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In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms

N. T. Sena, B. P. F. A. Gomes, M. E. Vianna, V. B. Berber, A. A. Zaia, C. C. R. Ferraz & F. J. Souza-Filho

Piracicaba Dental School, State University of Campinas, UNICAMP, Piracicaba, São Paulo, Brazil

Abstract

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Aim To investigate the antimicrobial activity of 2.5% and 5.25% sodium hypochlorite and 2.0% chlorhexidine gel and liquid as endodontic-irrigating substances against selected single-species biofilms.

Methods Single-species biofilms of *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, *Prevotella intermedia*, *Porphyromonas gingivalis*, *Porphyromonas endodontalis* and *Fusobacterium nucleatum* were generated on a cellulose nitrate membrane placed on agar medium. The biofilms were then immersed in the endodontic-irrigating substances for 30 s and also for 5, 10, 15, 30 and 60 min, with and without mechanical agitation. Sterile saline was used as control. After each time period, the membrane filters were then transferred to tubes containing 2 mL of fresh broth medium plus neutralizers (in order to prevent the residual action of the tested substances). The micro-organisms were suspended using a vortex, and the inoculum was serially diluted 10-fold. Aliquots of the dilutions were plated on 5% sheep blood agar medium, and incubated under adequate gaseous conditions. Colony-forming units were calculated. The samples were compared using the Friedman and Tukey test, when necessary, at a significance level of $P < 0.05$.

Results Mechanical agitation promoted the effectiveness of the antimicrobial agents, resulting in less time to eliminate the same micro-organisms, except for *S. aureus* with 2.5% NaOCl. Antimicrobial agents in liquid presentation, especially 5.25% NaOCl and 2% chlorhexidine, killed the tested micro-organisms more rapidly. Saline did not inhibit the growth of any of the tested micro-organisms, with or without agitation, being statistically different ($P < 0.05$) from NaOCl and chlorhexidine. *P. intermedia*, *P. gingivalis*, *P. endodontalis* and *F. nucleatum* were eliminated in 30 s by all antimicrobial agents, with our without agitation, in contrast with the facultative and aerobe strains.

Conclusions Mechanical agitation improved the antimicrobial properties of the chemical substances tested using a biofilm model, favouring the agents in liquid presentation, especially 5.25% NaOCl and 2% chlorhexidine.

Keywords: biofilm, chlorhexidine, endodontics, micro-organisms, sodium hypochlorite.

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Introduction

Bacteria play the primary aetiological role in the development of necrotic pulps, periapical pathosis and post-treatment disease following root canal treatment (Kakehashi *et al.* 1965). One of the crucial factors for the success of the treatment consists in the eradication

Correspondence: Dr B. P. F. A. Gomes, Department of Restorative Dentistry, School of Dentistry of Piracicaba, State University of Campinas, UNICAMP Av. Limeira, 901, Piracicaba, São Paulo, Brazil (Tel.: 0055 19 3412 5215; fax: 0055 19 3412 5218; e-mail: bpgomes@fop.unicamp.br).

of micro-organisms and their by-products from the root canal system (Gomes *et al.* 1996a,b, 2004, Peters *et al.* 2001).

Amongst the procedures involved in the control of endodontic infection, instrumentation and irrigation are important agents in eliminating the micro-organisms from the root canal system. (Sjögren *et al.* 1997, Siqueira 2001). However, mechanical debridement alone does not result in total or permanent reduction of bacteria (Byström & Sundqvist 1983). The use of antimicrobial agents has been recommended as an adjunct to mechanical instrumentation to reduce the numbers of micro-organisms (Byström & Sundqvist 1983, Gomes *et al.* 1996b, 2001).

The most popular irrigating solution is sodium hypochlorite (NaOCl). It is an effective antimicrobial agent (Byström & Sundqvist 1983, Gomes *et al.* 2001, Vianna *et al.* 2004) and an excellent organic solvent for vital, necrotic and fixed tissues (Grossman & Meiman 1941). However, it is highly irritating to periapical tissues, especially at high concentrations (Becking 1991, Ercan *et al.* 2004).

