

Original Article

Application of laser scanning microscopy for the analysis of oral biofilm dissolution by different endodontic irrigants

Aldo del Carpio-Perochena, Clovis Monteiro Bramante, Marco Antonio Hungaro Duarte, Flaviana Bombarda de Andrade, Bruno Cavalini Cavenago, Marcelo Haas Villas-Bôas, Ronald Ordinola-Zapata, Pablo Amoroso-Silva

Department of Dentistry, Endodontics and Dental Materials, Bauru Dental School, University of São Paulo, Bauru, São Paulo, Brazil

ABSTRACT

Background: Multi-specie biofilms are highly resistant to antimicrobials due to cellular interactions found in them. The purpose of this study was to evaluate, by confocal laser scanning microscopy, the biofilm dissolution effectiveness of different irrigant solutions on biofilms developed on infected dentin *in situ*.

Materials and Methods: A total of 120 bovine dentin specimens infected intraorally (30/group) were treated by the following solutions: 2% of chlorhexidine digluconate, 1%, 2.5% and 5.25% of sodium hypochlorite (NaOCl). The solutions were utilized for 5, 15 and 30 min with 2 experimental volumes 500 μ L and 1 mL. All the samples were stained using an acridine orange and the biofilm thickness before (control group) and after the experiments were evaluated, utilizing a confocal microscope at $\times 40$. The Mann-Whitney U and the non-parametric Kruskal-Wallis Dunns tests were utilized to determine the influence of the volume and to perform the comparisons among the groups respectively. The significance level was set at $P < 0.05$.

Results: Statistical differences were not found among the control and the 2% chlorhexidine digluconate groups at any experimental period ($P > 0.05$). The biofilm dissolution treated with 1% NaOCl was directly proportional to the exposure time ($P < 0.05$). The higher values of biofilm dissolution were found in 2.5% and 5.25% NaOCl groups ($P > 0.05$).

Conclusion: The higher exposure times and concentrations of NaOCl were not sufficient to dissolve 100% of the biofilm. However, all NaOCl solutions were more effective than 2% chlorhexidine digluconate to dissolve organic matter.

Key Words: Biofilm, chlorhexidine, confocal laser scanning microscopy, dentin, sodium hypochlorite

Received: July 2013

Accepted: October 2013

Address for correspondence:

Dr. Aldo del Carpio-Perochena,
Bauru Dental School,
University of São Paulo,
Al. Octavio Pinheiro
Brisolla, 9-75, 17012-901,
Bauru, São Paulo, Brazil.
E-mail: aldoelcp@usp.br

INTRODUCTION

The main objective of endodontic therapy is to remove pulp debris and bacterial populations from the root canal system. However, due to the complex anatomy of the root canal system, more than 50% of its walls remain uninstrumented during instrumentation.^[1] The

study of the relationship between endodontic therapy and microbial biofilms involves the observation of bacterial condensation in the root canal system, with or without endodontic therapy^[2] and the ability of irrigant solutions or endodontic procedures to dissolve or eradicate them.^[3]

The persistence of infection in the root canal with endodontic treatment may occur due to the existence of bacteria in the dentinal tubules or even by bacteria introduced into the root canal.^[4,5] Bacteria and their products in avascular and necrotic root canal systems are the main etiological factor of apical periodontitis.^[6,7] Conventionally, root canal disinfection is performed using procedures that include chemomechanical

Access this article online



Website: <http://drj.mui.ac.ir>

cleaning, shaping and the applications of chemical disinfectant solutions.^[8] Although this technique is the standard procedure to disinfect root canals with necrotic pulp, in many occasions, it may fail to completely eliminate bacterial biofilms, mainly due to microbiological and anatomic factors.^[2,9] Several studies have shown the antimicrobial effectiveness of sodium hypochlorite (NaOCl) and chlorhexidine,^[10-13] but currently there are limited information concerning the biofilm dissolution capabilities of these irrigants.^[11]

Although NaOCl is the irrigant commonly used in endodontic therapy, it is highly toxic to periapical tissues.^[14] In order to overcome this disadvantage, the chlorhexidine gluconate was suggested as an alternative endodontic irrigant solution, due to its low toxicity and relative biocompatibility.^[15]

