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RESEARCH ARTICLE OPEN ACCESS

EX VIVO STUDY OF DIFFERENT FINAL IRRIGATION PROTOCOLS AGAINST MATURE BIOFILMS IN ROOT CANAL SYSTEM DISINFECTION

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ABSTRACT

In endodontics the main objective is the debridement and disinfection of the root canal system. The purpose of this study was to evaluate the effectiveness of final irrigation protocols using different irrigation systems, with and without using sodium hypochlorite (NaOCl) as an irrigant, in promoting the disruption of mature biofilms in moderately curved root canals. Sixty mesial roots from extracted human mandibular molars were used. The root canals were contaminated with a standard strain of Enterococcus faecalis for 21 days. Before instrumentation, bacterial samples were collected by inserting a sterile paper point into the root canals. The root specimens were then randomly divided into 6 groups (n = 10) for instrumentation and final irrigation: NaOCl + MI: sodium hypochlorite + manual irrigation; NaOCl + PUI: sodium hypochlorite + passive ultrasonic irrigation; NaOCl + ECL: sodium hypochlorite + irrigation with EasyClean; SS + MI: saline + manual irrigation; SS + PUI: saline + passive ultrasonic irrigation; SS + ECL: saline + irrigation with EasyClean. After the final irrigation, a second sample collection was performed for viable bacterial counts following the same protocol used for the baseline collection. The results were analyzed using Wilcoxon and Friedman nonparametric tests at a significance level of 5%. All final irrigation protocols resulted in significant biofilm reduction within the root canal system ($p < 0.05$). It can be concluded that the irrigating solution influenced biofilm reduction in the root canal system, regardless of the final irrigation protocol used.

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INTRODUCTION

The main objective of endodontics is the debriment and disinfection of the root canal system. The mechanical cleansing by instruments associated with irrigation solutions has an important role on the residual bacterial biofilm removal in surfaces with hard access during the instrumentation (Joy et al., 2015; Caputa et al., 2019). The biofilm is more resistant to the antimicrobial agents, for this reason, the debriment, become harder, leading it to endodontic treatment failure (Pladisai et al., 2016). The principal cause of flop is the microorganism's resistance in the infected root canal, which can be related to the periradicular lesions (Ricucci et al., 2013). After the end of the instrumenatlization of the root canal system, there is a possibility of finding new areas that have not been touched by the endodontic's tools, harboring fragments of dentin, microorganisms and its subproducts. The irrigation can aid the cleaning of these areas (Jiang et al., 2010). Many irrigation solutions are used to diminish the residual debris, as well as the necrotic tissue, bacterias and a smear layer formed by the action of the instruments inside the root canal (Mancini et al., 2013). The penetration of the irrigant on the canal system will depend on the anatomy, the volume of the solution, the instrumentation, the physical-chemical characteristics of the irrigant, and mainly on the application's technique of it (Castelo-Baz et al., 2012; Boutsioukis & Nova, 2021). The application manner of the irrigant inside the canal can influence its action. The irrigation with the conventional syringe, and it is widely accepted, although its action

is not sufficient to remove irregular debris of the root canal, besides that it is not efficient in the apical third of the root canal and in the isthmus (Virdee et al., 2017). The Passive Ultrasonic Irrigation (PUI) has been used as an efficient method of removal of the smear layer (Shiavotelo et al., 2017). The Ultrasound has the potential to remove the dentin remains and organic tissue in areas with difficult access of the root canal (Rödig et al., 2010). The Sodium hypochlorite (NaOCI) is the root canal irrigant most used in endodontics due to its great antimicrobial spectrum and for its capability of organic tissue removal (Van der Sluis et al., 2007; Paqué et al., 2011). For this reason, adopting the same principals of optimization of the chemical agent actions through instruments that do not get in contact with the root walls, a plastic instrument made of acrylonitrile butadiene styrene (ABS), called Easy Clean was developed (Kato et al., 2016). Notwithstanding, the literature it is not conclusive as to the results of the sodium hypochlorite agitation using a manual instrument or ultrasonic to decrease the amount of microorganism in the interior of the root canal system. Therefore, the purpose of this study was to evaluate different protocols of final irrigation, with the use, or not, of the sodium hypochlorite (NaOCI), on the disinfection of the mature biofilm of the canal root system (SCR) with moderated curvature. The null hypothesis is that there is no difference in the disinfection of the biofilm after the application of the final irrigation protocols.