Chlorhexidine gluconate has been recommended as a root canal irrigant and medicament (Greenstein *et al.* 1986, Ferraz *et al.* 2001, Evans *et al.* 2003, Gomes *et al.* 2003). It is a potent antimicrobial agent (Jeansonne & White 1994, White *et al.* 1997, Gomes *et al.* 2001, 2003, Vianna *et al.* 2004), holds substantivity (Kuruvilla & Kamath 1998, Tanomaru Filho *et al.* 2002, Dametto *et al.* 2005), and has a low grade of toxicity (Tanomaru Filho *et al.* 2002). However, chlorhexidine is unable to dissolve pulp tissue and debris may remain on canal walls, obstructing the dentinal tubules (Kuruvilla & Kamath 1998).

Even after meticulous mechanical procedures associated with antimicrobial agents, bacteria can still be recovered from the canals. The genera that most frequently persist include enterococci (Engström 1964, Gomes *et al.* 1996b), staphylococci (Goldman & Pearson 1969, Gomes *et al.* 1996b) and gram-negative enteric rods (Haapasalo *et al.* 1983).

Much research has been undertaken to test the effectiveness of antimicrobial irrigants agents in the laboratory, using different methodologies, such as: (i) direct contact method: micro-organisms and materials tested are in close contact (Vianna *et al.* 2004); (ii) agar diffusion method: tested materials diffuse through the medium, producing the zones of inhibition of microbial growth around the tested substance (Siqueira *et al.* 1998) and (iii) the artificial infection of extracted teeth with the selected bacteria and *in situ* irrigation with the test antimicrobial agents (Sen *et al.* 1999, Siqueira 2001, Dametto *et al.* 2005). However, different results have been reported even when using the same micro-organisms and the same antimicrobial agents (Shih *et al.* 1970, Foley *et al.* 1983). These differences have been attributed to a variation in the contact between micro-organisms and the irrigant (Spratt *et al.* 2001).

The conditions used in laboratory tests do not reflect the *in vivo* conditions, where bacteria grow aggregated on the tooth surface producing a biofilm (Wilson 1996). A biofilm can be defined as communities of micro-organisms attached to a surface, embedded in an extra-cellular matrix of polysaccharides. Within these microcolonies, bacteria have developed into organized communities with functional heterogeneity (Costerton *et al.* 1999, O'Toole *et al.* 2000, Wimpenny *et al.* 2000). It constitutes a protected mode of growth that allows survival in a hostile environment. Bacteria in such an environment differ greatly in phenotype when compared with their planktonic counterparts, and are far less susceptible to antimicrobial killing (Costerton *et al.* 1999, Kimberly 2004). However, the clinical relevance of bacterial biofilm formation in endodontics has not been widely appraised.

The purpose of this study was to use a simple single species biofilm model for seven bacterial species to evaluate the effectiveness of commonly used antimicrobial irrigants.

Materials and methods

The methodology used was adapted from Spratt *et al.* (2001). The substances tested were NaOCl (2.5% and 5.25%), and two forms of chlorhexidine gluconate (gel and liquid) at a concentration of 2%.

The same manufacturer prepared all substances (Drogal Farmácia de Manipulação Ltda, Piracicaba, Sao Paolo, Brazil). The manufacturer diluted NaOCl and chlorhexidine liquid in sterile water without preservatives. The solutions were prepared 24 h before the beginning of the experiment, always in small portions. Chlorhexidine gel consisted of gel base (1% natrosol) and chlorhexidine gluconate. Sterile saline (0.89%) was used as a control.

The species of micro-organisms used in this experiment were: (i) *Enterococcus faecalis* ATCC 29212, (ii) *Candida albicans* NTCC 3736, (iii) *Staphylococcus aureus* ATCC 25923; all of them grown on 5% sheep blood–Brain Heart Infusion (BHI) agar plates (Lab M, Bury, UK) for 48 h at 37 °C; (iv) *Porphyromonas gingivalis*, (v) *Porphyromonas endodontalis*, (vi) *Prevotella*

intermedia and (vii) *Fusobacterium nucleatum* (all strict anaerobes *were* isolated from the root canal infections and identified using the conventional biochemical tests). For this study, the four strict anaerobic micro-organisms were previously subcultured on 5% sheep blood Fastidious Anaerobe Agar (FAA) plates (Lab M) for 48 h in anaerobic gaseous conditions (10%H_2, 10% CO_2 and 80% N_2) at 37 °C.