NaOCl has great organic tissue dissolution ability by saponification reaction and also, has a wide-spectrum antimicrobial efficacy produced by the acid neutralization and chloramination reactions that occur in the presence of microorganisms and organic matter.^[16]

Chlorhexidine gluconate is an antiseptic solution belonging to the biguanide group. It possesses a broad spectrum against Gram-positive and negative bacteria.^[17] Its bactericide effect is caused by the disruption of the microbial cell membrane of the bacteria. However, the main limitation of chlorhexidine is its inability to dissolve organic matter.^[13]

Confocal laser scanning microscopy (CLSM) presents advantages for the study of biofilms without the necessity of a specific treatment applied to the sample, such as dehydration or sputter coating, which are usually necessary when a conventional scanning electron microscope (SEM) is used.^[18,19] This advantage allows for the analysis of infected dentin samples both before and after the treatment with antimicrobial compounds. The aim of this study was to evaluate the biofilm dissolution of different concentrations of NaOCl and 2% chlorhexidine digluconate on biofilms developed on infected dentin *in situ*. The influence of volume and contact time was also studied. The null hypothesis of this study is as follows: The biofilm dissolution is affected by different variables, such as, exposure time, concentration and volume of the solution.

MATERIALS AND METHODS

This research was approved by the Human Ethical Committee of the Bauru Dental School (Protocol

064/2009). The irrigant solutions used in this study were 1% and 2.5% NaOCl (Cloro Rio, São José do Rio Preto, SP, Brazil), 5.25% NaOCl (Farmacia Especifica, Bauru, SP, Brazil) and 2% chlorhexidine digluconate (Villevie, Joinville, SC, Brazil). Freshly extracted bovine teeth were used. The crowns were sectioned using an Isomet saw (Buehler Ltd., Evanston, IL). The roots were then cut parallel to the tooth axis and the segments obtained were then cut perpendicular to the tooth axis. Through this dentin blocks were obtained (approximately 3 mm × 3 mm × 3 mm). The root canal remained intact and was discarded. The samples were autoclaved and treated with 2.5% NaOCl for 15 min and 17% ethylenediaminetetraacetic acid for 3 min.

In order to induce bacterial infection, 12 dentin samples were fixed on a removable orthodontic device with cavities. Each sample was attached into these cavities using sticky wax. The dentin surface in contact with the oral cavity was fixed approximately 1-2 mm above the surface in order to facilitate the accumulation of plaque and to avoid the “sweeping effect” produced by the tongue. This procedure was repeated until all the samples were completed. A healthy single volunteer used the intraoral device for 72 h to try to standardize the biofilm thickness as much as possible. During this time, the volunteer received a controlled diet and also maintained recommended oral hygiene practices. After this time period, the samples were removed and stained with 1 mL of 0.01% acridine orange for 15 min. Afterwards, the samples were rinsed with 100 mL of distilled water. The pre-irrigation samples were used as a control group.

One drop of oil objective (CLSM Leica TCS-SPE; Microsystems GmbH, Mannheim, Germany) was used to facilitate the biofilm visualization. This means that a thin layer of oil covered the biofilm. This oil lens application did not vary the biofilm morphology nor did it interfere with the action of the experimental solutions. This statement was previously demonstrated in a pilot study.

The specimens were immediately analyzed at ×40 via the CLSM technique. The biofilm was evaluated in several locations, to find the greatest points of thickness. Three segments per sample were analyzed. The Z-Stack of Leica Application Suite-Advance Fluorescence Software (LAS AF, Leica, Mannheim, Germany) was utilized to measure the thickness of the biofilm. The samples were scanned at intervals

of 1 μm , from the upper biofilm level to the dentin surface.

When the height of pre-irrigation biofilm was recorded, the samples were randomly divided into 3 groups ($n = 10$) according to the contact time (5, 15 and 30 min) and subdivided into 2 subgroups ($n = 5$) according to the volume of the solution (500 μL or 1 mL), totaling to thirty samples per irrigant. The samples were immersed in 24-well tissue culture plates, containing the experimental solutions. In the 15-min and 30-min groups the irrigants were refreshed every 5 min in order to simulate clinical conditions.

