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EFFECT OF RECIPROCATING INSTRUMENTATION ON CHLORHEXIDINE SUBSTANTIVITY ON HUMAN DENTIN: CHEMICAL ANALYSIS FOLLOWED BY CONFOCAL LASER MICROSCOPY

Matheus Albino SOUZA[1], Fernanda Duda BONATTO[2], Afonso Cristiano Fleck da SILVA[2], Ezequiel Santin GABRIELLI[1], Felipe Trentin MOTTER[2], Larissa PIUCO[2], Karen Barea de Paula DUARTE[3], Charise Dallazem BERTOL[4], Luciana Grazziotin ROSSATO-GRANDO[5], Carlo Theodoro Raimundy LAGO[6], Doglas CECCHIN[1], Juliane BERVIAN[7], João Paulo De CARLI[8], Francisco MONTAGNER[9]

[1] Department of Endontics, School of Dentistry, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[2] Private Practice, Passo Fundo, RS, Brazil.
[3] Postgraduate Program in Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.
[4] Department of Pharmacy, Pharmacy Course, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[5] Department of Toxicology, Pharmacy Course, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[6] Department of Restorative Dentistry, School of Dentistry, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[7] Department of Pediatric Dentistry, School of Dentistry, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[8] Department of Oral Medicine, School of Dentistry, University of Passo Fundo, Passo Fundo, Rio Grande do Sul, Brazil.
[9] Department of Endodontics, School of Dentistry, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil.

Corresponding author:
João Paulo De Carli
Email: joaodecarli@upf.br

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Abstract

The present research analyzed the reciprocating instrumentation associated to chlorhexidine (CHX) substantivity as its correlation with *E. faecalis* viability in *ex vivo* root canals. Eighty extracted single-rooted human teeth were used, being 40 to high-performance liquid chromatography (HPLC) and 40 to confocal laser scanning microscopy (CLSM). In both, teeth were decoronated and the cervical third was prepared. In the CLSM analysis, the root canals were inoculated with *E. faecalis* for 14 days. Samples were divided into 4 groups (n=10) according to instrumentation technique: no instrumentation and irrigation with distilled water (control); manual instrumentation (K-File); rotary instrumentation (ProTaper Next); and reciprocating instrumentation (Reciproc R25). Two percent chlorhexidine was applied as irrigating substance in experimental groups. Longitudinal grooves resulted in 2 halves root and 20 proof bodies in each group. Samples were divided by chance in two groups (n=10) and the outcomes were evaluated after two days and one week. The retained chlorhexidine and live cells after instrumentation techniques in each evaluation time was measured by HPLC and CLSM, respectively. Specific analysis was applied for experimental tests ($p \leq 0.05$). Both rotary as well as reciprocating techniques significantly reduced the amount of chlorhexidine on dentin in all observation periods ($p<0.05$). After evaluation times, all experimental groups presented lower live cells compared to control, but without statistically difference. Intragroup comparisons in times of evaluation showed no differences in instrumentation techniques, in chlorhexidine retention and number of live cells ($p>0.05$). Reciprocating instrumentation does not interfere on chlorhexidine substantivity.

Keywords: Chromatography. *Enterococcus faecalis*. *Ex vivo*. Human Tooth. Root Canal Preparation.

1. Introduction

Microorganisms are the primary etiologic agents of endodontic infection, promoting the progression of pulp and periapical diseases (Rôças and Siqueira 2010). Among these microorganisms, *Enterococcus faecalis* (*E. faecalis*) is a gram-positive and facultative bacteria, which is strongly resistant and usually associated with endodontic failure (Williams et al. 2006). So, auxiliary chemical substances as well as endodontic apparatus are very important in performing effective chemo-mechanical preparation, with the aim of providing endodontic decontamination (Dal Bello et al. 2019).

Different auxiliary chemical substances can be used for root canal disinfection. Chlorhexidine (CHX) has broad-spectrum antimicrobial action (Rôças et al. 2016) and ability to be adsorbed in the dentin walls, providing antimicrobial effects over time (substantivity) (Böttcher et al. 2015). On the other hand, the use of reciprocating files is recommended to perform root canal shaping, because this technique applies less stress to the instrument and increase resistance to flexural fatigue (Gavini et al. 2012), removes bacteria (Neves et al. 2016) and induces lower apical extrusion of bacteria and debris during instrumentation (Alves et al. 2018).