Tubes containing 5 mL of BHI sterile suspension were inoculated individually with aerobe strains (*C. albicans* and *S. aureus*) and a facultative strain (*E. faecalis*). The suspension was then adjusted spectrophotometrically according to Koo *et al.* (2000) who used the optical density at 800 nm (OD_{800}) to match the turbidity of 1.5×10^8 CFU mL^{-1} (colony forming unit, CFU), which is equivalent to 0.5 McFarland standard.

Tubes containing 5 mL of Fastidious Anaerobe Broth (FAB, Lab M) sterile suspension were individually inoculated with strict anaerobic micro-organisms, which were suspended spectrophotometrically at 800 nm (OD_{800}) to match the turbidity of 3.0×10^8 CFU mL^{-1} (equivalent to 1 McFarland standard).

Single-species biofilms of *E. faecalis*, *S. aureus*, *C. albicans*, *P. intermedia*, *P. gingivalis*, *P. endodontalis* and *F. nucleatum* were generated on a cellulose nitrate membrane (0.2-μm pore size, 13-mm diameter – Whatman International Ltd, Maidstone, UK). The membranes were placed on the surface of 5% defibrinated sheep blood BHI agar plates (for aerobic and facultatively anaerobic micro-organisms) and on 5% defibrinated sheep blood-FAA plates (for strict anaerobes) and further inoculated with 20 μL of each test micro-organism suspension. The plates, each containing four membrane filters, were incubated at 37 °C again under the appropriate gaseous conditions: aerobes and facultative anaerobes in a CO_2 incubator (Jouan, Saint Herblain, France) and anaerobes in an anaerobic chamber (Don Whitley Scientific, Bradford, UK) in an atmosphere of 10% H_2, 10% CO_2 and 80% N_2.

The efficiency of the method for biofilm generation was observed in a pilot study, visually and by SEM, where it was possible to verify the presence of biofilm after 10 days of incubation (Fig. 1).

The membrane filters were removed aseptically from the agar plate and transferred carefully to tubes containing 5 mL of the selected antimicrobial test agent and saline for the control group, which were incubated for 30 s, and also for 5, 10, 15, 30 and 60 min with and without mechanical agitation.

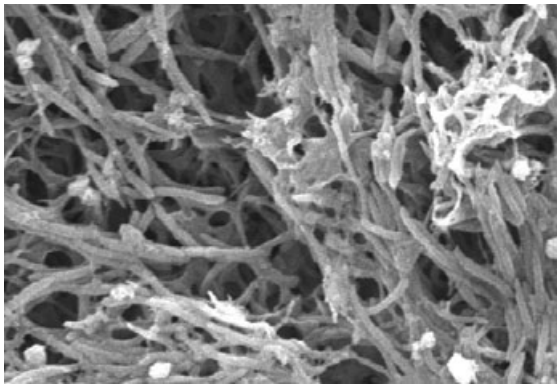

Figure 1 *Fusobacterium nucleatum* biofilm.

After each period of time, the membrane filters were then transferred to tubes containing 2 mL of freshly prepared broth medium plus neutralizers (Tween 80 plus 0.07% lecithin was used for chlorhexidine and 0.06% sodium thiosulphate for NaOCl) in order to prevent the residual action of the substances (Gomes *et al.* 2001). These were then vortexed for 30 s to resuspend the micro-organisms. Tenfold serial dilutions were made up from the bacterial suspension and plated out on blood agar plates (BHI or FAA according to the micro-organism). The plates were then incubated at 37 °C under the appropriate gaseous conditions for 24 h (aerobes), 48 h (facultative anaerobes) and 7 days (strict anaerobes). The number of CFU per membrane was calculated. The tests were carried out in triplicate for each antimicrobial agent and micro-organism, and the survival curve was calculated.

Samples were statistically analysed, demonstrating that the data were nonparametric. Because of the high SD of the CFU means, a rank transformation was indicated. This is a statistical tool that produces a table containing the ordinal rank of each value in a data set, in other words, rank transforms the dependent variable. In the present analysis, high rank averages indicate great CFU means. Then, the samples were compared using the Friedman and Tukey test, when necessary, at a significance level of $P < 0.05$. All data were converted to seconds to make the comparisons.

