The effect of sodium hypochlorite concentration and irrigation needle extension on biofilm removal from a simulated root canal model

Abstract

To investigate the effect of sodium hypochlorite concentration and needle extension on removal of *Enterococcus faecalis* biofilm, sixty root canal models were 3D printed. Biofilms were grown on the apical 3 mm of the canal for 10 days. Irrigation for 60s with 9 mL of either 5.25% or 2.5% NaOCl or water was performed using a needle inserted either 3 or 2mm from the canal terminus and imaged using fluorescence microscopy and residual biofilm percentages were calculated using imaging software. The data were analysed using analysis of covariance and two-sample t-tests. A significance level of 0.05 was used throughout. Residual biofilm was less using 5.25% than with 2.5% NaOCl. Statistically significant biofilm removal was evident with the needle placed closer to the canal terminus. A greater reduction of available chlorine and pH was noted as the concentration increased. One-minute irrigation was not sufficient for complete biofilm removal.

Key words: Biofilm, concentration, e*nterococcus faecalis,* sodium hypochlorite, 3D printing model.

Introduction

Root canal treatment is a procedure designed to resolve or prevent the development of apical periodontitis (1) which is caused mainly by bacteria (2). The bacteria adhere to surfaces and rapidly form biofilms (3) which are defined as a community of microorganisms (e.g. bacteria, protozoa, fungi) of one or more species embedded in an extracellular polysaccharide matrix that is attached to a solid substrate (4). The aim of root canal treatment is therefore to remove the biofilm, bacterial toxins, and tissue remnants that may serve as microbial substrate (5). The root canal therapy consists of root canal debridement and enlargement by instruments to a size sufficient to deliver an irrigant solution (6); this helps to degrade and remove bacterial biofilms. The root canal is then obturated with a filling material (e.g. gutta-percha) together with a sealer that may actively kill bacteria (e.g. Tubliseal) to trap remaining microbiota (6).

The irrigation process is the most important step in root canal treatment for the removal of bacteria from the infected walls of the root canal system (7). The efficacy of irrigation procedure depends on chemical and mechanical (shear stress) effects of the irrigant solution (8). The chemical effect depends on irrigant type and concentration (9), the surface area of contact (10), and the duration of interaction between the irrigant and the infected material (11). The physical effect may be limited by the taper geometry of the root canal system that affect the flow rate of the irrigant, as well as the closed-end behaviour of the root canal, which is related to the periodontal tissue and bone socket that encloses the root (12). Two phenomena are responsible for limiting an irrigant penetration in the root canal system: First, the stagnation of the irrigant that exists between 0.5 to 3 mm beneath the needle tip depending on fluid flow rate, needle size and tip design, and apical size of the canal

(13). Second, the vapour lock effect ahead of the advancing front of the irrigant (14). It has been reported that irrigant penetration increased by increasing its temperature, or adding a surfactant that lowers its surface tension (15). Irrigant agitation has been recommended to improve the irrigant penetration and mixing within the root canal system (9).

Sodium hypochlorite (NaOCl) is the most popular irrigant used in root canal treatment (10). Increased pH contributed by hydroxyl ions (OH^-) and available chlorine represented by hypochlorite ion (OCl^-) and hypochlorous acid ($HOCl^-$) are responsible for the antimicrobial action of NaOCl (16). It has been recommended to use a concentration of NaOCl between 0.5% and 5.25% (wt/v) as an irrigant solution (17). Although the efficacy of NaOCl is enhanced by an increase in its concentration (9), and frequent application or replenishment (18), there is no consensus of optimum concentration. Several studies have recommended the use of 5.25% NaOCl (11, 17). In contrast, others have suggested a concentration of 2.5% which provided adequate antibacterial activity (7), as well reducing the risks of physical damage to dentine (19). Measurement of the rate of biofilm removal during irrigation by NaOCl in the root canal system may help identify the factors that may interfere with the efficacy of NaOCl irrigant which may improve and affect clinical outcomes.