MATERIAL AND METHODS

The Ethics Committee approved this paperwork by the legal opinion number 2.220.848

Sample Selection: 60 human extracted molars were used, after the Free and Enlightened Consent Term signature (FECT) that remain stored in saline solution until the beginning of this research. The criteria of inclusion were teeth with mesial vestibular roots with independent foramina, with the absence of fractures, dilacerations, uncompleted rhizogenesis or resorption; and canals with the moderated curvature in between 10° and 20° (Schneider, 1971). The number of 10 samples by group was obtained from the sample calculation made after the pilot procedure. It was used the ANOVA statistical test for the sample calculation, with a minimum difference amongst the treatment average of 0.06, error standard deviation of 0.035, treatment number 6, test power of 0.80 and 0.05 alpha. The number of required samples calculated was 10.

Experimental procedures: The teeth crowns were sectioned to the junction-cement-enamel level (JCE) and the mesial root removed with the double-sided diamond disc (KG Sorensen, Cotia, SP, Brazil) in low rotation (Kavo Dental Excellence, Joinville, SC, Brazil). The root was standardized to the length 18 mm with a digital caliper (MTX, China). The length of the work (CT) was like a 17mm, being determined with an instrument K#15 (VDW, Munique, Germany) trespassing the foramen canal and retreating 1mm. Subsequently, the mesial vestibular canal was handled to an endodontic file tipo K#20 (VDW, Munique, Germany) with enlargement movement until the apical foramen with 5ml irrigation os saline solution JP, Ribeirão Preto, SP, Brazil) in each change of file, using the sterile hypodermic syringe (SR, Manaus, AM, Brazil) conected to a sterile hypodermic and disposable needle 22G (Descarpack, Jiangsu, Nanquim, China) until the medium third of the root. The roots were waterproofed with resin epóxi (Araldite, Joinville, SC, Brazil) in the forame extension, and the cosmetic polish (Colorama, Diadema, SP, Brazil) in all the surface, except the canal access. The mesial root of the tooth was pressured and placed in this region, until a great part of it were retained in the interior of the Eppendorf tube Eppendorf do Brazil, SP, Brazil). Around the root specimen were inserted two layers of cyanoacrylate (Ciano, Valinhos, SP, Brazil). The tubes that contained the roots were autoclaved (Cristofoli, Campo Mourão, PR, Brazil) to 121^0 C during 15 minutes.

Biofilm confection: The contamination of the root canal was made with standard strain of *Enterococcus faecalis* ATCC 29212 (LabCenter, Campinas, SP, Brazil). The strain was defrosted and reactivated in a brain infusion and sterile heart broth (Brain Heart Infusion- sterile, Acumedia Manufacturers, Michigan, USA) and put in a incubator with 5% of CO2 and 37°C for 24 hours for the bacterium growth. After being activated, it was made a suspension with turbidity corresponding to 10 in a McFarland scale to contaminate the root canal with 10 ml, using the micropipette (Probac Do Brazil, São Paulo, Brazil). During 21 days, once a day, it was made a renovation of the sterile to obtain a mature biofilm. The viability and pureness of the microorganisms observed inside the canal were confirmed weekly by the sample collection randomly from two teeth using #15 paper cones Endopoints, Rio de Janeiro, Brazil). After the microbial growth, it was confirmed the bacterial morphology using the Gram coloration. To the initial collection, it was inserted a cone to the absorbent paper #15 points endo, Rio de Janeiro, Brazil) to the root canal. The cone was maintained to the interior of the canal during 1 minute and transferred like a pincher to the flask of polypropylene (Eppendorf do Brazil, SP, Brazil). It was made the homogenization for 30 seconds in the tube stirrer Vortex AD 56; Phoenix, Araraquara, Brazil). Series of dilution were prepared from this suspension to a concentration of 10^{-5}. Aliquots of 0.1 mL of the suspension and each one of the dilutions were seeded in Petri Dishes containing BHI agar (Kasv, São José do Pinhais, PR, Brazil). The sown dishes were included in a atmosphere of CO_2 to 5% and 37º C during 24 hours.