Results

Table 1 shows the contact time in seconds and the mean rank required for NaOCl and chlorhexidine (liquid and gel formulations) to produce negative cultures against all micro-organisms tested using mechanical agitation. All strictly anaerobic micro-organisms

Table 1 Contact time in seconds and mean rank required for chlorhexidine (CLX liquid and gel formulations) and sodium hypochlorite (NaOCl) to produce negative cultures against all tested micro-organisms using the mechanical agitation

Antimicrobial agents	Micro-organisms							Mean rank
	Prevotella intermedia	*Porphyromonas endodontalis*	*Porphyromonas gingivalis*	*Fusobacterium nucleatum*	*Candida albicans*	*Enterococcus faecalis*	*Staphylococcus aureus*	
2% CLX gel	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	900 (4.0)	300 (4.0)	600 (4.0)	3.14 B
2.5% NaOCl	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	300 (3.0)	30 (2.0)	300 (3.0)	2.14 AB
2% CLX liquid	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	30 (1.5)	30 (2.0)	30 (1.5)	2.14 A
5.25% NaOCl	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	30 (1.5)	30 (2.0)	30 (1.5)	2.57 A
Saline solution	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	5.00 C

Different letters (from A to B) mean significant differences (Tukey's test $P < 0.05$) amongst mean rank.
Values in brackets: ranks for the Tukey's test.

were killed within 30 s by all antimicrobial substances tested. All micro-organisms were killed in 30 s by 5.25% NaOCl and 2% liquid chlorhexidine. *C. albicans* and *S. aureus* took 300 s (5 min) to be killed by 2.5% NaOCl, while *E. faecalis* was eliminated in 30 s. *C. albicans* was the most resistant micro-organism to 2% chlorhexidine gel, taking 900 s (30 min) to be totally eliminated, followed by *S. aureus* (600 s = 15 min) and *E. faecalis* (300 s = 5 min). There was no statistically significant difference between 5.25% NaOCl, 2.0% liquid chlorhexidine and 2.5% NaOCl. Chlorhexidine gel needed more time to eliminate all bacteria and yeast (mean rank 3.14) than the other irrigants. However, it was statistically different ($P < 0.05$) from the control group (saline).

Table 2 shows the contact time in seconds required for NaOCl and chlorhexidine (liquid and gel formulations) to produce negative cultures against all micro-organisms without mechanical agitation. All strictly anaerobic micro-organisms tested were eliminated within 30 s by all antimicrobial substances tested. *E. faecalis* was the most resistant micro-organism to the 2.5% NaOCl, surviving even after 60 min. *S. aureus* was also very resistant to 2% chlorhexidine gel, which was able to kill all cells within 60 min. No statistically significant difference was observed amongst the antimicrobial substances tested. However, all of them were statistically different ($P < 0.05$) from the control group (saline).

Comparing Table 1 (treatment with mechanical agitation) with Table 2 (without mechanical agitation), it can be observed that mechanical agitation promoted the effectiveness of the antimicrobial agents, resulting in less time to eliminate the same micro-organisms, except for *S. aureus* for 2.5% NaOCl. Antimicrobial substances in liquid presentation, especially 5.25% NaOCl and 2% chlorhexidine, killed more rapidly the tested micro-organisms. Saline did not inhibit the growth of any of the tested micro-organisms, with or without agitation, being statistically different ($P < 0.05$) from NaOCl and chlorhexidine. *P. intermedia*, *P. gingivalis*, *P. endodontalis* and *F. nucleatum* were eliminated in 30 s by all antimicrobial agents, with or without agitation, in contrast with the facultative and aerobe strains.

Discussion

Micro-organisms and their products are considered the main cause of pulpal and periapical diseases (Kakehashi *et al.* 1965), and can attach to the root canal walls

Table 2 Contact time in seconds and mean rank required for chlorhexidine (CLX) in liquid and gel formulations and NaOCl to produce the negative cultures against all tested micro-organisms without mechanical agitation

Antimicrobial agents	Micro-organisms							Mean rank	
	P. intermedia	*P. endodontalis*	*P. gingivalis*	*F. nucleatum*	*C. albicans*	*E. faecalis*	*S. aureus*		
2% CLX gel	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	1800 (4.0)	1800 (3.0)	3600 (4.0)	3.00	A
2.5% NaOCl	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	300 (2.5)	>3600 (4.5)	30 (2.0)	2.71	A
2% CLX liquid	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	300 (2.5)	30 (1.5)	30 (2.0)	2.29	A
5.25% NaOCl	30 (2.5)	30 (2.5)	30 (2.5)	30 (2.5)	30 (1.0)	30 (1.5)	30 (2.0)	2.07	A
Saline solution	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (5.0)	>3600 (4.5)	>3600 (5.0)	4.93	B

Equivalent letters mean lack of significant differences (Friedman's test $P < 0.05$) amongst rank mean.
Values in brackets: ranks for the Friedman's test.

forming biofilms (Nair *et al.* 1990). Because of the difficulty of ensuring adequate removal of the biofilm on the dentine wall using the mechanical procedures only, it is necessary to use the substances with antimicrobial properties (Ercan *et al.* 2004).