After the experimental periods, the samples treated with NaOCl were washed with 100 mL of 5% sodium thiosulfate for 5 min. Chlorhexidine-treated samples were washed with 100 mL of distilled water. The samples were then immediately stained with the acridine orange dye and the post-treated biofilm was measured for thickness. Representative images of the samples both before and after treatment with the experimental solutions can be observed in Figure 1.

The Mann-Whitney U-test was utilized to determine the influence of the volume. The non-parametric Kruskal-Wallis and Dunns tests were utilized for comparisons among the groups and times because the data did not show a normal distribution. The level of significance was set at $P < 0.05$. The Prisma 5.0

(GraphPad Software Inc., La Jolla, CA) was utilized as the analytical software.

RESULTS

A total of 120 samples were evaluated. The Mann-Whitney's U test showed statistically, that there were no significant differences between the volumes (1 mL and 500 μL) of the experimental solutions ($P > 0.05$). As a consequence, the data was combined to provide a single mean of 10 samples (30 scans) per group.

The medians and 25-75% percentiles of the percentage values of the thickness of the biofilm in μm , both before and after contact with the irrigating solutions, are shown in Table 1.

Nearly 2% chlorhexidine digluconate showed no effect on biofilm thickness, in comparison to the other evaluated irrigant solutions. No significant statistical differences were found between the 2% chlorhexidine digluconate groups and the pre-irrigation samples (control) at any time ($P > 0.05$). The biofilm thickness treated with 1% NaOCl for 5 min, was significantly higher than the 15 and 30 min groups. The 15 min group was significantly higher than the 30 min group ($P < 0.05$).

No significant statistical differences were found between the 2.5% NaOCl groups, at any time ($P > 0.05$). Almost 5% NaOCl groups showed the same results.

DISCUSSION

In the present study, the CLSM technique was used because it allows an optical sectioning of the sample. This eliminates the possibility of physical sectioning, as in conventional light and electron microscopic techniques. In addition, the optical sectioning can be used to record data from three axes (x, y and z), allowing to analyze the biofilm in depth or laterally.^[20]

Regarding to *in vitro* studies, they are important to determine bacterial interaction that occurs in biofilm,^[21] but are not able to accurately simulate bacterial growth conditions in the oral cavity such as, the different varieties of nutrients, saliva, pH and temperature changes. In order to solve these limitations, an *in situ* model developed to study tooth decay,^[22] was modified for this kind of endodontic methodology.^[23-25]

Currently, there is not enough substantial information, indicating that the bacterial growth within the root

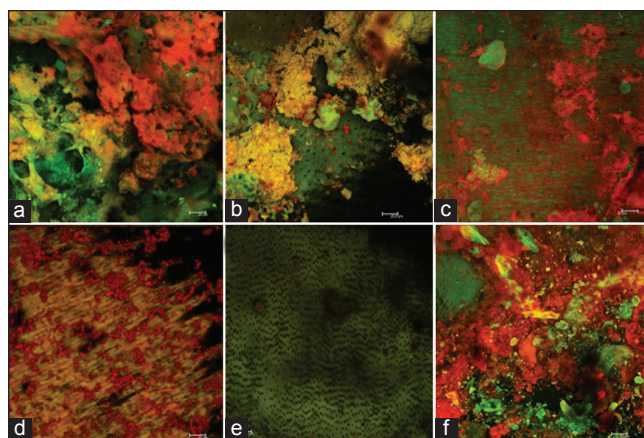


Figure 1: Representatives confocal pictures of biofilm before and after treatment. Preoperative picture is shown in (a). A great-undisturbed biomass after 5 min of 1% NaOCl on biofilm can be seen in (b). An evident disorganization of the biofilm after 15 min of contact with 2.5% NaOCl is shown in (c and d). Only few isolated biofilm areas are visible after 30 min of contact with 2.5% NaOCl (e). The biofilm structure remains intact after treatment with 2% chlorhexidine digluconate during 30 min (f). All bars represent 20 μm

Table 1: Medians and (25-75%) percentiles of the percentage of biofilm thickness in μm before/after contact with 2% chlorhexidine (A), 1% NaOCl (B), 2.5% NaOCl (C), 5.25% NaOCl (D)

