

ORIGINAL ARTICLE

The antimicrobial effect of new and conventional endodontic irrigants on intra-orally infected dentin

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Abstract

Objectives. To evaluate if the incorporation of antimicrobial compounds to chelating agents or the use of chelating agents with antimicrobial activity as 7% maleic acid and peracetic acid show similar disinfection ability in comparison to conventional irrigants as sodium hypochlorite or iodine potassium iodide against biofilms developed on dentin. **Materials and methods.** The total bio-volume of live cells, the ratio of live cells and the substratum coverage of dentin infected intra-orally and treated with the irrigant solutions: MTAD, Qmix, Smear Clear, 7% maleic acid, 2% iodine potassium iodide, 4% peracetic acid, 2.5% and 5.25% sodium hypochlorite was measured by using confocal microscopy and the live/dead technique. Five samples were used for each irrigant solution. **Results.** Several endodontic irrigants containing antimicrobials as chlorhexidine (Qmix), cetrimide (Smear Clear), maleic acid, iodine compounds or antibiotics (MTAD) lacked an effective antibiofilm activity when the dentin was infected intra-orally. The irrigant solutions 4% peracetic acid and 2.5–5.25% sodium hypochlorite decrease significantly the number of live bacteria in biofilms, providing also cleaner dentin surfaces ($p < 0.05$). **Conclusions.** Several chelating agents containing antimicrobials could not remove nor kill significantly biofilms developed on intra-orally infected dentin, with the exception of sodium hypochlorite and 4% peracetic acid. Dissolution ability is mandatory for an appropriate eradication of biofilms attached to dentin.

Key Words: biofilms, dentin, bacteria, antimicrobials, root canal irrigants

Introduction

Biofilms can be defined as bacterial cells attached to a surface embedded in an exopolysaccharide matrix [1]. Because the root canal space is sterile under normal conditions, establishment of biofilms in the root canal system will directly progress to a periapical inflammatory response.

Although eradication of intra-radicular infection should be ideally performed by instrumentation process, this procedure cannot affect considerable areas of the root canal of complex anatomies such as isthmuses, lateral canals or fins [2,3]. After the chemo-mechanical process several phenomena occurs: elimination of the major bulk of biofilms predominantly in the main root canal [4], formation of a smear layer on shaped root canal walls [5] and accumulation of hard tissue debris (AHTD) in non-instrumented areas [6].

Irrigant solutions such as sodium hypochlorite (NaOCl) are commonly used to dissolve necrotic tissue and kill biofilms situated in the root canal space. However, NaOCl does not remove the smear layer nor prevents the accumulation of hard tissue debris in isthmuses and fins [7]. Dentin debris and smear layer may act as a physical barrier limiting the correct diffusion of NaOCl into accessory anatomy or by inactivating/decreasing its antimicrobial activity [8].

It has been reported that EDTA can decrease AHTD after the instrumentation process [7]. This final rinse theoretically can expose contaminated areas not previously treated by the primary chemo-mechanical process. In fact, when NaOCl was used after EDTA the antimicrobial effect was improved in comparison to the use of NaOCl as the only irrigant [9]. In spite of the fact that this method appears reasonable from the microbiological point of view,

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the use of 5.25% NaOCl after an EDTA irrigation causes dentin erosion, which may also lead to a decrease of the physical properties of dentin [10].

Despite the high success rate of endodontic treatments, clinical data has shown that the presence of biofilms after the chemomechanical process in complex anatomies is not uncommon [11]. In this context, the use of irrigants that eliminate smear layer and present antimicrobial activity appear highly desirable because EDTA has limited antimicrobial effects [12]. Many irrigant solutions such as MTAD, Qmix, Smear Clear or Tetraclean contain EDTA or citric acid in combination with antiseptics or antibiotics such as chlorhexidine, cetrимide or doxycycline. The objective of these combinations is to provide antimicrobial activity and elimination of the smear layer in one-step.