In the laboratory, micro-organisms are generally grown planktonically, but the utopian microcosms created in culture vessels are designed to maximize microbial growth rates and not to replicate the natural growth conditions of the micro-organisms, which usually utilize the biofilm mode to grow outside the laboratory (Kimberly 2004).

Most of the *in vitro* tests use planktonic cultures for testing the antimicrobial efficacy of endodontic irrigants. Depending on the concentration of the substance tested and the susceptibility of the micro-organism, the latter can be eliminated in seconds using the planktonic cells and the direct contact method. Such a killing effect may not happen clinically (Ohara *et al.* 1993, D'Arcangelo *et al.* 1999). Organization of bacteria within biofilms confers a range of phenotypic properties that are not evident in their planktonic counterparts and amongst other characteristics, and confers a reduced susceptibility to antimicrobial agents (Wilson 1996, Abdullah *et al.* 2005). Therefore, the use of a biofilm model could reproduce more precisely the *in vivo* conditions.

The biofilm model in this study was used to evaluate the antimicrobial efficacy of substances used during the chemo-mechanical preparation, against selected micro-organisms commonly found in root canals. The methodology performed was based on the one used by Spratt *et al.* (2001), which allows the biofilm to grow on cellulose nitrate membranes. The antimicrobial agent was tested in direct contact with the single biofilm. However, differently from Spratt *et al.* (2001), in the present study, the time taken to produce consistent biofilm was longer, which may explain the greater biofilm resistance, probably because of the biofilm organization. The more fastidious anaerobic species (*P. gingivalis* and *P. endodontalis*) required a longer incubation period (approximately 10 days) for the development of the biofilm. Therefore, this period of time was used as a pattern for the other micro-organisms.

Also, in this study, all substances were tested with and without mechanical agitation, with better results in the groups with agitation. It could be possible that the agitation of the substance may have improved the contact between the antimicrobial agent and the micro-organism on the biofilm. Such agitation also favoured the antimicrobial agents in liquid presentation. In the present investigation, NaOCl and chlorhexidine liquid killed the microbial cells more rapidly than chlorhexidine gel, which is in accordance with our previous work (Gomes *et al.* 2001). NaOCl and the chlorhexidine liquid mixed very well with the microbial suspension, exerting rapidly its antimicrobial action, whereas the gel formulation, which is more difficult to mix, prevented the direct contact between the microbial cells and chlorhexidine, thus requiring a longer time to act against the micro-organisms.

The protocol used in this study is practical and easy to reproduce, allowing a primary screening to test the antimicrobial effect of the substances against biofilms.

This study showed that different micro-organisms are susceptible to different degrees to the antimicrobial agents tested, and the time of exposure could be decisive in the efficacy of the substance. All micro-organisms grew in contact with saline, with or without agitation (positive control).

The 5.25% NaOCl, with/without mechanical agitation, eliminated all micro-organisms in 30 s. Similar results were found by Spratt *et al.* (2001). Vianna *et al.* (2004), using 5.25% NaOCl, reported that *P. intermedia*, *P. gingivalis*, *P. endodontalis*, *C. albicans*, *S. aureus* and *E. faecalis* were eliminated within 15 s using the direct contact method. Gomes *et al.* (2001) and Senia

et al. (1975) also found that 5.25% NaOCl killed *E. faecalis* cells in 15 s using the direct contact method.

Radcliffe *et al.* (2004) and Vianna *et al.* (2004) found that 2.5% NaOCl inhibited the growth of all tested micro-organisms in 5 and 10 min, respectively. In the present study, 2.5% NaOCl, without mechanical agitation, was effective against all micro-organisms in 5 min except against the *E. faecalis* biofilm. However, the latter was eliminated under the mechanical agitation.

