

# An *In Vitro* Evaluation of the Influence of Concentration and Duration of Exposure on the Antimicrobial Efficacy of Sodium Hypochlorite on *Enterococcus Faecalis*

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## ABSTRACT

**Objectives:** To evaluate, *in vitro*, the effectiveness of different concentrations of sodium hypochlorite [NaOCl] (0.5 %, 1.0 %, 2.5% and 5.25% w/v) in the elimination of *Enterococcus faecalis* E10e.

**Methods:** *E. faecalis* E10e was grown overnight and allowed to grow until the 'mid-exponential' phase. 0.2 ml of the inoculum that was then transferred into 9.8 ml of NaOCl solution. One ml samples were removed and poured in 9 ml of sodium thiosulphate [Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>] solution (neutralisation) after 30 sec, 1 min, 2 min, 5 min, 10 min and 30 min. Serial dilution of each neutralised solution was carried out to give a dilution factor of 10<sup>-6</sup>. Subsequently, 0.02 ml of each dilution was plated onto an agar plate, and then incubated at 37°C in an aerobic incubator for 24 hrs. The number of viable colonies (Colony Forming Units / ml) was determined for each plate.

**Results:** The results showed that 5.25 % NaOCl was the most effective irrigant assessed, killing 100% of bacterial cells in 2 min. However, the time required by 2.5 %, 1.0 % and 0.5% concentrations was 5 min, 10 min and 30 min, respectively.

**Conclusion:** There was a statistically significant difference between NaOCl concentrations with respect to the mean number of viable counts recovered, with 5.25 % NaOCl being the most effective irrigant assessed. There was a strong curvilinear relationship between NaOCl concentrations and time taken to attain zero viable counts (100 % killing).

**Key words:** *E. faecalis*, Sodium hypochlorite, Viable colonies.

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## Introduction

Sodium Hypochlorite (NaOCl) has been widely used as an endodontic irrigant since its introduction by Walker in 1936.<sup>(1)</sup> When NaOCl is added to water, hypochlorous acid (HOCl), which contains active chlorine, a strong oxidizing agent, is formed. Substantial evidence suggests that chlorine exerts its antibacterial effect by the irreversible oxidation of sulfhydryl (-SH) groups of essential enzymes, which leads to disturbance of important metabolic functions of the bacterial cell.<sup>(2)</sup> Chlorine may also

combine with cytoplasmic components to form N-chloro compounds, which are toxic complexes and destroy micro-organisms.<sup>(3)</sup>

The chemical removal of organic tissue by NaOCl is mediated by the release of hypochloric acid, which reacts with insoluble proteins to form soluble polypeptides, amino acids and other by-products.<sup>(4)</sup> The clinical efficacy of NaOCl is due to its ability to oxidise, hydrolyse and osmotically draw fluids out of tissues.<sup>(5)</sup>

NaOCl is highly effective in destroying bacteria;

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however, it does not penetrate well into confined areas of the root canal system. In addition, it remains in the canals for only a short period of time, which may limit its effectiveness against bacteria located in and near the main root canal.<sup>(6)</sup> Micro-organisms, such as *Enterococcus faecalis*, are resistant to NaOCl, especially at low concentrations.<sup>(3)</sup>

NaOCl is a caustic material which makes it an effective solvent of both necrotic and vital tissues, but as it is non-specific agent; it is toxic to the surrounding tissues. Accidental injection of NaOCl beyond the root apex can cause severe pain, a rapidly developing oedema, haematoma, necrosis and abscesses.<sup>(7)</sup>

Oral enterococci, predominantly *Enterococcus faecalis*, have been recovered from dental plaque, saliva, dorsum of tongue, dental caries, osseointegrated dental implants and persistent root canal infections.<sup>(8)</sup> The genus *Enterococcus* consists of Gram-positive, facultative anaerobic organisms that are ovoid in shape, non-sporing and may appear on smear in short chains, in pairs or as single cells.<sup>(9)</sup> It has the ability to grow in 6.5 % NaCl and pH 9.6, to grow at 10°C and at 45°C. Its optimum growth temperature is 35°C. It has a strong reducing ability. It survives at 60°C for 30 minutes and produces ammonia from peptone.<sup>(10)</sup>