This study aimed to compare between the *in situ* biofilm removal by 5.25% and 2.5% NaOCl delivered using a syringe and needle, using a percentage of canal wall coverage with residual biofilm over time as the outcome measurement. The rate of biofilm removal was assessed. Also, the effect of needle extension within the root canal system on the efficacy of NaOCl (5.25% & 2.5%) was assessed. Finally, the outcomes of chemical interaction between NaOCl irrigants (5.25% & 2.5%) and

bacterial biofilm was investigated, using the available chlorine and pH of outflow of the irrigant.

Materials and Methods

Construction of transparent root canal models and distribution between experimental groups

The root canal models (n = 60) were manufactured using 3D printer (Formlabs Inc., Somerville, MA, USA) as previously described (20), creating a straight simple canal model of 18 mm length, apical size 30, and a .06 taper. The models were then divided into two main groups. The models where irrigation needle was placed at 3 mm from the canal terminus comprising group 1 (n = 30) and those where irrigation needle was placed at 2 mm comprising group 2 (n = 30). The models within each group were subdivided into three subgroups (n = 10) (A, B, and C) according to the type of irrigant (5.25% NaOCl, 2.5% NaOCl, and demineralized water respectively).

Generation of biofilm on the surface of the canal models

Biofilm generation on the canal models was demonstrated in our previous study (20). Briefly, *E. faecalis* strain ATCC 19433 was plated onto a BHI plate with 5% defibrinated horse blood (E&O Laboratories, Scotland, UK) and incubated at 37 °C in the 5% CO_2 incubator for 24 hours. Inoculum concentration was 1.1 x 10^8 CFU/mL, which was confirmed using six ten-fold serial dilutions.

The model halves were sterilised in 7 mL plastic bijou bottle using gas plasma. 1mL of *E. faecalis* inoculum was delivered into a sterilised plastic bottle containing the sterilised half model to immerse the 3 mm apical portion of the half model. The samples were then incubated at 37 °C in the 5% CO_2 incubator for 10 days. Every

two days, half of the inoculum that surrounded the sample was discarded and replaced with fresh BHI broth.1 µL of CV stain (Merck, Darmstadt, Germany) was applied to the part of the canal half where the biofilm had been generated (3 mm). The two halves of the model were then held in position using four brass bolts (size 16 BA) and nuts.

Irrigation experiments

The apical end of each canal was blocked using a sticky wax. Each model was fixed to a plastic microscopic slide. The model half with the biofilm faced the slide. The microscopic slide was placed on the stage of an inverted fluorescence microscope (Leica, UK). Commercial NaOCl (Sigma–Aldrich, Germany) irrigant without surfactants was used. Nine mL per-minute of irrigant were delivered using a 10 mL syringe with a 27-gauge side-cut open-ended needle. In group 1, the needle was inserted 3 mm from the canal terminus, and in group 2 the needle was inserted 2 mm from the canal terminus. The port opening of the needle always faced the model half containing the biofilm. The syringe was attached to a programmable precision syringe pump (NE-1010) to deliver the irrigant at a flow rate of 0.15 mL s^{-1}. Outflow irrigant was collected in a 15 mL plastic tube. The amount of available chlorine (%) and pH of the outflow NaOCl were measured using iodometric titration and a pH calibration meter (HANNA pH 211, Hanna Instrument, UK) respectively.

Removal of biofilm was recorded using a high-resolution CCD camera connected to the 2.5 × lens of a fluorescence microscope (Leica, UK). An image was obtained for each second of the one-minute irrigation (60 images). The canal surface coverage by biofilm present after every second of irrigation (0.15 mL) was visualised and automatically quantified using Image-pro Plus 4.5 software (Figure 1).