The acting time of 2% chlorhexidine gluconate in liquid and gel presentation forms was different. The liquid form, under the mechanical agitation, showed better antimicrobial activity, eliminating all micro-organisms in 30 s. However, without mechanical agitation, it took 15 min to be totally effective.

Analysing the performance of 2% chlorhexidine gluconate gel, it took 30 min with mechanical agitation, and 60 min without mechanical agitation, to inhibit the growth of all tested micro-organisms.

Vianna *et al.* (2004) and Gomes *et al.* (2001), who used planktonic cells, showed that the time required for 2% chlorhexidine gluconate liquid/gel to produce negative cultures was 1 min for aerobic and facultative micro-organisms.

In agreement with our results, Vianna *et al.* (2004) found that all agents tested, with or without mechanical agitation, were completely effective within 30 s against the strict anaerobes: *P. intermedia*, *P. gingivalis* and *P. endodontalis*. These species are not resistant micro-organisms but they probably survive because of their association with other persistent micro-organisms (Gomes *et al.* 1996a, Spratt *et al.* 2001). However, it should be emphasized that the anaerobic strains used in this study were root canal isolates and the results may be strain-dependent.

In clinical studies, *F. nucleatum* has been found to persist after endodontic treatment (Gomes *et al.* 1996b). However, Ohara *et al.* (1993) and Sassone *et al.* (2003), using the *in vitro* tests, showed that this micro-organism was very susceptible, being easily eliminated by antimicrobial agents. In the present study, this species was eliminated in 30 s by all antimicrobial substances tested, agreeing with the latter authors. On the other hand, Spratt *et al.* (2001) found that this species was significantly resistant to all irrigants for up to 15 min.

Candida albicans is the fungal species most commonly isolated from infected root canals (Siqueira & Sen 2004), being associated with cases of post-treatment disease (Nair *et al.* 1990). In this study, *C. albicans* required, under the mechanical agitation, from 30 to 900 s (15 min) to be eliminated by all tested antimicrobial agents. On the other hand, the growth of this species was observed, without mechanical agitation, from 30 to 1800 s (30 min). Radcliffe *et al.* (2004) using the direct contact method found that after 10 s of contact between *C. albicans* and 0.5% NaOCl, no cells could be detected. Vianna *et al.* (2004) reported a maximum time for growth inhibition of 30 min for NaOCl and 10 min for all chlorhexidines tested (gel and liquid presentation).

Facultative anaerobic and gram-positive bacteria, such as *Enterococcus* and *Staphylococcus*, are more resistant to instrumentation and to antiseptic agents, and therefore can be expected to persist more frequently in the root canal after inadequate root canal preparation and obturation (Cavalleri *et al.* 1989, Gomes *et al.* 1996b, Molander *et al.* 1998). Persisting micro-organisms or their by-products can maintain an infectious process and cause treatment failure. According to Molander *et al.* (1998), *E. faecalis* can survive in a quiescent phase with low metabolic activity for a period of time, and factors such as coronal leakage can change the nutritional conditions and contribute to bacterial growth. The potential factor of virulence is its ability to survive inside of polymorphonuclear leucocytes (PMN) and macrophages. Aggregation substance (AS) has been indicated as being responsible for the internalization of *E. faecalis* within PMN, making it resistant to killing (O'Toole *et al.* 2000, Distel *et al.* 2002, Baldassarri *et al.* 2004). The results from this study also found that *S. aureus* and *E. faecalis* were the most resistant micro-organisms tested.

This biofilm model seems to be more realistic than the direct contact method to test antimicrobial agents, as it allows micro-organisms to grow as biofilms on the nitrate cellulose membrane, which are more resistant to therapy. Further studies should test the susceptibility of antimicrobial substances against the mixed biofilms.

Within the limitations of this *in vitro* study, the biofilm model gave a simple means of determining the antimicrobial efficacy of irrigants used in root canal treatment. This methodology may be more clinically representative than the methods, which do not consider the micro-organism in biofilms. However, it still does not reproduce what happens clinically in the root canal. In such an environment, several mechanisms allow the growth and selection of several micro-organisms, even after the treatment.

Conclusion

Mechanical agitation improved the antimicrobial properties of the chemical substances tested using a biofilm

model, favouring the agents in liquid presentation, especially 5.25% NaOCl and 2% chlorhexidine,

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