*Enterococcus faecalis* usually enters the canal during treatment, survives the antibacterial agents and then persists after obturation.<sup>(11)</sup> When *E. faecalis* is present in low numbers initially, it can be eliminated. However, once established in root canal system, it is a difficult organism to eradicate. It can remain viable, maintains the capability to invade the dentinal tubules, adheres to collagen in the presence of human serum, acts as a pathogen in failed root canal treatment.<sup>(12)</sup> and is one of the few micro-organisms that have been shown to resist antibacterial effect of calcium hydroxide.<sup>(13,14)</sup> The buffering capability of the hydroxyapatite might protect *E. faecalis* from the pH rise by calcium hydroxide.<sup>(15)</sup> Tissue fluid from the periodontal ligament and alveolar bone bathing the root of a tooth may provide sufficient nutrition to *E. faecalis* within radicular dentinal tubules or the obturated root canal.<sup>(12)</sup> This can enable *E. faecalis* to grow and gain nourishment for a long period of time and may account for the presence of *E. faecalis* in failed endodontically treated cases.<sup>(16)</sup> Recognising the potential role of *Enterococcus faecalis* in the failure of root canal therapy makes it important to develop

strategies to control infections caused by this organism.

This study was conducted to evaluate, *in vitro*, the effectiveness of different concentrations of sodium hypochlorite [NaOCl] (0.5 %, 1.0 %, 2.5 % and 5.25 % w/v) in the elimination of *Enterococcus faecalis* E10e.

## Methods

The tested micro-organism was cultured by placing 50 ml of a fresh nutrient of Fastidious Anaerobe Broth (FAB; Lab M, Burry, UK) in a sterile 100 ml conical flask. The broth was then inoculated with *Enterococcus faecalis* E10e (a patient isolate that was grown at 37°C for 24 hrs in an aerobic incubator obtained from the University Dental Hospital of Manchester) using a sterile cotton swab, which was dipped in the broth before few colonies of the micro-organism were, harvested from the culture plates. The inoculum was grown overnight in a shaking water bath at 120 rev/min, 37°C.

A sterile 50 ml conical flask containing 19.8 ml of (FAB) was inoculated with 0.2 ml of overnight grown culture (of the same medium) of *E. faecalis* E10e and was shaken on a Spinmix (Gallenkamp, Philip Morris Scientific, Manchester, UK) to ensure thorough mixing. Aliquots (0.1 ml) of this inoculum was immediately taken and poured into the first glass bottle containing 0.9 ml of sterile Phosphate Buffered Saline solution (PBS, Sigma Chemical Co., Steinheim, Germany) to give a dilution factor of 10<sup>-1</sup>. A further five serial dilutions were made to give a dilution factor of 10<sup>-6</sup>. This was taken as time zero, where 0.02 ml of each dilution was removed and plated on Columbia Agar Base (CBA; Lab M, Burry, UK) plate that had already been divided into six sections, and then incubated at 37°C for 24 h.

Every hour, 0.1 ml samples of inoculum from the 50 ml flask (containing the liquid growth medium plus 0.2 ml of overnight grown culture) in the shaking water bath were taken, serially diluted, plated as above, and then incubated at 37°C for 24 h. The number of colonies was recorded as 'viable colony' with the aid of a plate microscope (Olympus VN, Japan).

The Colony Forming Units per ml (CFU/ml) were then calculated for each time interval:

CFU/ml = Number of viable colonies × Dilution factor × 50.

Exposure to test chemical (NaOCl):

A sterile 50 ml conical flask containing 19.8 ml of (FAB) was inoculated with 0.2 ml of overnight

grown culture of the micro-organism tested. The flask was then placed in a shaking water bath at 120 rev/min, 37°C and the microbial culture allowed growing until the 'mid-exponential' phase. At that time, 0.2 ml of the inoculum was removed and poured into a sterile 20 ml plastic bottle containing 9.8 ml of 0.5 % NaOCl (Sigma-Aldrich Co. Ltd, Gillingham, UK). One ml samples were removed and poured in 9 ml of 1.93 g/100 ml sodium thiosulphate solution ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ , Sigma Chemical Co., Steinheim, Germany) for neutralisation after 30 sec, 1 min, 2 min, 5 min, 10 min and 30 min. Immediately, 0.1 ml of each neutralised solution was taken, serially diluted, plated and then incubated at 37°C in an aerobic incubator for 24 h. (CFU/ml) were then calculated for each time interval.