Data analyses

The amount of residual *E. faecalis* biofilm after one-minute irrigation using three irrigants was assessed using line plots. An analysis of covariance (ANCOVA) was used to examine the effect of concentration and needle extent (2 & 3 mm) from the canal terminus on the area percentage of canal covered with residual biofilm. A similar analysis was performed to compare the effect of time on the area covered with residual biofilm. A two-sample t-test was used to compare the mean difference in available chlorine and pH of the outflow NaOCl before and at the end of irrigation. A significance level of 0.05 was used throughout. The data were analysed by SPSS (BM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp).

Results

The mean (95% CI) percentages of the canal surface area covered with residual bacterial biofilm against duration of irrigation(s) are presented in Figure 2.

The data showed that in a canal where the needle was placed at 3 mm from the canal terminus (Figure 2a), the interaction of both NaOCl concentrations with biofilm was highest during the first 22 seconds. From then on, the removal declined, but with greater removal associated with 5.25% than that with 2.5%. The greatest residual biofilm was associated with water irrigant. However, in a canal where the needle was placed at 2 mm from the canal terminus (Figure 2b), the interaction was consistent throughout the irrigation procedure and was at its maximum during the first 31 seconds.

Regardless of needle position, the results showed (Table 1) that the difference between the amount of biofilm before and after irrigation was greater in the group

where 5.25% NaOCl irrigant was used as opposed to the group using 2.5% NaOCl irrigant.

In general, one-minute irrigation was insufficient for complete removal of bacterial biofilm.

When the needle was placed at 3 mm, the results (Table 2) revealed that the type of irrigant had an influence on the percentage of surface-area of the canal covered with biofilm. The residual biofilm after a 60-second irrigation protocol using 5.25% NaOCl and 2.5% NaOCl was 10.8% (± 0.3) and 7.5% (± 0.3) respectively less than that using water ($p = 0.001$). Moreover, the residual biofilm using 5.25% NaOCl irrigant was 3.3% (± 0.3) less than that using 2.5% NaOCl ($p = 0.001$).

When the needle was placed at 2 mm, the results (Table 3) revealed that residual biofilm using 5.25% NaOCl and 2.5% NaOCl was 29.7% (± 0.3) and 29.4% (± 0.3) respectively less than that using water ($p = 0.001$).

The results of ANCOVA test showed that there were correlations between extent of the irrigation needle and the percentages of residual biofilm. At 3 mm (group 1) from the canal terminus, the residual biofilm after irrigation using 5.25% and 2.5% NaOCl were (28.9%, 95% CI: 28.4, 29.5) and (25.9%, 95% CI: 25.3, 26.9) respectively, more than that at 2 mm (group 2) ($p = 0.001$). However, none of the needle positions examined could completely remove the bacterial biofilm.

The results of ANCOVA test (Table 4) showed that at 3 mm, the biofilm was significantly reduced at a rate of 0.6% s^{-1} using 5.25% NaOCl and 0.4% s^{-1} using 2.5% NaOCl, ($p = 0.001$). At 2 mm, the biofilm was significantly reduced at a rate of 1.3% s^{-1} using both 5.25% and 2.5% NaOCl ($p = 0.001$).

The results of two-sample t-test revealed the mean difference (before and after irrigation) in values of available chlorine of 5.25% NaOCl were 0.3% (95% CI: 0.1, 0.5) and 0.2% (95% CI: 0.1, 0.2) more than that that of 2.5% NaOCl respectively ($p < 0.001$). Regarding the pH values, the mean difference in values of pH of 5.25% NaOCl were 0.06% (95% CI: 0.1, 0.01) and 0.04% (95% CI: 0.03, 0.05) more than that of 2.5% NaOCl respectively ($p < 0.001$).

Discussion

The *in vitro* experiments presented herein investigated the rate of bacterial biofilm (*E. faecalis*) removal using two concentrations of sodium hypochlorite, and water (control) irrigant. NaOCl irrigant was selected for the irrigation procedure as it is the most frequently used irrigant in root canal treatment and has been proven to be effective against a broad spectrum of bacteria (21). NaOCl used in this study had either a 5.25% or 2.5% concentration, based on the hypothesis that NaOCl concentration may affect the amount of biofilm removal in the most apical part of the canal. The purpose of using distilled water (control group) was to assess the mechanical flushing effect of an irrigant without antibacterial or tissue-dissolving properties.