However, CHX substantivity is time dependent (Souza et al. 2012) and reciprocating method is faster, providing less time of contact of the auxiliary chemical substance with the root dentine (Guelzow et al. 2005), reducing the antimicrobial properties of these chemical agents during endodontic therapy (Souza et al. 2018a). In addition, the use of reciprocating instrumentation results in lower CHX retention in the root dentin (Souza et al. 2018b). Finally, there are no studies correlating CHX retention in the root dentin with bacterial viability in the literary search carried out by the authors, in root canals prepared using the reciprocating technique.

Thus, the objective of this research was to analize the reciprocating instrumentation on chlorhexidine substantivity and its correlation with *E. faecalis* viability in *ex vivo* human root canals. The null hypotheses were (i) reciprocating instrumentation does not interfere on CHX substantivity and (ii) there is no correlation between the amount of retained CHX and bacterial viability in infected root canals.

2. Material and Methods

The experiment was analized by the Research Ethical Committee of UPF (process 669.390). Eighty freshly extracted single-rooted human teeth of upper central and lateral incisors group were elected. The dental organs belonged to patients aged 20-30 years. The methodology of this work was based on previous studies (Souza et al. 2012; Ran et al. 2015). Each sample was prepared by sectioning its coronary portion, establishing the root remnant at a length of 15mm. The pulp tissue remaining in the root canals was removed with distilled water (DW) and mechanical instrumentation with a #08 K file (Dentsply Maillefer, Ballaigues, Switzerland). The cervical third was prepared using Largo drill #3 (Dentsply Maillefer) and DW. The working length (WL) was established by introducing #10 K-file up to the apical foramen and subtracting 1.0 mm from the length. Root canals with an apical diameter larger than the K # 15 file were not included. The methodology was divided into two sections: CHX quantification with High-performance Liquid Chromatography (HPLC) and bacterial viability analysis by Confocal laser scanning microscopy (CLSM).

CHX quantification with HPLC: chemo-mechanical preparation

The 40 samples were randomly divided into four groups (n=10), according to the instrumentation technique:

(1) Control group - Irrigation with 5 mL of DW was performed, using 5-mL syringe with 19-G needle centered within the canal, 3 mm short of the WL. Then, root canals were dried with sterile paper points.

(2) Manual instrumentation - The step-back technique was used for widening the root canals. Manual K files was worn, starting with file # 15 and ending with file # 30 in WL. The step-back instrumentation was performed using four K files larger than the last file used for apical preparation. It started with file k # 35 ending with file # 50, gradually decreasing 1mm of WL with each increase in diameter of the file.

(3) Rotary instrumentation. ProTaper Next instruments (Dentsply Tulsa Dental Specialties, Johnson City, TN, USA) were used in a handpiece powered by electric engine (VDW Silver Reciproc Motor, VDW), at

speed of 300 rpm in a crown-down manner, using a gentle in-and-out motion, according to manufacturer's instructions. X1 shaping file moved apically to 2 mm before reaching WL, followed by reaching full WL. Files X2 and X3 were used to finish the preparation of the apical third in the WL. Instrumentation was performed in pecking movements. The instruments were regularly cleaned to remove debris. When the instrument reached the WL, and showed free rotation in the root canal, it was removed. At this point, the next instrument of the sequence was used.

(4) Reciprocating instrumentation. Reciproc R25 (VDW GMBH, Munich, Germany) was inserted into the root canal, in pecking movements the instrumentation was recommended in thirds, starting with the cervical third. After 3 movements in and out of the root canal, the file was removed for cleaning. Irrigation was performed interspersed with instrumentation, until the file reached the WL. Finally, the file was inserted up to 1 mm short of the WL with brushing motion against the lateral walls and irrigation was performed. The file was activated in an electric engine (VDW GMBH), using the predefined set recommended by the manufacturer.

CHX quantification with HPLC: irrigation regimen

CHX was the auxiliary chemical substance used for root canal preparation. The applied formulation was Natrosol-based gel at 2% concentration (Natupharma, Passo Fundo, RS, Brazil). DW was the irrigation solution.

Irrigation was performed with DW in a volume of 5mL in all samples from the experimental groups. The root canal was previously filled with CHX until extravasation before using each manual instrument (8 times) and rotary instrumentation (3 times) and also before progression in each third of the reciprocal instrumentation (3 times). After the use of each instrument or progression in each third in the reciprocating instrumentation, irrigation with 5 mL of DW was performed.

Final irrigation with 3 mL of 17% EDTA (Natupharma, Passo Fundo, RS, Brazil) for 1 minute followed by irrigation with 5 mL of DW was performed in all groups in order to remove the smear layer. Then, root canals were dried with sterile paper points.