The above experiment was repeated for other concentrations of NaOCl (1.0 %, 2.5 % and 5.25 %) with the use of corresponding solutions of  $\text{Na}_2\text{S}_2\text{O}_3$  (1.93 g/100 ml, 1.93 g/100 ml and 3.86 g/100 ml, respectively). The experiment was repeated four times to achieve statistical significance.

Three controls were included in this study. The first control was carried out to test if  $\text{Na}_2\text{S}_2\text{O}_3$  could neutralise NaOCl. A sterile 20 ml plastic bottle containing 9.8 ml of 2.5 % NaOCl solution was inoculated with 0.2 ml of fresh sterile medium (FAB). Subsequently, 0.98 ml of this mixture was added to a sterile 20 ml plastic bottle containing 9 ml of 1.93 g/100 ml  $\text{Na}_2\text{S}_2\text{O}_3$ . Then, 0.02 ml of the 'mid-exponential' phase inoculum was added to the bottle, followed by mixing. Aliquots (0.1 ml) of each neutralised solution was removed from the 20 ml plastic bottle after 30 sec, 1min, 2min, 5min, 10min and 30 min, serially diluted, plated and then incubated at 37°C for 24 h. The number of colonies and CFU/ml were calculated for each time interval.

The second control was the negative chemical control (no NaOCl solution used) to test if  $\text{Na}_2\text{S}_2\text{O}_3$  by itself was toxic to the micro-organisms used. Aliquots (0.2ml) of the mid-exponential phase inoculum was removed and poured into a sterile 20 ml plastic bottle containing 9.8 ml of 1.93 g/100 ml  $\text{Na}_2\text{S}_2\text{O}_3$ . 1ml samples were removed and poured in 9 ml of 1.93 g/100 ml  $\text{Na}_2\text{S}_2\text{O}_3$  solution after 30 sec, 1 min, 2 min, 5 min, 10 min and 30 min. Serial dilution was made to give a dilution factor of  $10^{-6}$ . Subsequently, 0.02 ml of each dilution was removed and plated on an agar plate and then incubated at 37°C in an aerobic incubator for 24 h. (CFU/ml) were then calculated for each time interval.

The third control was the negative microbiological control (no micro-organisms used) to test if the growth media used were contaminated. Uninoculated broth (0.2 ml) was removed from a sterile 50 ml conical flask containing 20 ml of (FAB) and poured into a sterile 20 ml plastic bottle containing 9.8 ml of 2.5 % NaOCl solution. One ml samples were removed and poured in 1.93 g/100 ml  $\text{Na}_2\text{S}_2\text{O}_3$  (neutralisation) after 30 sec, 1 min, 2 min, 5 min, 10 min and 30 min. aliquots (0.1 ml) of each solution was taken immediately, serially diluted, plated out as above and then incubated at 37°C for 24 h. The number of colonies and CFU/ml was then calculated for each time interval.

### Statistical Analysis

The data collected were entered onto a spreadsheet and statistically analysed using the software packages (Stata and SPSS/PC + version 10.0).

A regression model was fitted to the dependent variable viable count, for the independent variables time and concentration, including an interaction term. In order to take the clustering of the samples into account, the regression analysis was conducted using the software package Stata. This package provides robust estimates of the standard errors of the time coefficient.

The variables time and viable count were transformed by taking natural logs in base e (and adding one to avoid taking logs of zero), and this transformation produced a linear relationship between  $\log_e(\text{time} + 1)$  and  $\log_e(\text{count} + 1)$ , for each concentration. The rest of the analysis was conducted using SPSS/PC + version 10.0, where descriptive data and plots of the data were produced.

## Results

The number of viable colonies was recorded and the CFU per ml calculated. The viable counts were the mean of four repeated experiments performed for *E. faecalis* E10e. The number of CFU/ml for *E. faecalis* E10e at each time interval is shown in Table I.

Exposure to test chemical (NaOCl):

As the concentration of NaOCl was increased, the time necessary to reduce CFU decreased. At a concentration of 5.25 %, NaOCl was very effective in killing 100 % of the tested micro-organism within only 2 min. The CFU/ml for *E. faecalis* E10e recovered after exposure to NaOCl is shown in Table II.