In this study, root canal models were created using 3D printing techniques. Advantages of *in vitro* biofilm models were described in our previous study (20). The model is acrylate base polymers consisting of a mixture of methacrylic acid and a photo-initiator, which is designed to work with 3D Printer to produce solid plastic parts on curing. One criticism of this model is that the adhesion of bacterial biofilm on to root canal dentine may differ from that on a synthetic material (resin) of the model used here. However, our previous study which investigated the potential of the

material for suitable *in vitro* biofilm models illustrated that it allowed for adhesion and growth of *E. faecalis* biofilm on their surface in a similar manner to dentine (22).

In this study, the model was created with an apical size 30 with .06 taper as it has been suggested that the minimum apical size necessary to deliver irrigant to the canal terminus is size 30 (23). A side cut 27-gauge endodontic needle was used in this study, as it is commonly used in clinical practice, and also to avoid greater pressure required to deliver the irrigant at a rate of 9 mL per minute, as is the case when a flat-ended 30-gauge needle is used (24). 9 mL per minute (0.15 mL s^{-1}) irrigant flow was used in an attempt to improve the solution penetration (25). This rate also falls within the range of 0.01–1.01 mL s^{-1} reported in previous study (26). One criticism may be the high flow rate which may increase irrigant extrusion (27); however, it has been argued that periapical tissue creates a barrier against the apical extrusion (28).

Biofilm growth over ten days was used as it has been previously confirmed in our laboratory (22) that this period allowed microbial colonisation and standardised biofilm models were developed. The relevant model allowed for controlled investigation and comparison of the antimicrobial protocols (29).

Resistance of generated biofilms over time has been extensively explored. Wang *et al.* (2012) showed that young biofilm was more sensitive to intracanal medicaments, and bacteria were more easily eliminated than in old biofilm. It has been suggested that biofilms become increasingly resistant to antibacterial agents between 2 and 3 weeks (31). However, another study found that biofilm resistance is inherent and it is possible to generate mature wild bacterial biofilm (*Pseudomonas aeruginosa*) after 5 days incubation (32).

In this study, fluorescence microscopy was selected to observe and record biofilm removal by NaOCl. The main advantage of this method is that it allowed direct vision of biofilm removal without the need for sample fixation. However, the high-resolution imaging proved difficult due to the steeply curved sides of the canal walls, which interfered with light reflection from these areas. Furthermore, single bacterial cell degradation could not be observed in the biofilm because the lens of the microscope used was a 2.5-x objective lens.

The use of crystal violet stain to visualise the biofilm under microscope was problematic, as the stain may have affected the oxidative capability of NaOCl. Therefore, trial experiments were performed examining the effect of crystal violet stain on the oxidative capacity of NaOCl. The results showed that crystal violet, which displays fluorescence capacity, had a neutral effect on NaOCl most likely due to the alkaline property of the stain, or concentration, which was not high enough to affect the oxidative capacity of NaOCl.

Image-Pro Plus software was used to analyse the images, as it has been used in another study to quantify biofilms (33). As measurement of the biofilm areas can be subjective, the software was used to manually draw the biofilm outlines prior to washing and the same template was used to calculate the biofilm area after washing, without further interference of the examiner. This approach allowed the quantitative results to be examiner-independent.

Our findings showed that NaOCl was more effective than distilled water in biofilm removal from the walls of the canal models likely due to the organic tissue dissolution capacity of NaOCl (chemical action) (16) and enhanced by flow dynamics (mechanical action) (8). Nevertheless, it has been shown that 9 mL/min of NaOCl was insufficient for the removal of a biofilm from the walls of the root canal models.