CHX quantification with HPLC: CHX quantification

Longitudinal grooves were performed on root free surfaces with diamond disk, avoiding the inner part of the root canal. Fracture was made with chisel and hammer, providing 2 halves of each root and 20 samples per group. Samples from each group were randomly divided into 2 subgroups (n=10) and substantivity was evaluated after 48 hours and 7 days. Samples were stored at 37 °C and 100% relative humidity during evaluation times.

The collected samples were stored in a 1 and 5 mL microtube, and an extracting solution (acetonitrile: 1% formic acid, 20:80) was added. The microtubes were heated in a water bath at 80º C for 20 minutes and sonicated for 10 minutes. Contents were transferred to 1.5-mL microtubes and centrifuged at 6.000 rpm for 15 minutes. Supernadants were diluted 10 times, and 20 µL were injected into the HPLC system (Flexar, Perkin Elmer, Burnsville, MN, USA). CHX was assayed by using isocratic separation with methanol:water (63:37, v/v). Triethylamine (0.4%) was added to the mobile phase, and the pH was adjusted to 3.7 with chloridric acid. Flow rate of 1.0-mL/min was maintained with diode array detector set at 260 nm, ayielding a total run time of 8 minutes. Twenty microliters of each sample were injected into HPLC system equipped with isocratic pump, diode array detector, degasser, and manual injection system (all HPLC components and Chromera WorkStation software were from Perkin Elmer, Burnsville, MN, USA). Chromatographic separations were performed using reverse-phase column (250 x 4mm, 5-mm LiChrospher 100 RP-18). The column was protected by a guard column (4 x 4mm, 5-mm LiChrospher 100 RP-18; Merck Millipore, Frankfurt, Germany) and maintained at temperature of 22 ± 2 °C.

Bacterial viability analysis by CLSM: Specimen preparation

Two longitudinal grooves were made on the outer root surface of the samples. The grooves extended close to the root canal. In addition, two circumferential grooves around the entire root were made at a distance of 5mm and 10mm from the root canal entrance. These preparations were performed with a diamond disk, avoiding touching the inner portion of the root canal.

Bacterial viability analysis by CLSM: Culture and inoculum preparation

The samples were standardized as to the inner diameter of the canal. WL was established as described above. Samples were increased in the WL up to a #15 file with renewed irrigation of DW at each instrument change. Finally, the canals were filled with 17% EDTA, which remained inside for 1 minute, followed by final irrigation with 5 mL of DW and drying samples with absorbent paper tips.

Roots were sterilized at 120 ^{o}C in autoclave (Kavo, Joinville, SC, Brazil) for 30 minutes. Four samples were randomly selected for sterilization control. Sterile paper point was placed in contact with canal walls for 15 seconds and transported to microtube containing 1 mL of 0.9% saline solution (Basso, Caxias do Sul, RS, Brazil). The material was homogenized and 100 µL aliquot was cultivated on blood agar. Samples were incubated at 37º C for 48 hours and showed no bacterial growth.

The reference strain was *E. faecalis* (ATCC 19433), which was cultivated in brain-heart infusion (BHI) broth (Acumedia–Neogen, Lansing, MI, USA) for 24 h at 37 ^{o}C in bacteriological incubator (Kavo, Joinville, SC, Brazil). The turbidity degree was adjusted to McFarland's 1.0 scale, corresponding to $3.0x10^{8}$ CFU/mL and optical density from 0.25 to 550 nm. A 100-µL culture aliquot was inoculated into the root canal of each sample at WL. The bacterial contamination process was maintained for 14 days. Every 48 hours, intercalating with the inoculum, an aliquot of sterile BHI was inserted into the samples.

The control of contamination by E. faecalis was performed with an aliquot of a specimen randomly collected from each group once a week. Gram stain test, blood agar culture, catalase, and esculin tests were carried out, verifying the absence of other microorganisms.

Bacterial viability analysis by CLSM: chemo-mechanical preparation

The 40 samples were irrigated with 5 mL of DW and randomly distributed into four groups (n=10): control group (DW), manual, rotary and reciprocating instrumentation. Procedures previously described for control and experimental groups, as well as for the irrigation regimen, were adopted in this evaluation.