**Table I.** Viable count determination for *Enterococcus faecalis*. E10e (CFU/ml)\*

Time (h)	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
0	1.015×10 <sup>8</sup>	5.350×10 <sup>8</sup>	1.600×10 <sup>9</sup>
1	1.075×10 <sup>8</sup>	6.650×10 <sup>8</sup>	2.650×10 <sup>9</sup>
2	1.120×10 <sup>8</sup>	7.950×10 <sup>8</sup>	4.300×10 <sup>9</sup>
3	1.335×10 <sup>8</sup>	9.150×10 <sup>8</sup>	4.650×10 <sup>9</sup>
4	1.415×10 <sup>8</sup>	1.055×10 <sup>9</sup>	5.750×10 <sup>9</sup>
5	++	1.170×10 <sup>9</sup>	7.050×10 <sup>9</sup>
6	++	1.220×10 <sup>9</sup>	7.650×10 <sup>9</sup>
7	++	1.420×10 <sup>9</sup>	9.450×10 <sup>9</sup>
8	++	++	1.140×10 <sup>10</sup>
9	++	++	1.305×10 <sup>10</sup>
10	++	++	1.370×10 <sup>10</sup>

++: Number of colonies too many to count

\*At 10<sup>-1</sup>, 10<sup>-2</sup>, 10<sup>-3</sup> dilutions, the number of colonies too many to count

**Table II.** Viable count (CFU/ml)\* for *Enterococcus faecalis* E10e recovered after exposure to (0.5%, 1.0%, 2.5% and 5.25%) NaOCl

NaOCl	Start (time zero)	30s	1min	2min	5min	10min	30min
0.5%	4.900×10 <sup>9</sup>	4.050×10 <sup>9</sup>	3.050×10 <sup>9</sup>	2.450×10 <sup>9</sup>	1.600×10 <sup>9</sup>	6.500×10 <sup>8</sup>	0
1.0%	8.900×10 <sup>9</sup>	4.750×10 <sup>9</sup>	3.150×10 <sup>9</sup>	2.250×10 <sup>9</sup>	8.500×10 <sup>8</sup>	0	0
2.5%	9.500×10 <sup>9</sup>	3.300×10 <sup>9</sup>	1.950×10 <sup>9</sup>	3.000×10 <sup>8</sup>	0	0	0
5.25%	1.045×10 <sup>10</sup>	2.200×10 <sup>9</sup>	5.000×10 <sup>8</sup>	0	0	0	0

\* Average at a dilution factor of 10<sup>6</sup>

**Table III.** Viable count (CFU/ml) for *Enterococcus faecalis* E10e recovered after exposure to 2.5% NaOCl and 1.93g/100ml Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (Control 1)

Time	CFU/ml*	% Survivors
Start	4.700×10 <sup>9</sup>	100
30s	4.650×10 <sup>9</sup>	99
1min	4.650×10 <sup>9</sup>	99
2min	4.650×10 <sup>9</sup>	99
5min	4.600×10 <sup>9</sup>	98
10min	4.600×10 <sup>9</sup>	98
30min	4.500×10 <sup>9</sup>	96

\* Average at a dilution factor of 10<sup>6</sup>

Control experiments revealed a very high percentage of survivors even after 30min. The CFU/ml of the organism tested and the percentage of survivors recovered after exposures to the mixture (NaOCl + Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solutions at each time interval were determined (Tables III and IV). All incubated plates showed no microbial growth following exposure to both NaOCl and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solutions, indicating that there was no contamination of either the growth plates or the liquid growth medium (FAB).

The results for *Enterococcus faecalis* E10e showed a strong relationship between the mean of viable count and time, for each concentration of NaOCl solution (Fig. 1). Significant differences between the concentrations were also apparent for the longer time intervals (p<0.001), with the highest concentration (5.25 %) reducing the viable counts to zero in 2 min compared to 30 min for the lowest concentration (0.5 % NaOCl).