Although the results showed significant differences between 5.25% and 2.5% NaOCl, this study did not identify any complete biofilm removal using a higher concentration. This may be due to a lack of adequate contact between the antimicrobial agent and the biofilm due to irrigant stagnation in the apical portion of the canal (34), or due to air bubble formation during irrigation (14). Both these phenomena are related to the closed nature of the root canal system, which interferes with the flushing action of the irrigant as well as limiting its dissolving action (34). In addition, the accumulation of bubbles results from biofilm dissolution may hinder irrigant penetration into the biofilms and thus decrease the rate of removal (9). Another possible reason is the space between the needle and the canal wall not being large enough for irrigant replacement or the extracellular substance of the biofilm, which acts as a physical barrier against the penetration of depleted NaOCl irrigant (35).

Previous study which has compared the efficacy of sodium hypochlorite in eliminating *E. faecalis*, reported that NaOCl was unable to render the root canal completely sterile in bovine tooth models infected with *E. faecalis* (36). However, the complete eradication of an *E. faecalis* biofilm has been demonstrated by Ross-fedele in bovine models. This difference in efficacy may be related to a difference in volume, since a greater irrigant volume was used twice in the latter study. Estrela *et al.* reported that the use of 2.5% NaOCl for 20 minutes was not completely effective against *E. faecalis* after 60 days of incubation. Halford *et al.* showed that the ultrasonic agitation of 5.25% NaOCl reduces viable *E. faecalis* bacteria in root canal at 3-mm and 1-mm apical levels. Spratt *et al.* studied the bactericidal effect of different irrigants on single-species biofilms, including *E. faecalis*. They identify

NaOCl as the most effective agent tested, stating that its efficacy is dependent on the nature of the organism in the biofilm and contact time.

The results of this investigation are consistent with a previous study, which showed incomplete biofilm removal after NaOCl was used in the root canal system (36). Regarding irrigant concentration, the results are in agreement with Sena *et al.* who showed 5.25% NaOCl destroyed *E. faecalis* more rapidly than 2.5% NaOCl.

Interesting observations related to a small reduction in the total remaining amount of available chlorine and pH of NaOCl. Significant differences ($p < 0.001$) in the values of the available chlorine and pH of NaOCl between the two concentrations were identified. These differences can be explained by the consumption of OCl^-, $HOCl^-$ and OH^- ions (40). However, the reduction was less than originally expected, which indicates that the interactions between NaOCl and the biofilm were short. A possible explanation might be related to the small area of contact between the irrigant and the test targets (10) or due to the short duration of the irrigation process (11).

Further research is essential for an understanding of ways to improve the apical penetration of irrigants within the root canal system (for example, irrigant agitation).

Conclusion

Within the limitation of the present study, both concentration and position of the irrigation needle affect the efficacy of NaOCl to remove *E. faecalis* biofilm. Although 5.25% NaOCl was more effective than 2.5%, one-minute irrigation using higher concentration was not enough for complete biofilm removal.

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Tables

Table 1: Mean value of the biofilm (%) covering the root canal surface before and after one-minute irrigation using different irrigants (5.25% NaOCl, 2.5% NaOCl, water) delivered at flow rate of 0.15 mL s^{-1} (Total n = 60, n = 10 per group).

Group	Type of irrigation	Mean value of the biofilm covering the root canal surface before irrigation (%)	Mean value of the biofilm covering the root canal surface after irrigation (%)	Difference (Range) (%)
Group (1) Irrigation needle at 3 mm from the canal terminus (n = 30)	5.25% NaOCl	99.08	54.58	44.50
	2.5% NaOCl	97.04	60.04	37.00
	Water	96.80	78.91	17.89
Group (2) Irrigation needle at 2 mm from the canal terminus (n = 30)	5.25% NaOCl	98.08	10.05	88.03
	2.5% NaOCl	97.04	7.05	89.99
	Water	96.80	70.02	26.78

Table 2: Analysis of covariance (ANCOVA) comparing the mean amount of residual biofilm (%) remaining onto the surface of the root canal, over time (1 to 60 seconds) of irrigation using three irrigants (5.25 % NaOCl, 2.5% NaOCl, and water) delivered by syringe and needle placed at 3 mm (group 1) from the canal terminus and at flow rate of 0.15 mL s^{-1} (Total n = 30, n = 10 per group).