In vitro experiments using planktonic bacteria of aerobic and anaerobic species have shown effective antimicrobial activity for several compounds such as: cetrимide [13,14], chlorhexidine [13,15], sodium hypochlorite [15], iodine potassium iodide [16] or MTAD [17]. However, previous research has suggested that this method could not reflect the real effectiveness of the solutions [14], because biofilm mode is the most common colonization form of bacteria in the root canal space [11,18]. For this reason, a polymicrobial biofilm formed from subgingival plaque is commonly used to provide a more realistic representation of the antimicrobial performance of different irrigants [14,19,20].

To date, biofilm experiments have studied the efficiency of irrigants containing either antibiotics or antiseptics [14]. However, little research has been performed comparing the efficiency of different categories of agents such as antibiotics, antiseptics, oxidative compounds or newly-proposed irrigant solutions such as peracetic acid [21], Qmix (a mixture of chlorhexidine, cetrимide and EDTA) [22] or 7% maleic acid [23]. Stojicic et al. [22] claim that Qmix is as effective as 2% NaOCl in killing plaque biofilm bacteria. However, the authors only compared the percentage of dead cells after chemical treatment without taking into consideration the amount of biovolume of live cells or the substratum coverage parameters and, thus, this result remains questionable.

Furthermore, it is unknown if these new proposed irrigants containing detergents such as cetrимide (Qmix, Smear Clear) or tween-80 (MTAD) have the ability to improve the cleaning of infected dentin or to remove the biofilm. As a result, direct comparisons addressing three-dimensional quantification of the affected biomass attached to dentine after exposure to new irrigant solutions, in comparison to common irrigants or medicaments such as sodium hypochlorite or iodine compounds have yet to be completed.

Thus, we evaluated the effect of MTAD, Qmix, Smear Clear, 7% maleic acid, 2% iodine potassium iodide, 4% peracetic acid and 2.5% and 5.25% sodium hypochlorite on the architecture and viability of mixed biofilms attached to dentin by measuring the

total bio-volume of live cells, the ratio of dead cells and the substratum coverage. The null hypothesis was that mixed biofilms treated with the evaluated irrigant solutions do not result in different amounts of bio-volume, substratum coverage or ratio of live cells.

Materials and methods

The irrigant solutions evaluated were: 2.5% sodium hypochlorite (CloroRio, São José do Rio Preto, SP, Brazil), 5.25% sodium hypochlorite (Clorox industria química, Peru), MTAD (Dentsply Tulsa Dental, OK, USA), Qmix (Dentsply, Tulsa Dental, OK, USA), Smear Clear (SybronEndo, Orange, CA), 7% Maleic Acid (Sigma, MI), 2% iodine Potassium iodide (Farmacia Especifica, Bauru, SP, Brazil) and 4% Peracetic acid (Peresal, Ecolab, Düsseldorf, Germany). Peracetic acid solution contains 26% hydrogen peroxide in its composition. Distilled water was used for control purposes.

Forty-five sterile bovine dentin sections ($2 \times 2 \times 2$ mm) were used. The samples were treated with 1% NaOCl and 17% EDTA for 3 min to eliminate the smear layer produced during the sectioning process. To induce the dentin infection an *in situ* model was selected [24]. Nine dentin samples were inserted into the cavities of a Hawleys orthodontic device with sticky wax. The dentin surface in contact with the oral cavity was fixed 1 mm above the surface to favour the accumulation of plaque. The device was used by one volunteer for 48 h in order to induce the dentin infection. Regular oral hygiene practices were maintained (Human committee and ethic research (CEP134/210)). After the intra-oral infection process, each sample was incubated in 2 mL of BHI at 37°C for 24 h in aerobic conditions. Then, 1 mL of distilled water was used to eliminate the culture medium and non-adherent cells. For the contact direct test, the dentin samples were immersed for 5 min in 1 mL of the irrigant solution by using 24-well tissue culture plates. Five independent experiments were performed for each irrigant solution.

