed, less time was required to attain zero viable counts of *E. faecalis* colony.

In contrast with the present study, Nagayoshi *et al.* (12) found that killing ability of ozonated water and 2.5% of sodium hypochlorite was almost comparable when the specimen was irrigated with sonication. However, in this study, microorganisms in dental plaque such as *Streptococcus salivarius* and *Streptococcus sanguis* were used. These microorganisms were proven not as resistant as *E. faecalis*. Furthermore, they did not exist as biofilm form during testing, as sonication resulted in bacterial in biofilm to be converted into planktonic form.

Study by Hems *et al.* (25) however found that NaOCl was superior to ozonated water in killing *E. faecalis* in broth culture and in biofilm. In this study, sparging ozone with and without stirring was used to detach the bacteria from biofilm model. In a separate test, biofilm was also exposed to gaseous ozone. It was found that gaseous ozone had no significant effect on *E. faecalis* viability in biofilm. Biofilm incubated for 240 seconds with ozonated water showed no significant reduction in cell viability attributable to ozone alone compared to NaOCl (control) over same condition. They concluded that ozone had an antibacterial effect on planktonic *E. faecalis* cells and those suspended in fluid, but little effect when embedded in biofilm. The conclusion of this study was similar with the finding of the present study although different method in ozone application was employed.

Abdullah *et al.*, (10) compared susceptibility of different *E. faecalis* presentation i.e. planktonic, biofilm and pellet form to various root canal irrigants and medications. One of their findings was irrigant and medication which have tissue dissolving capabilities such as NaOCl and $\text{Ca}(\text{OH})_2$ respectively, showed effective antimicrobial activity regardless of the bacterial presentation compared to those that did not possess the tissue dissolving property such as chlorhexidine and iodine. These former medications may be less inhibited in its antimicrobial action by the extracellular polysaccharide matrix in biofilm. In agreement with this study, Hems *et al.*, (25) and the present study found that ozone had no effect on *E. faecalis* cells in the biofilm. The resistance of the bacterial cells in the biofilm must be attributed to the depletion of ozone as it diffuses into the biofilm by virtue of its organic composition. The biofilm extracellular polysaccharide matrix, therefore, probably protects against ozone.

In this study it was found that combination of 1.31% NaOCl & 0.1ppm of ozonated water was shown to reduce CFU count than 1.31% NaOCl alone. The oxidation effect of ozone might interfere with chloramination of NaOCl. Abdullah *et al.*, (10) stated that tissue dissolving property of NaOCl at e¹% may enhance the antibacterial effects of other agents. Likewise in the present study, 1.31% NaOCl might allow passage of oxidation of ozonated water, thus increase their antibacterial effect compared to 1.31% NaOCl or ozonated water alone.

The stock solution of NaOCl (Clorox®) was 0.48% less concentrated (4.77%) than the expected concentration (5.25%) when tested upon initial receipt. Thus NaOCl should ideally be assayed/titrate before dilution prior to testing if accurate concentrations are required to preserve its available chlorine. Nevertheless, pilot studies on effect of storage on concentration of available chlorine in NaOCl revealed that there was no significant difference in available chlorine at baseline after storage at room temperature for 6 months.

CONCLUSION

In conclusion, the antimicrobial effect of NaOCl reduced with concentration below 1.31%, however increased with an increased time but only fully effective at 15 min for 2.62% and 60 min for 1.31%. 0.1ppm of ozonated water can reduce the viable bacteria colony number but cannot totally eliminate *E. faecalis* biofilm even after 60 minutes of exposure time. 0.1ppm of ozonated water was proven not comparable with NaOCl in the antimicrobial effect against *E. faecalis* biofilm.

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