Experimental variable	*Mean difference in residual biofilm (%) (SE)	95% CI for mean difference	p value
5.25% NaOCl vs water	10.8 (± 0.3)	11.4, 10.2	**0.001**
2.5% NaOCl vs water	7.5 (± 0.3)	8.1, 6.9	**0.001**
5.25% NaOCl vs 2.5% NaOCl	3.3 (± 0.3)	3.9, 2.7	**0.001**

* The mean difference is significant at the 0.05 level, SE = standard Error CI = confidence interval.

Table 3: Analysis of covariance (ANCOVA) comparing the mean amount of residual biofilm (%) remaining onto the surface of the root canal, over time (1 to 60 seconds) of irrigation using three irrigants (5.25% NaOCl, 2.5% NaOCl, and water) delivered by syringe and needle placed at 3 mm (group 1) from the canal terminus and at flow rate of 0.15 mL s^{-1} (Total n = 30, n = 10 per group).

Experimental variable	*Mean difference in residual biofilm (%) (SE)	95% CI for mean difference	p value
5.25% NaOCl vs water	29.7 (± 0.3)	30.3, 29.1	**0.001**
2.5% NaOCl vs water	29.4 (± 0.3)	30.1, 28.8	**0.001**
5.25% NaOCl vs 2.5% NaOCl	0.3 (± 0.3)	0.9, 0.4	0.3

* The mean difference is significant at the 0.05 level, SE = standard Error, CI = confidence interval.

Table 4: Analysis of covariance (ANCOVA) analysing the effect of time on the mean percentage of canal surface coverage with residual biofilm for each experimental groups delivered by syringe and needle placed at 2 mm (group 1) from the canal terminus and at flow rate of 0.15 mL s^{-1} (Total n = 30, n = 10 per group).