After the contact test, NaOCl and peracetic acid (PAA) samples were treated with 1 mL of 5% sodium thiosulfate for 5 min. A pilot study revealed that sodium thiosulfate did not have dissolution abilities and improved the quality of the staining process when the dentine was treated with oxidizing agents. The other irrigant solutions were washed with distilled water.

For biofilm analysis the Syto-9/Propidium iodide technique was used on dentin specimens as previously described [12,22] (Live/Dead, BacLight, Invitrogen, Eugene, OR); SYTO-9 is a green-fluorescent stain, labelling both live and dead micro-organisms. PI is a red-fluorescent nucleic acid stain and penetrates only the cells with damaged membranes (dead microbes).

All the samples were observed using a confocal laser-scanning microscope (Leica TCS-SPE, Mannheim,

Germany). Five confocal 'stacks' from random areas that presented between 20–30 μm of thickness were obtained from each sample using the $40 \times$ oil lens, 1 μm step-size and a format of 512×512 pixels. Each picture represented an area of $275 \times 275 \mu\text{m}$. At least 5 μm of the scanning included the sub-surface level of the dentin. For quantification purposes the *bioimage_L* software was used [25]. The parameters evaluated in each stack were the total Biovolume and green population (live cells) and substratum coverage expressed in percentages. Biovolume was determined according to Heydorn et al. [26] as volume per unit area ($\mu\text{m}^3/\mu\text{m}^2$). The substratum coverage parameter shows horizontal expansion of the biofilm. Ratio of live cells was also calculated [20].

Statistical analysis of the substratum coverage, total, green biovolume and ratio of live cells were performed using the non-parametric Kruskal-Wallis and Dunn tests ($p < 0.05$) because of the absence of normal distribution confirmed in the preliminary analysis. The relation between the biovolume and ratio of live cells was determined by using the Spearman correlation test (r). Prisma 5.0 (GraphPad Software Inc, La Jolla, CA) was used as the analytical tool.

Results

A total of 225 confocal operative fields (3D stacks) were evaluated, 25 for each irrigant solution. Overall biovolume of all the irrigant solutions tested showed values between 0.7–9, except for NaOCl and PAA solutions. The Spearman test showed no correlation between biofilm biovolume and ratio of live cells in distilled water, Qmix, MTAD, Smear clear, 2% IKI and 7% Maleic acid, showing that the increase or decrease of biovolume does not influenced the ratio of live cells in these groups. The results of 5.25% NaOCl and 4% PAA showed an inverse correlation between the residual biovolume and ratio of live cells ($r = -0.51$, $r = -0.46$), respectively ($p < 0.05$). In these solutions higher ratio values of live cells were found when lower values of biovolume were present. See Figure 1.

The median, 25–75% percentile and range of total and green biovolume (live cells) are shown in Table I. No differences of total and green biovolume in comparison to control were found in Qmix, Smear clear, MTAD, 7% maleic acid and 2% IKI irrigant solutions ($p > 0.05$). Lower biovolume values were found in NaOCl solutions and PAA ($p < 0.05$), 5.25% and 2.5% NaOCl irrigant solutions showed 99.4% and 84.33% lower biovolume values in comparison to control. Peracetic acid total biovolume was 90% less than the control group.

The parameter ratio of live cells is shown in Table I. This *in situ/in vitro* model provided 89% of viability when exposed to the control (water). Overall, the lower values were found in 5.25% NaOCl and 4% PAA ($p < 0.05$). No statistical significances were

found between the Qmix and NaOCl solutions ($p > 0.05$).

A box-plot of substratum coverage (%) is shown in Figure 2. Significant lower values of this parameter were found in 4% PAA, 2.5% and 5.25% NaOCl treated dentin in comparison to the others solutions ($p < 0.05$). Confocal pictures showed an intense colonization of dentin substrate without visible evidence of structural alterations or dissolution effects when distilled water, MTAD, Qmix, Smear Clear, 2% IKI or 7% maleic acid were used. Evidence of biofilm disorganization was evident in NaOCl and PAA treated dentin. In these groups residual biofilms layers were evident at the entrance of dentinal tubules and usually associated with the presence of live cells. Representative pictures of the treated biofilms are shown in Figure 3.