Bacterial viability analysis by CLSM: bacterial viability analysis

The complete fracture of longitudinal grooves was made with a chisel and hammer, providing 2 samples of 5 mm from the middle third of each root and 20 samples per group. Samples from each group were randomly divided into 2 subgroups (n=10) and bacterial viability was evaluated after 48 hours and 7 days. Samples were stored at 37 °C under 100% relative humidity during the evaluation periods.

After each evaluation period, sample were placed over a glass coverslip (0.17-mm thick) (Precision Glass Line; CRAL Ltda, Cotia, Brazil). The Live/Dead BacLight Bacterial Viability kit L-13152 (Molecular Probes, Invitrogen, Eugene, OR) was used to determine bacterial viability. SYTO9 probe (excitation at 488 nm and emission at 525 nm) labels all bacteria in a population, whereas propidium iodide probe (excitation at 488 nm and emission at 560 nm) penetrates only bacteria with damaged plasmatic membrane. Thus, bacteria incubated in the presence of the 2 fluorescent markers simultaneously in green color if alive and in red color if dead. The dye was applied over the dentin sample for 15 minutes at 1:1 ratio (total volume of 20 mL). Subsequently, topographic analysis of sample was performed. Fluorescence from the stained cell was viewed using a CLSM (Olympus Europa Holding GmbH, Hamburg, Germany). Simultaneous dual-channel imaging was used to display green and red fluorescence. The confocal ‘‘stack’’ with 1-mm step size and format of 512x512 pixels from a random area was obtained from each sample, using 60x oil lens. Images were acquired through Olympus FluorView Version 1.7 software (Olympus Europa Holding GmbH). At least 10 mm of the scanning included the subsurface level of the dentin. For the viability evaluation, quantitative analysis was performed with the bio Image L software (The MathWorks Inc, Natick, MA). Briefly, the software produces

information on the total biofilm population as well as on independent subpopulations represented by green and red fluorescent colors. The software evaluated the subpopulation of green and red cells as living and dead cells in each sample of each group, assigning a value for this sample. To remove the green background, the noise reducing factor was adjusted to 0.9.

Statistical analysis

In the HPLC evaluation, means and standard deviations of CHX substantivity for all groups were calculated in milligrams per milliliter (mg/mL), representing the volume of extracted solution containing CHX retained on the dentinal surface. Normal data distribution was confirmed by the Kolmogorov-Smirnov test ($p>0.05$). Results were statistically analyzed by two-way ANOVA and Scheffe post-hoc tests ($\alpha=0.05$).

In the CLSM evaluation, normal distribution was confirmed by the Lilliefors test. The mean and standard deviations of the amount of live cells were analyzed by two-way ANOVA followed by Tukey test ($p<0.05$).

Correlation between amount of retained CHX and live cells was performed by Spearman rank correlation co-efficient.

All data were analyzed using SPSS 11.0 software (IBM SPSS Statistics 20; SPSS, Chicago, IL).

3. Results

Data about substantivity (in mg/mL) are presented in table 1. Manual instrumentation presented higher CHX retention, whereas rotary and reciprocating instrumentation induced significantly lower CHX retention, in all observation periods ($p<0.05$). There was no statistical significant differences in all instrumentation techniques, presenting similar amount of CHX retained in 48 hours and 7 days ($p>0.05$).

Table 1. Means (standard deviation) of substantivity of all groups (in mg/mL) on human dentin according to instrumentation techniques and evaluation times.

Instrumentation technique	48 hours	7 days
Control	0.000 (0.000)^{A,a}	0.000 (0.000)^{A,a}
Manual	0.137 (0.015)^{C,a}	0.104 (0.010)^{C,a}
Rotary	0.083 (0.018)^{B,a}	0.067 (0.019)^{B,a}
Reciprocating	0.082 (0.019)^{B,a}	0.060 (0.016)^{B,a}

Different capital letters in each column indicate statistically significant difference ($p<0.05$). Different lowercase letters in the row indicate statistically significant difference ($p<0.05$).

Mean and standard deviation of the amount of live cells (green) of all groups are presented in table 2. After 48 hours and 7 days, all experimental groups presented lower number of live cells compared to the control group, with no statistically significant difference between them ($p>0.05$). There was no significant difference when periods were compared within the same group. Representative images of each group on different evaluation times are shown in figure 1.

The Spearman rank correlation co-efficient revealed no correlation between CHX retaining (HPLC evaluation) and live cells (CLSM evaluation) ($p>0.05$).

Table 2. Mean and standard deviation of live cells according to instrumentation techniques and evaluation times.