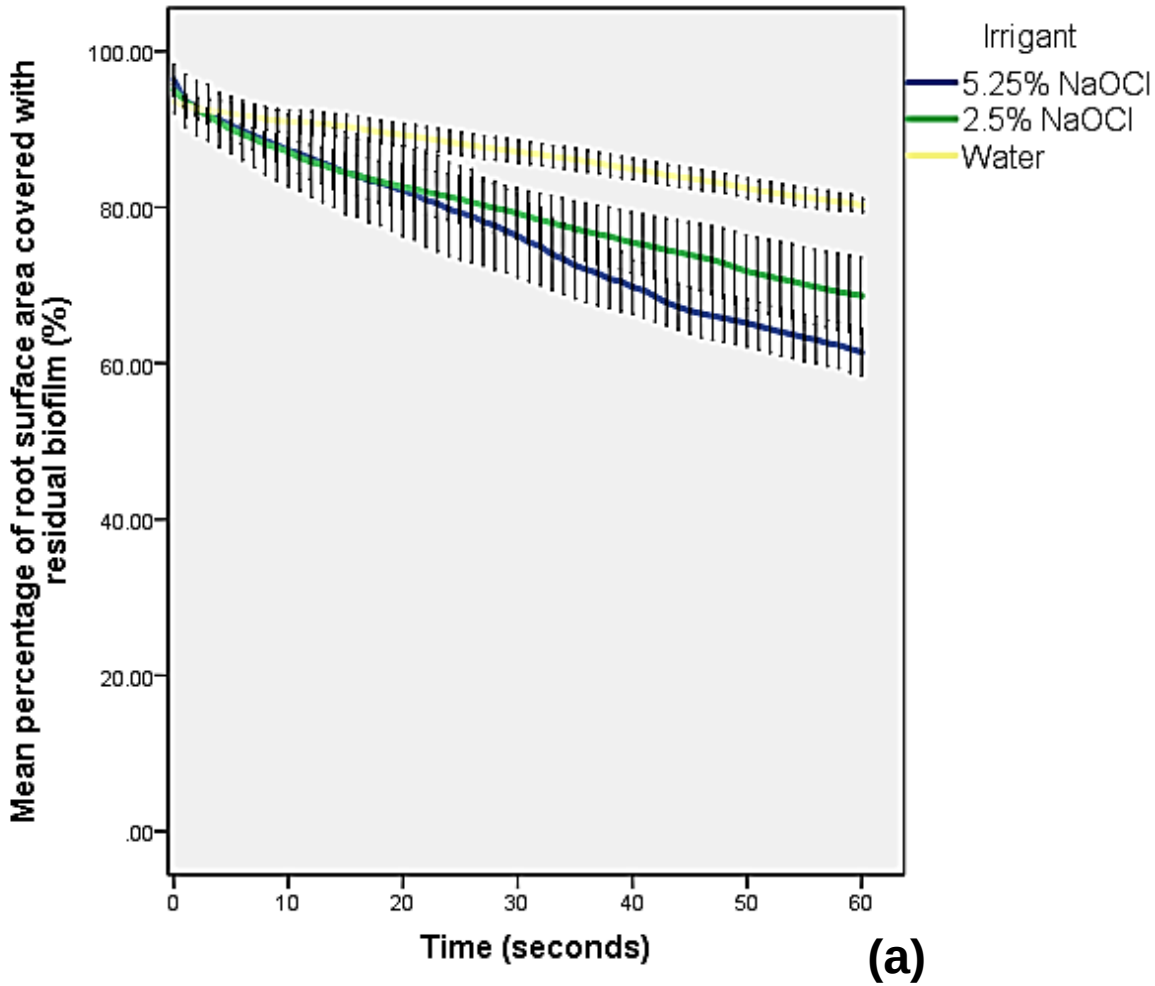
Experimental variable (reference category)	*Coefficient (% s^{-1}) (SE)	95% CI	*p* value	*Coefficient (% s^{-1}) (SE)	95% CI	p value
5.25% NaOCl (time)	-0.6 (± 0.02)	-0.7, -0.5	**0.001**	-1.3 (± 0.01)	-1.3, -1.3	**0.001**
2.5% NaOCl (time)	-0.4 (± 0.02)	-0.5, -0.3	**0.001**	-1.3 (± 0.02)	-1.3, -1.3	**0.001**
Water (time)	-0.02 (± 0.01)	-0.03, -0.01	0.61	-0.02 (± 0.02)	-0.02, -0.02	0.67

*Coefficient for time effect represents the rate of biofilm removal, SE = standard error CI = confidence interval.