Groups	5 min		15 min		30 min	
	Baseline	Treatment	Baseline	Treatment	Baseline	Treatment
A	36.0 (30.0-49.0) ^a	30.5 (23.0-43.0) ^a	30.0 (25.0-44.5) ^a	28.0 (16.0-36.0) ^a	30.00 (24.5-36.0) ^a	28.5 (19.5-35.0) ^a
B	28.0 (23.0-60.0) ^a	18.0 (14.0-24.0) ^b	39.0 (28.0-45.5) ^a	14.0 (12.0-17.0) ^b	37.00 (23.0-44.0) ^a	10.0 (8.0-13.5) ^b
C	31.0 (28.0-41.0) ^a	15.5 (13.0-21.5) ^{bc}	30.5 (26.0-36.0) ^a	13.5 (9.0-18.0) ^b	37.50 (29.5-45.0) ^a	12.0 (10.0-16.0) ^b
D	32.0 (28.0-37.0) ^a	13.0 (10.0-16.0) ^c	37.5 (30.0-50.0) ^a	14.0 (13.0-18.0) ^b	40.00 (32.0-47.5) ^a	12.0 (10.0-16.0) ^b

Different superscript letters in each column represent significant differences ($P < 0.05$); ($n = 10$)

canal biofilm is significantly different than the biofilms found in dental plaque. Thus, based on the fact that root canal biofilm is formed by several species^[26] and considering that mono-infections occur rarely in nature, the authors believe that the induction of bacterial growth *in situ* on dentin is a good alternative to test the dissolution effect of endodontic solutions.

It is worth noting that the anatomical variations of the root canal system can act as protection for bacteria lodged in it, hence, preventing the penetration of antimicrobial agents.^[2] For this reason, the lack of anatomic variations of the samples was a limitation of this study.

In addition, the topography of the substrate is an important item when discussing the survival of biofilm. On the initial phases of biofilm formation, the rough surfaces will increase the bacterial adhesion and retention because it provides anchor points for microorganisms and their nutrients.^[27,28] In a pilot study using SEM, it was verified that the samples that were cut transversal or longitudinally showed the mentioned rough surfaces. However, we believe that this factor is not really important in this particular case because the focus of the present study was to measure the bacterial biomass from the dentinal surface to the highest part of the mature biofilm.

Laboratory studies have showed that the organic tissue dissolution is directly proportional to the NaOCl concentration.^[11,16] It is also known that this dissolution ability is dependent on factors such as: Amount of organic matter, irrigation frequency and surface size where the bacteria are adhered.^[29] In the present study, no statistical differences were found between the exposure times of 2.5% and 5% NaOCl groups respectively. Furthermore, none of NaOCl solutions tested were able to completely dissolve the biofilm. These results could be due to the bacterial colonization induced *in situ* conditions,

which present variable bacterial growth for each sample and high resistance to the antibacterial agents due to the bacterial synergism presented in biofilm multispecies.^[30,31] In the line with this statement, Retamozo *et al.*^[32] showed that 5% NaOCl was not effective in eradicating the *Enterococcus faecalis* biofilms when the contact times were less than 40 min. Similarly, Clegg *et al.*^[11] demonstrated that 6% NaOCl-15 min was the only irrigant capable of physically removing the biofilm.

NaOCl and chlorhexidine show antimicrobial capacity, but the ability of dissolution of organic matter is unique to NaOCl. This solution dissolves organic tissue by a chemical reaction called "saponification." This reaction degrades fatty acids, transforming them into fatty acid salts (soap) and glycerol (alcohol).^[16] In addition, NaOCl is dissociated into HOCl and NaOH when reacting with organic material. This chemical reaction is responsible for the liquification of organic compounds, such as biofilms or necrotic tissue.^[29] In the present study, it was observed a directly proportional relationship between the NaOCl concentration, exposure time and organic matter dissolution. Similar results were found by Clegg *et al.*,^[11] who analyzed the effect of different concentrations of NaOCl and 2% chlorhexidine. The results showed that 6% NaOCl was able to remove 100% of the biofilm, while, 1% NaOCl partially removed it. The biofilm remained intact when it was irrigated with 2% chlorhexidine.