Instrumentation technique	48 hours	7 days
Control	720.0 (182.4)^{A,a}	514.5 (138.3)^{A,a}
Manual	255.9 (172.4)^{B,a}	133.3 (84.0)^{B,a}
Rotary	136.6 (121.0)^{B,a}	88.3 (40.0)^{B,a}
Reciprocating	120.2 (71.4)^{B,a}	124.4 (64.5)^{B,a}

Different capital letters in each column indicate statistically significant difference ($p<0.05$). Different lowercase letters in the row indicate statistically significant difference ($p<0.05$).

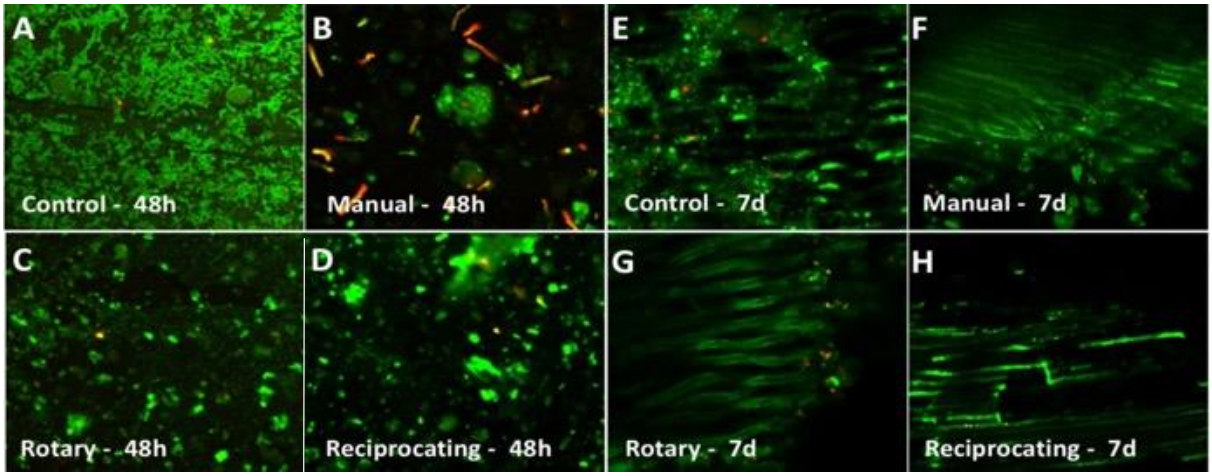

Figure 1. Representative images of confocal laser scanning microscopy of each group on different evaluation times. A – control group (48 hours); B – manual instrumentation (48 hours); C – rotary instrumentation (48 hours); D – reciprocating instrumentation (48 hours); E – control group (7 days); F – manual instrumentation (7 days); G – rotary instrumentation (7 days); H – reciprocating instrumentation (7 days).

4. Discussion

During root canal instrumentation, some areas are not touched by endodontic instruments (Versiani et al. 2018), requiring auxiliary chemical substances to eliminate microorganisms (Böttcher et al. 2015; Rôças et al. 2016). However, some microorganisms remain (Souza et al. 2018a) and penetration of fluids occurs when the seal fails (Ricucci et al. 2013), leading recontamination. Chlorhexidine adsorbs to surfaces, being released in the form of active cation, allowing antimicrobial activity over time (Böttcher et al. 2015). Then, the prevention of recontamination may be achieved (Gomes et al. 1996), justifying the importance to evaluate CHX substantivity and the influence of reciprocating instrumentation on this property in the present study.

CHX substantivity has been measured in previous studies through HPLC (Souza et al. 2012; Souza et al. 2018b), as it provides an accurate estimative of the CHX amount that is retained on root dentin (Souza et al. 2012). However, it does not assess whether retention is effective in reducing microorganisms. For this reason, CLSM was used in the present study to assess the relationship between CHX retention and its antimicrobial action. This method evaluates the viability of microorganisms on the infected dentin by assessing membrane permeability without disturbing attached cells. Furthermore, this assessment is compatible with clinical conditions that demonstrate that no inactivation of antimicrobial substances is achieved during endodontic treatment. (Böttcher et al. 2015). The high sensibility and costs for performing can be a limitation of this kind of test.

The use of alternative systems is indicated due to the ease of preparation of the root canal and providing a reduced number of endodontic instruments and clinical time (Rasimick et al. 2010). Moreover, these systems reduce the contact time of CHX with root canal walls and its retention on the dentinal surface (Souza et al. 2018b). However, there are no studies in literature showing if lower CHX retention interferes on its antimicrobial activity over time. Böttcher et al. (2015) revealed that CHX was detected for 48 hours and 7 days with low percentage of viable cells, also presenting increase in the number of viable cells after 30 days, becoming similar to saline. Thus, the present study evaluated the retention and antimicrobial activity of CHX associated with reciprocating instrumentation after 48 hours and 7 days, comparing with manual and rotary techniques.