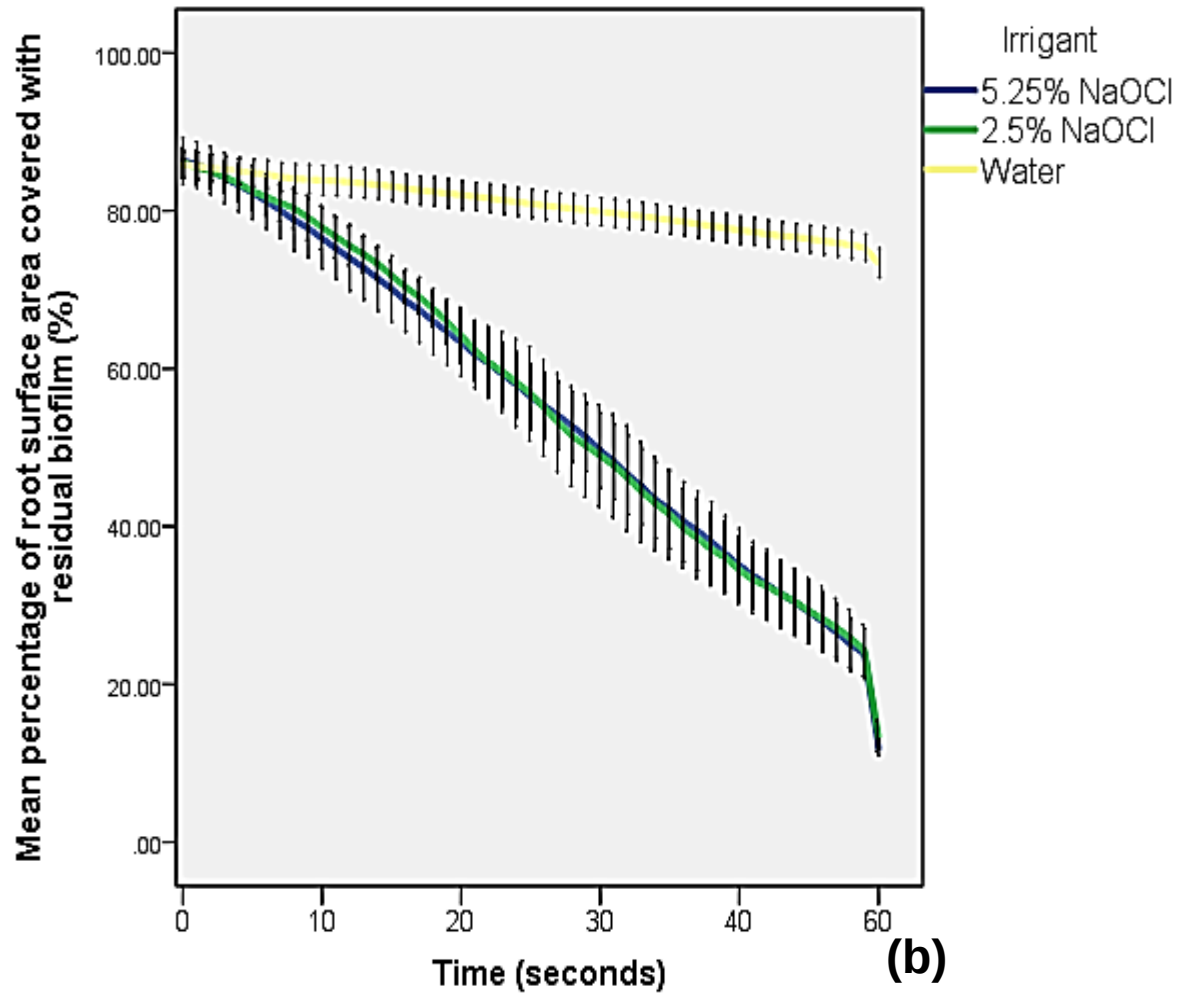
Figure legends

Figure 1: Schematic diagram illustrating the set-up of the equipment for recording residual biofilm by irrigant delivered at flow rate of 0.15 mL s^{-1} using an inverted fluorescence microscope.

Figure 2: Mean percentages (95% CI) for root canal surface-area coverage with biofilm over duration (s) of canal irrigation using needle place at (a) 3 mm or (b) 2 mm from the canal terminus and delivered at flow rate of 0.15 mL s^{-1} for each group, stratified by type of irrigant (Total n = 60, n = 10 per group).


Mean percentage of root surface area covered with residual biofilm (%)
100.00
80.00
60.00
40.00
20.00
.00
0
10
20
30
40
50
60
Time (seconds)
Irrigant
5.25% NaOCl
2.5% NaOCl
Water

(a)


Mean percentage of root surface area covered with residual biofilm (%)
100.00
80.00
60.00
40.00
20.00
.00
0
10
20
30
40
60
Time (seconds)
Irrigant
5.25% NaOCl
2.5% NaOCl
Water

(b)