Moreover, in the present study, we observed that NaOCl, in its lower concentration and exposure time (1% for 5 min), was more effective than the 2% chlorhexidine digluconate for biofilm thickness reduction. These results are in agreement with the results found by Bryce *et al.*^[33] and Chavez de Paz *et al.*^[10]

Finally, although several studies have shown that chlorhexidine has antibacterial capacities, there is

evidence to show its lack of dissolution capacity.^[10,11,13,34] This statement is similar to the results found in the present study. Similarly, Shen *et al.*,^[35] using a CLSM, observed that 2% chlorhexidine digluconate was not able to dissolve the biofilm in any of the 3 time periods studied (1, 3 and 10 min). In accordance with this statement, the major implication of residual biofilms is that they can act as a protective shield for bacteria within the dentinal tubules.^[36] Therefore, residual biofilms can be considered as an organic layer, which may interfere with the adaptation and intratubular penetration of sealing materials.

CONCLUSION

Although the NaOCl solutions showed significant ability to dissolve biofilm, 30 min of exposure time was insufficient to completely remove organic matter regardless of the irrigant concentration. 2% chlorhexidine was not able to dissolve the biofilm at any time.

REFERENCES

- Peters OA, Laib A, Göhring TN, Barbakow F. Changes in root canal geometry after preparation assessed by high-resolution computed tomography. *J Endod* 2001;27:1-6.
- Nair PN, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2005;99:231-52.
- Williamson AE, Cardon JW, Drake DR. Antimicrobial susceptibility of monoculture biofilms of a clinical isolate of *Enterococcus faecalis*. *J Endod* 2009;35:95-7.
- Buck RA, Eleazer PD, Staat RH, Scheetz JP. Effectiveness of three endodontic irrigants at various tubular depths in human dentin. *J Endod* 2001;27:206-8.
- Ringel AM, Patterson SS, Newton CW, Miller CH, Mulhern JM. *In vivo* evaluation of chlorhexidine gluconate solution and sodium hypochlorite solution as root canal irrigants. *J Endod* 1982;8:200-4.
- Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surg Oral Med Oral Pathol* 1965;20:340-9.
- Silva LA, Leonardo MR, Assed S, Tanomaru Filho M. Histological study of the effect of some irrigating solutions on bacterial endotoxin in dogs. *Braz Dent J* 2004;15:109-14.
- Siqueira JF Jr, Paiva SS, Rôças IN. Reduction in the cultivable bacterial populations in infected root canals by a chlorhexidine-based antimicrobial protocol. *J Endod* 2007;33:541-7.
- Kvist T, Molander A, Dahlén G, Reit C. Microbiological evaluation of one- and two-visit endodontic treatment of teeth with apical periodontitis: A randomized, clinical trial. *J Endod* 2004;30:572-6.
- Chávez de Paz LE, Bergenholtz G, Svensäter G. The effects of antimicrobials on endodontic biofilm bacteria. *J Endod* 2010;36:70-7.
- Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms *in vitro*. *J Endod* 2006;32:434-7.
- Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. *Dent Clin North Am* 2010;54:291-312.
- Okino LA, Siqueira EL, Santos M, Bombana AC, Figueiredo JA. Dissolution of pulp tissue by aqueous solution of chlorhexidine digluconate and chlorhexidine digluconate gel. *Int Endod J* 2004;37:38-41.
- Gernhardt CR, Eppendorf K, Kozłowski A, Brandt M. Toxicity of concentrated sodium hypochlorite used as an endodontic irrigant. *Int Endod J* 2004;37:272-80.
- Jeanson MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. *J Endod* 1994;20:276-8.
- Estrela C, Estrela CR, Barbin EL, Spanó JC, Marchesan MA, Pécora JD. Mechanism of action of sodium hypochlorite. *Braz Dent J* 2002;13:113-7.
- Parsons GJ, Patterson SS, Miller CH, Katz S, Kafrawy AH, Newton CW. Uptake and release of chlorhexidine by bovine pulp and dentin specimens and their subsequent acquisition of antibacterial properties. *Oral Surg Oral Med Oral Pathol* 1980;49:455-9.
- Lawrence JR, Korber DR, Hoyle BD, Costerton JW, Caldwell DE. Optical sectioning of microbial biofilms. *J Bacteriol* 1991;173:6558-67.
- Brunk U, Collins VP, Arro E. The fixation, dehydration, drying and coating of cultured cells of SEM. *J Microsc* 1981;123:121-31.
- Chávez de Paz LE. Image analysis software based on color segmentation for characterization of viability and physiological activity of biofilms. *Appl Environ Microbiol* 2009;75:1734-9.
- Standar K, Kreikemeyer B, Redanz S, Münter WL, Laue M, Podbielski A. Setup of an *in vitro* test system for basic studies on biofilm behavior of mixed-species cultures with dental and periodontal pathogens. *PLoS One* 2010;5:1-14.
- Koulourides T, Phantumvanit P, Munksgaard EC, Housch T. An intraoral model used for studies of fluoride incorporation in enamel. *J Oral Pathol* 1974;3:185-96.
- Barthel CR, Zimmer S, Zilliges S, Schiller R, Göbel UB, Roulet JF. *In situ* antimicrobial effectiveness of chlorhexidine and calcium hydroxide: Gel and paste versus gutta-percha points. *J Endod* 2002;28:427-30.
- Virtej A, MacKenzie CR, Raab WH, Pfeffer K, Barthel CR. Determination of the performance of various root canal disinfection methods after *in situ* carriage. *J Endod* 2007;33:926-9.
- Del Carpio-Perochena AE, Bramante CM, Duarte MA, Cavenago BC, Villas-Boas MH, Graeff MS, *et al.* Biofilm dissolution and cleaning ability of different irrigant solutions on intraorally infected dentin. *J Endod* 2011;37:1134-8.
- Chavez de Paz LE. Redefining the persistent infection in root canals: Possible role of biofilm communities. *J Endod* 2007;33:652-62.