Discussion

This study showed the limitations of several irrigant solutions to eradicate biofilms in a direct contact test in which other confounding factors were eliminated. Overall, with the exception of PAA and NaOCl solutions, an area of $2 \times 2 \text{ mm}$ (4 mm^2) of infected dentin could not be significantly decontaminated by a reasonable amount of irrigant solution (1 mL or 1000 mm^3). Whereas a variety of substratum such as nitrocellulose [27], hidroxyapatite [19] or polystyrene have been used to test the antimicrobial activity of endodontic medications. The advantage of dentin is that it better simulates the micro-irregularities of the surfaces found in root canals.

The use of biofilm models to test endodontic irrigants formed from dental plaque [14,19] or developed intra-orally has been used to test endodontic antimicrobials or lasers [28–30]. With the exception of sodium hypochlorite, chelating agents containing antimicrobials are used after mechanical treatments and aims to kill residual microflora that is basically composed from facultative bacteria. For this reason, anaerobic conditions were avoided because strict anaerobic bacteria do not represent the microflora found in chemo-mechanically treated teeth [31]. This biofilm model appears to be notable, considering that direct contamination of radicular dentin to the oral environment is feasible under clinical conditions [32].

In vitro biofilm models used in endodontics does not necessarily resemble the limited nutrient conditions found in necrotic pulp in root canals. The use of additional incubation of the intra-orally developed biofilms in BHI was necessary in an attempt to standardize a baseline value of live cells in all the evaluated groups because the proportion of dead bacteria in oral biofilms can be considered variable [24,33].

Biovolume variations were found in this experiment. However, with the exception of oxidant agents, this variability appeared to be irrelevant since higher