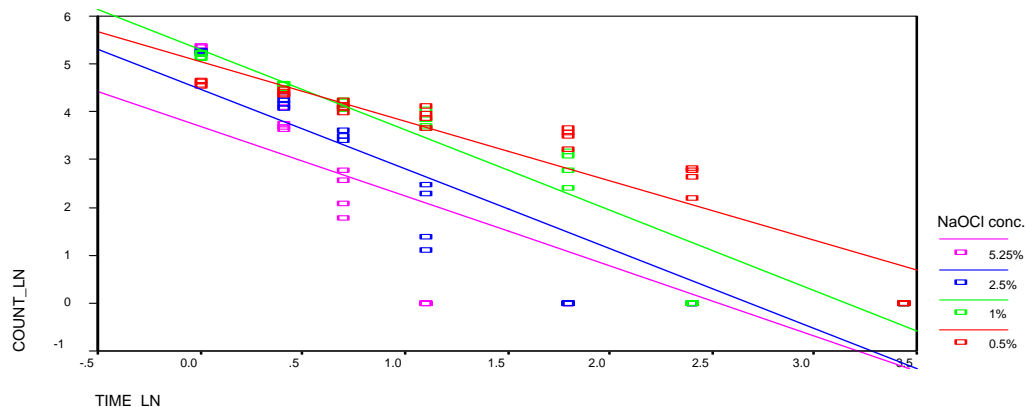
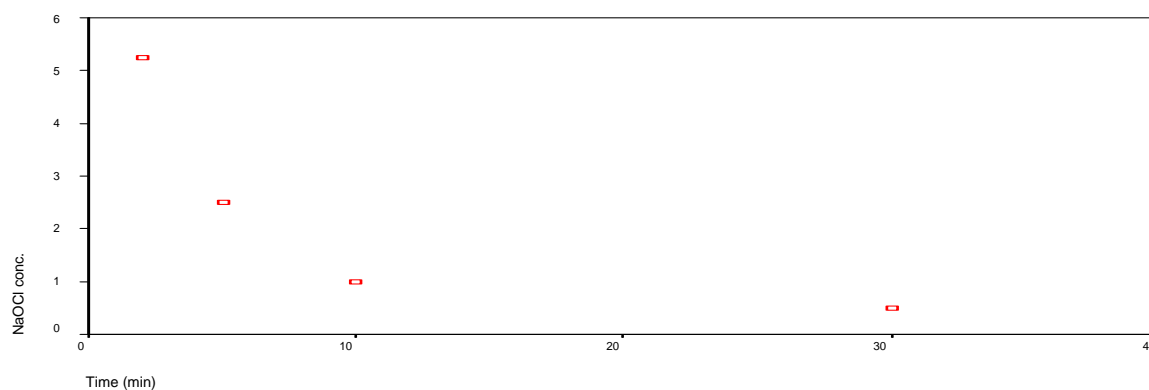
There is, therefore, a strong curvilinear relationship between concentration and time taken to attain zero viable counts (Fig. 2).

## Discussion

*Enterococcus faecalis* was chosen for use in this investigation for various reasons. It is probably the most significant species in persistent endodontic infections and is the species most frequently found in cases of apical periodontitis requiring retreatment.<sup>(16)</sup> It also exhibits resistance to a wide range of antimicrobial agents, including calcium hydroxide and Tetracycline.<sup>(17,18)</sup> and it is easy to grow and identify.<sup>(19)</sup> Endodontic infections with *E. faecalis* usually constitute a problem with treatment because this micro-organism is difficult to eliminate.<sup>(14)</sup> Sodium hypochlorite is the most popular agent for endodontic irrigation, even though its optimum working concentration has not been universally agreed.<sup>(20)</sup>

**Table IV** Effect of 1.93g/100ml Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> on *Enterococcus faecalis* E10e (Control 2)

Time	CFU/ml*	% Survivors
Start	8.650×10 <sup>9</sup>	100
30s	8.600×10 <sup>9</sup>	99
1min	8.500×10 <sup>9</sup>	98
2min	8.350×10 <sup>9</sup>	97
5min	8.350×10 <sup>9</sup>	97
10min	8.250×10 <sup>9</sup>	95
30min	8.250×10 <sup>9</sup>	95

\*Average at a dilution factor of 10<sup>-6</sup>**Fig. 1.** The relationship between log<sub>e</sub> (time + 1) and log<sub>e</sub> (count + 1) for *Enterococcus faecalis* E10e at (0.5%, 1.0%, 2.5% and 5.25%) NaOCl**Fig. 2.** The relationship between NaOCl concentration and time necessary to attain zero viable counts for *Enterococcus faecalis* E10e

The choice of concentration of NaOCl has been a matter of debate, the range extending traditionally from 0.5% to 5.25%, and a 10% concentration has also been advocated.<sup>(21)</sup> The desirable concentration should be one that possesses low toxicity and adequate antibacterial effects.