27. Pickup RW, Rhodes G, Bull TJ, Arnott S, Sidi-Boumedine K, Hurley M, *et al.* *Mycobacterium avium* subsp. paratuberculosis in lake catchments, in river water abstracted for domestic use, and in effluent from domestic sewage treatment works: Diverse opportunities for environmental cycling and human exposure. *Appl Environ Microbiol* 2006;72:4067-77.
28. Whitehead KA, Rogers D, Colligon J, Wright C, Verran J. Use of the atomic force microscope to determine the effect of substratum surface topography on the ease of bacterial removal. *Colloids Surf B Biointerfaces* 2006;51:44-53.
29. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. *Int Endod J* 1982;15:187-96.
30. Elias S, Banin E. Multi-species biofilms: Living with friendly neighbors. *FEMS Microbiol Rev* 2012;36:990-1004.
31. Ozok AR, Wu MK, Luppens SB, Wesselink PR. Comparison of growth and susceptibility to sodium hypochlorite of mono- and dual-species biofilms of *Fusobacterium nucleatum* and *Peptostreptococcus* (micromonas) micros. *J Endod* 2007;33:819-22.
32. Retamozo B, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. Minimum contact time and concentration of sodium hypochlorite required to eliminate *Enterococcus faecalis*. *J Endod* 2010;36:520-3.
33. Bryce G, O'Donnell D, Ready D, Ng YL, Pratten J, Gulabivala K. Contemporary root canal irrigants are able to disrupt and eradicate single- and dual-species biofilms. *J Endod* 2009;35:1243-8.
34. Naenni N, Thoma K, Zehnder M. Soft tissue dissolution capacity of currently used and potential endodontic irrigants. *J Endod* 2004;30:785-7.
35. Shen Y, Qian W, Chung C, Olsen I, Haapasalo M. Evaluation of the effect of two chlorhexidine preparations on biofilm bacteria *in vitro*: A three-dimensional quantitative analysis. *J Endod* 2009;35:981-5.
36. Marley JT, Ferguson DB, Hartwell GR. Effects of chlorhexidine gluconate as an endodontic irrigant on the apical seal: Short-term results. *J Endod* 2001;27:775-8.

How to cite this article: del Carpio-Perochena A, Bramante CM, Hungaro Duarte MA, de Andrade FB, Cavenago BC, Villas-Bôas MH, *et al.* Application of laser scanning microscopy for the analysis of oral biofilm dissolution by different endodontic irrigants. *Dent Res J* 2014;11:442-7.

Source of Support: This work was supported by FAPESP process 2009/03217-5, 2011/08184-8.. **Conflict of Interest:** None declared.