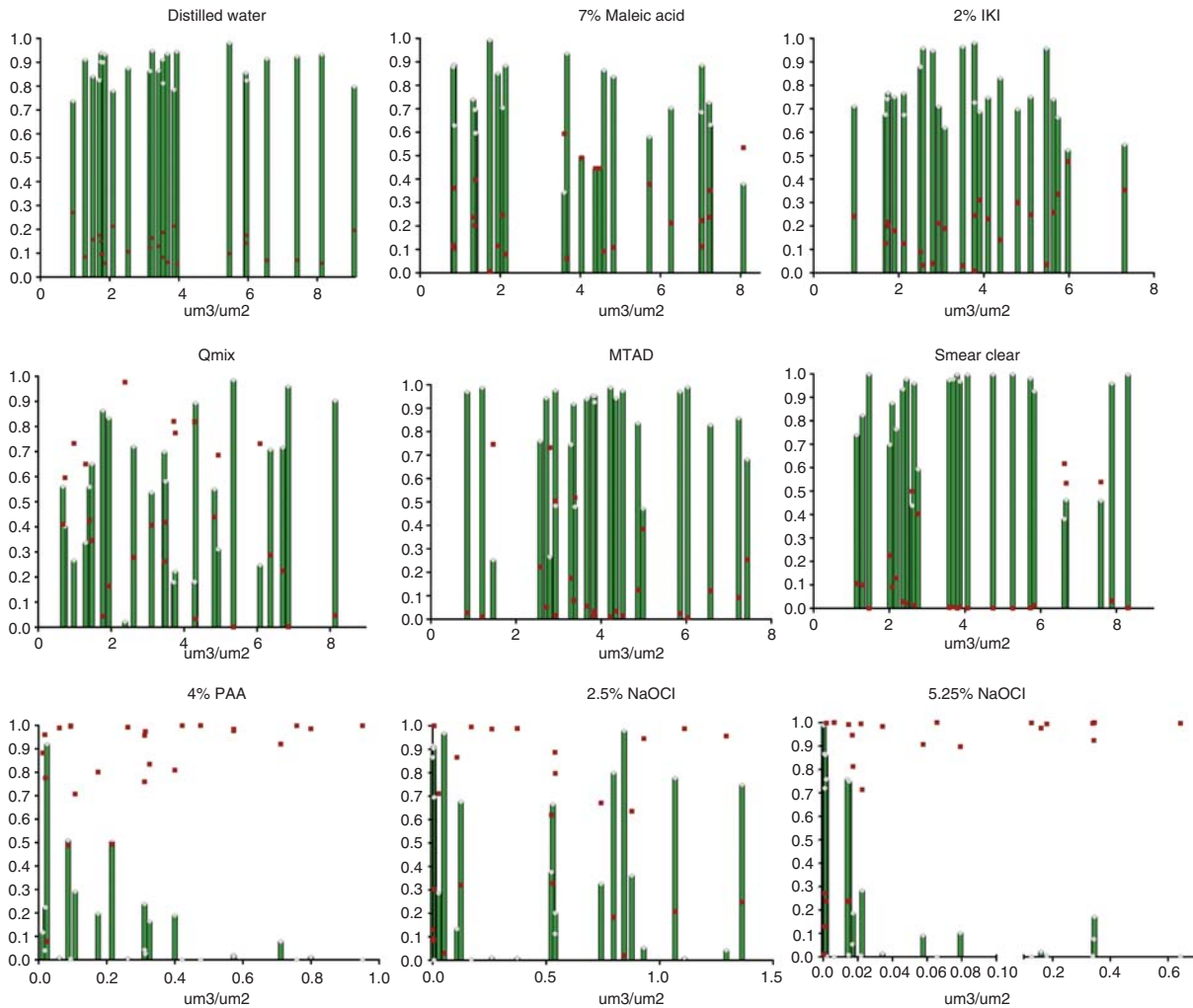


Figure 1. The relationship between the ratio of live cells [green/(Red+green)] (green bars) and the biovolume $\mu\text{m}^3/\mu\text{m}^2$ in the evaluated operative fields is presented. Red points represent the ratio of dead cells. Lower biovolume values were found in 2.5–5.25% NaOCl and 4% peracetic acid ($p < 0.05$). The results of 5.25% NaOCl and 4% PAA show an inverse correlation between the residual biovolume and ratio of live cells.

or lower biovolume levels had a poor correlation with the ratio of live cells (see Figure 1). The variability in this biofilm model can be explained by the common characteristics of biofilm architecture, which includes the presences of channels, cavities or voids.

Previous research and reviews in the biofilm area have shown that a great percentage of cells in the biofilm are not resistant to antimicrobial agents in comparison to the planktonic mode [34,35]. When biofilm cells are dispersed, common antibiotic agents rapidly kill them [36]. Despite the reported presence of persisters cells in biofilms [37], the resistance of biofilms can also be attributed also to other factors: (1) Reaction of the antimicrobial components with organic biofilm components in a stoichiometric fashion. Thus, the antimicrobial effect is consumed [38]; and (2) Cells in the deep layers of the biofilm are more resistant due to the antimicrobial limitation to diffuse through the organic structure [38].

Neutralizing activity of organic compounds such as dead cells or biofilm extracellular matrix (EPS) on

antimicrobial agents is well-documented [13,39–41]. In spite of the fact that 90% of EPS is water, the presence of organic compounds such as cells, polymers, proteins or DNA can act as a diffusive barrier to the antimicrobial agent which leads to a decline of the antimicrobial activity [38]. Thus, it is expected that cells inside the biofilm are exposed to dissimilar concentrations of the antimicrobial agent. This depends also on their spatial localization [37,42]. This well accepted finding in biofilm research [42] can explain the irregular patterns of antimicrobial activity found in Qmix (a mixture of chlorhexidine, edta and cetrimide) and maleic acid, which affects some operative fields. In other areas the effect was found negligible (see Figure 1). Another explanation could be that the contact time was insufficient to allow a better distribution of the antimicrobials into the biofilm. However, the 5 min exposure time appears reasonable considering that some solutions are used for a time that varies from 60–90 s, as recommended by the Qmix manufacturer.