According to the present methodology, human dentin was infected with *E. faecalis* for two weeks, as described by Souza et al. (2018a). This time is sufficient for the formation of biofilm and an adequate parameter to observe decontamination protocols (Guerreiro-Tanomaru et al. 2013). In this scenario, the present results show high prevalence of live *E. faecalis* cells in all experimental groups after both evaluation times. These results are in agreement with a previous study, in which the isolated use of the chemo-mechanical preparation also resulted in high prevalence of *E. faecalis* (Souza et al. 2018a). In addition to its

ability to penetrate dentinal tubules, *E. faecalis* is highly resistant and presents several virulence factors, ability to survive with limitation and lack of nutrients (Ran et al. 2015), which can help explain the high levels of living cells in all tested groups.

The present results revealed higher CHX retention on human dentin after manual instrumentation. This finding is not in accordance with a previous study, in which manual and rotary instrumentation resulted in higher CHX retention compared to reciprocating preparation (Souza et al. 2018b), which can be explained by the experimental design of the rotary instrumentation in each study. In the present study, Pro-Taper Next composed of 3 files was used, renewing CHX 3 times, whereas in the previous study Pro-Taper Universal composed of 5 files was used, renewing CHX 5 times. Thus, the lower number of CHX applications the lower the chlorhexidine molecules into the tooth, using this rotary technique.

Some mitigating factors can become shorter the CHX lifespan on human dentin, reducing the effectiveness of chemical agents in root canals. Proteins, collagen, dead microorganisms and dentin debris tend to have influence in this effectiveness (Rasimick et al. 2010). However, it could not be observed in the present results, since a significant reduction in the concentration of retained chlorhexidine was not observed after one week in all instrumentation techniques, when compared to a period of two days. These results are in accordance with other studies, in which CHX retention after 7 days was similar when compared to the first evaluation time, and whose significant reduction was observed only after 30 days (Souza et al. 2012; Souza et al. 2018b). Thus, CHX retention on human dentin provides antimicrobial activity in a satisfactory clinical time.

Regardless of instrumentation technique, the association with CHX resulted in lower levels of live *E. faecalis* cells after CLSM evaluation, when compared to control group in the present study. This antimicrobial activity was preserved over time, after 7 days. The interaction of CHX positive molecular charge and negative charge of microbial cell walls induces modification in the osmotic equilibrium of the bacterial cell. It increases cell permeability, which allows chlorhexidine molecules to penetrate and eliminate bacteria (Dametto et al. 2005). This mechanism is observed over time by the gradual releasing of CHX molecules, providing antimicrobial activity after its use in root canals (Böttcher et al. 2015). In addition, CHX maintains the integrity of the dentine substrate for root canal filling, being another way to prevent recontamination by effective material adhesion and sealing of the root canal space (Moreira et al. 2009). Thus, the use of CHX in association with instrumentation techniques represents a significant alternative to be used in chemo-mechanical preparation.

Böttcher et al. (2015) revealed that higher amount of CHX retained after HPLC was associated with low percentage of viable cells in *E. faecalis* biofilms. This finding is not in accordance with results of the present study. Despite the lower CHX retention on root dentin after rotary and reciprocating instrumentation, it did not interfere on the effectiveness against *E, faecalis*, since the amount of live cells was similar when compared to manual instrumentation. These results confirm the first (i) and second (ii) hypotheses of the present study. The association of instrumentation techniques with CHX can be the explanation for differences between studies, with rotary and reciprocating instruments exerting some effect on *E. faecalis* reduction, even with lower CHX retention. Our results show that conventional chemo-mechanical preparation is not enough to provide adequate decontamination of root canals. The present study suggests the use of auxiliary decontamination resources, such as ultrasonic activation and photodynamic therapy, in order to improve the bacterial reduction, as shown by Souza et al. (2018a). Thus, favorable environment is created, providing better conditions to endodontic therapy success and healing of apical tissues.

5. Conclusions

Under the study limitations, it was observed that reciprocating instrumentation does not interfere on CHX substantivity, maintaining the antimicrobial activity against *E. faecalis* in the same effectiveness level when manual or rotary instrumentation is used.

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