Published work related to the antimicrobial effectiveness of various NaOCl concentrations has also revealed conflicting results. Some clinical studies have found no significant difference in antibacterial effect between 0.5% and 5.25% NaOCl.<sup>(22)</sup> By contrast, another study has reported that the antibacterial effectiveness of NaOCl is significantly reduced after dilution.<sup>(23)</sup>

The exponential phase of growth is the period of

most rapid reproduction and the one in which the typical characteristics of the active cell are usually observed.<sup>(24)</sup> In this study, the “mid-exponential” phase was selected because microbial population is most uniform in terms of chemical and physiological properties during this phase.<sup>(25)</sup>

Viable count has been developed to count only live cells. The spread plate method was used in this study for viable count. With this method, a volume of an appropriately diluted culture is spread over the surface of an agar plate using a sterile spreader. The plate is then incubated until the colonies appear, and the number of colonies counted. This method is simple, sensitive and widely used technique.<sup>(26)</sup> The method has the virtue of high sensitivity; samples

containing very few cells can be counted, thus permitting sensitive detection of microbial contamination of products or materials.

The experimental method used in this study was incubation of broth cultures of selected bacteria with the antimicrobial agent. According to this method, 0.2 ml of the "mid-exponential" phase inoculum was transferred into bottles containing 9.8ml of 0.5 %, 1.0 %, 2.5 % and 5.25 % w/v NaOCl, respectively. 1ml samples were then placed in bottles containing 9 ml of neutraliser (sodium thiosulphate) after time intervals of either 30 s, 1 min, 2 min, 5 min, 10 min or 30 min to prevent residual action of NaOCl solutions.

Following neutralisation of NaOCl, 0.1 ml of each neutralised solution was removed, serially diluted (up to a dilution factor of  $10^{-6}$ ) and then 0.02 ml of each dilution was subcultured in blood agar plate incubated at 37° C for 24 h. The CFU/ml was then calculated.

The results showed that 5.25% NaOCl was the most effective irrigant assessed, killing 100% of bacterial cells in 2 min. However, the time required by 2.5%, 1.0 % and 0.5% concentrations was 5 min, 10 min and 30 min, respectively.

The direct exposure method used in this study appears to be correlated with the irrigant effectiveness and its direct contact with the micro-organisms. It seems to be independent of other variables and appears to be a simple, straightforward and practical laboratory test.<sup>(27)</sup> However, the clinical efficacy of NaOCl should be considered in light of the complex root canal anatomy and the polymicrobial nature of root canal infections.<sup>(28)</sup> An irrigating solution that is effective against a single micro-organism tested in the laboratory may not be effective against a mixed infection in the root canal.<sup>(29)</sup>

The presence of the smear layer may prevent the irrigant from penetrating into dentinal tubules in which micro-organisms may be harboured.<sup>(22)</sup> Furthermore, interaction with other factors such as organic material, tissue fluids, blood and dentine can influence the antibacterial effectiveness of irrigants.<sup>(2)</sup> Because chemo-mechanical preparation is a short-time procedure, and NaOCl remains in the canal for only few minutes, it would appear that the antibacterial effectiveness of NaOCl inside the root canal might be highly dependent on its concentration and the time of contact with dentine, organic matter and pulpal remnants.<sup>(6)</sup>

Finally, it must be stressed that the antimicrobial action of NaOCl was tested in this study, but this is not the only requirement for an endodontic irrigant. Root canal irrigants should also have other characteristics such as low tissue toxicity, high detergent power, low surface tension, ease of handling and high proteolytic and tissue dissolving ability.<sup>(30)</sup>

The results of controls 1 and 2 showed that high percentage of survivors was recovered even after a contact period of 30 min. Therefore, sodium thiosulphate solutions were very effective in the neutralisation of various concentrations of NaOCl and had a negligible toxic effect on *E. faecalis*. The results of the third control showed that there was no microbial growth following exposure to NaOCl and Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> solutions. Therefore, no contaminants were found in any of the incubated plates used in this study.

## Conclusions

Under the conditions of this study, it was concluded that:

- All concentrations of NaOCl solution were effective in the elimination of *Enterococcus faecalis* E10e, but at different time intervals. 5.25 % NaOCl being the most effective irrigant assessed, producing 100 % killing in 2 min, followed by 2.5 % (5 min), 1.0 % (10 min) and 0.5 % (30 min), respectively.
- There was a strong curvilinear relationship between concentration and time taken to attain zero viable counts (100 % killing).
- There was a statistically significant difference between the concentrations with respect to the mean number of viable counts recovered, with 5.25 % NaOCl being the most effective irrigant used, producing 100 % killing of bacterial cells in 2 min compared with 0.5 % NaOCl which took 30 min.

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