Table I. Median, 25–75% percentile and range of total and green biovolume defined as $\mu\text{m}^3/\mu\text{m}^2$.

	Biovolume $\mu\text{m}^3/\mu\text{m}^2$		Ratio Green
	Total	Green	Ratio[green/(green+red)]
Distilled water	3.32 (1.79–5.58) ^a [0.94–9.07]	2.91 (1.58–4.94) ^a [0.68–7.59]	0.89 (0.82–0.92) ^a [0.72–0.98]
MTAD	3.80 (2.86–4.92) ^a [0.85–7.43]	3.44 (1.78–4.28) ^a [0.37–6.20]	0.92 (0.71–0.97) ^{ab} [0.25–0.99]
Qmix	3.49 (1.62–5.14) ^a [0.67–8.11]	1.53 (0.72–3.26) ^a [0.05–8.04]	0.56 (0.28–0.77) ^{bc} [0.02–0.99]
Smear Clear	3.69 (2.27–5.77) ^a [1.14–8.30]	3.09 (1.65–4.39) ^a [0.84–8.29]	0.90 (0.72–0.97) ^a [0.38–0.99]
7% Maleic acid	3.60 (1.37–5.27) ^a [0.85–8.07]	1.89 (0.98–4.01) ^a [0.53–5.24]	0.70 (0.58–0.84) ^{ab} [0.34–0.99]
2% IKI	3.50 (2.12–4.94) ^a [0.95–7.30]	2.68 (1.52–3.68) ^a [0.67–5.24]	0.74 (0.67–0.85) ^{ab} [0.29–0.98]
4% PAA	0.31 (0.09–0.52) ^b [0.01–0.95]	0.01 (0–0.03) ^b [0–0.10]	0.04 (0–0.21) ^d [0–0.92]
2.5% NaOCl	0.52 (0.10–0.87) ^b [0–1.36]	0.05 (0–0.31) ^b [0–1.02]	0.36 (0.05–0.77) ^{cd} [0–0.97]
5.25% NaOCl	0.02 (0.01–0.17) ^b [0–0.64]	0 (0–0.01) ^b [0–0.24]	0.07 (0–0.68) ^{cd} [0–0.86]

Different letters in each column represent statistical significance ($p < 0.05$).

Inactivation of a mixture of 0.1% cetrimide and 0.1% chlorhexidine, IKI and MTAD by organic compounds (bovine serum) or even by the dentin substrate [43] is in accordance with the results of this study. Limited antimicrobials activity has been reported for chlorhexidine [44–46], MTAD [22,44,47,48] or 2% IKI, especially when short time periods were used [49].

It is also accepted that biofilm cells present in the depth layers are in an altered metabolic state and are more difficult to eradicate by antibiotics (persisters) [37,50]. Mathematical biofilm models have shown

two stages of chemical disruption of biofilms [51]. The first stage promotes rapid dissolution of the major part of the biofilm, followed by a longer period to remove residual thin layers that are in the proximity to the substrate. It is also reported that the detachment speed decreases as the biofilm thickness decreases [51]. As a result, it was not surprising that a higher ratio of live cells were found in 5.25% NaOCl and PAA treated biofilms in conjunction with reduced biomass close to the proximity of the dentin interface (See Figures 1 and 2).

Sodium hypochlorite is a very reactive oxidant that has a well-documented dissolution effect against biofilms [41,52,53]. It is possible that the dissolution reaction neutralized or consumed the amount of free available chlorine faster than it diffuses to the deep biofilm layers [54]. As a result, a higher amount of live cells was observed in 2.5% NaOCl in comparison to 5.25% concentration. The presence of viable cells could also be facilitated by the natural irregular characteristic of dentin, which includes dentinal tubules. A recent study showed that 1–2% NaOCl destroyed *in vitro* *E. faecalis* biofilms in 3 min [22]. Similar to clinical reports [55], our results showed that full elimination of survival cells cannot be achieved in all the cases using 5.25% NaOCl. This data suggests that the *in vitro/in situ* conditions of biofilm development may also affect the resistance of microorganisms inside the biofilm.

It is important to state that, although a similar ratio of live cells was found in NaOCl solutions and Qmix, the amount of biovolume of live cells was significantly

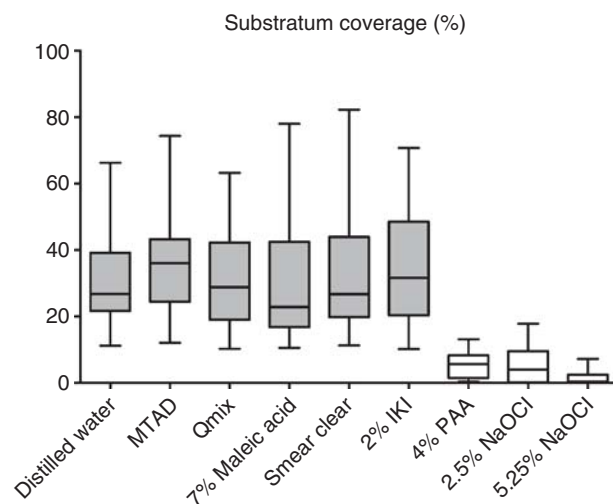


Figure 2. Box-plots of substratum coverage values in terms of percentage after 5 min of contact with the irrigant solutions.

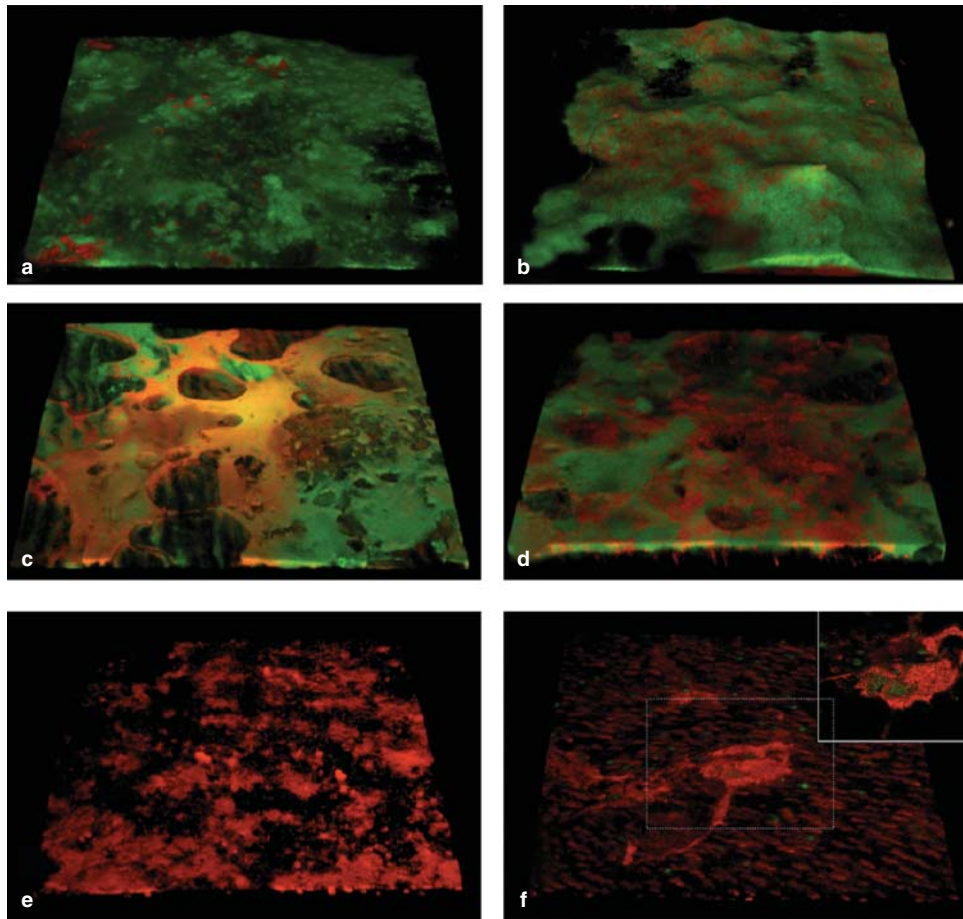


Figure 3. Representative three-dimensional constructions after the treatment with: distilled water (A), 7% Maleic acid (B), Qmix (C), 2% IKI (D) and 4% Peracetic acid (E). A decrease of the amount of microbial cells and a middle dissolution effect is visible in 4% peracetic acid (PAA) treated dentin. A detailed examination of 2.5% NaOCl treated dentin shows remaining live cells (green fluorescence) inside residual biofilms (F). A strong disorganization of the biofilm structure is evident. Three-dimensional constructions represent an area of $275 \times 275 \mu\text{m}^2$.

lower in NaOCl solutions. This demonstrates that the isolated parameter ratio of live cells does not take into account the dissolution effect. Thus, this parameter can over-estimate the antimicrobial activity because it does not take into account the biovolume [27]. These results show that several parameters are necessary for an adequate interpretation of the data. In this context, volumetric values were supported by the significantly lower values of the substratum coverage found in NaOCl and PAA treated dentin. The values of the other solutions showed a similar degree of attached cells when compared to control; thus, rejecting the null hypothesis.

It has been reported that main components of Smear Clear, MTAD or Qmix include EDTA and citric acid. These chelating agents do not improve the elimination or dissolution of biofilms in a short time period [12]. Despite reports showing antimicrobial activity in 7% maleic acid [23] or Iodine potassium iodide [43], this solution also fails to display lower values of substratum coverage in comparison to the control. Research in the biofilm area has shown that the more effective strategy to eliminate biofilms is to dissolve its structure [41]. Not surprisingly, the best

results were obtained when irrigants with dissolution abilities were used.

Previous studies have shown that NaOCl irrigation does not prevent the growth of residual bacteria in root canals [9,55]. Thus, the use of intra-canal medications and the filling of the canal system are necessary. In this perspective, elimination of the smear layer becomes important. Because NaOCl does not remove the smear layer, possible alternatives to reduce the bacterial load are: (1) the use of a soft-chelating solution as an etidronate-NaOCl mixture that maintains antimicrobial activity and avoids the formation of smear layer [5,56] or (2) the use of 1–2.5% NaOCl after smear layer elimination with a chelating solutions to improve dissolution of remaining biofilms without decreasing the mechanical properties of the dentin [57,58].

An interesting result was found using 4% PAA. The PAA solution used in this study contains 24% hydrogen peroxide in its composition and is indicated for disinfection and decalcification of haemodialysis equipments. A previous report showed that 5% hydrogen peroxide has the ability to dissolve 89% of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

biofilm matrix within 1 min in comparison to 66% dissolution ability of 1% NaOCl [41]. The fact that PAA kills and dissolves significantly mixed biofilms merits further research because it is also capable of eliminating the smear layer [21,59]. However, an adequate balance among toxicity, antimicrobial activity and smear layer elimination using this compound needs to be clarified in future studies.

Conclusion

Several chelating agents containing antimicrobials could not remove nor kill significantly biofilms developed on intra-orally infected dentin with the exception of sodium hypochlorite and 4% peracetic acid. Dissolution ability is mandatory for an appropriate eradication of biofilms attached to dentin.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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