Chemical-mechanical prepare: The procedures of handling and irrigating of the specimens were made always by the same endodontic specialist operator. Each file, syringe, needle and sucker were used for the preparation of 1 canal and then posteriorly discarded. All the materials used in this research were sterilized. The preparation chemical-mechanical was accomplished with Easy ProDesign Logic #. 25.01 files, torque 1 and speed 350 rpm and #.25.06, toque 4 e speed 950 rpm (Easy, Belo Horizonte, MG, Brazil) coupled with a motor with a remote of torque and rotatory movement. All the roots followed the same sequence of instrumentation according to the system protocol provided by the producer: exploitation of the cervical middle third with k # .10 file. Instrumentation with Logic #.25.01 file, until it reaches the work length. During the preparation, each one of the specimens was irrigated in each instrument change until the end of the preparation, with 6ml of saline solution (JP, Ribeirão Preto, SP, Brazil) adding up a total volume of irrigation by canal 18 ml, being the irrigation made with syringe and needle Luer Luer Look 10 mL (Injex, São Paulo, Brazil) 15mm measure of the root canal. Three groups received the final irrigation with saline solution JP, Ribeirão Preto, SP, Brazil) the other three groups received the final irrigation with Sodium Hypochlorite 2,5% Asfer, São Caetano do Sul, SP, Brazil). The specimens were distributed randomly in each group. After the preparation, the teeth were divided arbitrarily in the webpage in six, experimental groups (n=10), accordingly to the Table 1.

Table 1. Experimental draw

FINAL IRRIGATION	GROUPS (N=10)
Sodium hypochlorite (HP) + manual irrigation (IMN)	G1
Sodium hypochlorite (HP) + manual irrigation (PUI)	G2
Sodium hypochlorite (HP) + irrigation with easyclean (ECL)	G3
Saline Solution (SR) manual irrigation (IMN)	G4
Saline Solution (SR) + ultrasonic irrigation (PUI)	G5
Saline Solution (SR) + irrigation with easyclean (ECL)	G6

The solution was elevated to the canals through an irrigation needle 30G (Navitip; Ultradent, South Jordan, UT, USA) setted up to 2 mm shorter than the length of the work linked to a disposable syringe with 10ml like luer lock (Injex, Ourinhos-SP, Brazil) properly identified with the name of the irrigation sucker. The process of suction was made with an endodontic sucker Angelus Dental Products Industry S.A., Londrina-PR, Brazil) embedded to a sucker terminal of the dental equipment.

Final irrigation protocol: It was done the final irrigation of each specimen of the six groups following the PUI protocol and the Easy Clean recommendation (Simezo et al., 2017).

Table 2. Medians, interquartile deviations and statistical test (CAI) and final post-irrigation (AIF) of the sample groups (log10)

	SR/PUI	HP/PUI	SR/MN	HP/MN	SR/ECL	HP/ECL	(p)
CAI	6.12 (0.94) ^{A1}	6.47 (0.30)^{A1}	6.18 (0.30)^{A1}	6.27 (0.13)^{A1}	6.45 (0.31)^{A1}	6.50 (0.32)^{A1}	0.1753
AIF	4.90 (0.82) ^{A1}	0.00 (0.00)^{B2}	4.36 (0.72)^{B1}	0.00 (0.00)^{B2}	4.88 (0.21)^{B1}	0.00 (0.00)^{B2}	<0.0001
(p)	0.0745	0.0117	0.0051	0.0051	0.0077	0.0051	

CAI: microbial count before the instrumentation; AIF: after the final irrigation; SR/PUI: saline solution/ irrigation passive ultrasonic; HP/HUI: sodium hypochlorite/ passive ultrasonic irrigation; SR/MN: saline solution/ manual irrigation: HP/MN: sodium hypochlorite/manual irrigation; SR/ECL: saline solution/ easyclean; HP/ECL: sodium hypochlorite/easyclean, uppercase letters differs in the vertical way and different numbers in the horizontal way: different statistically significant.

The canals were irrigated with 2 ml of each solution and submitted to cycles of steering of 20 seconds each. In each period, it was dispensed 2ml of the irrigation solution with a syringe, after that the canals were irrigated with 5 ml of EDTA 17% during 3 minutes. Each group received 26 ml of the irrigation solution and 5 ML of EDTA 17%, summing up to 31ML of the solution volume. The ultrasonic activation passive was realized through an inserted type of a slick probe E1 Irrisonic (Helse, Capelli e Fabris, São Paulo, Brazil) with diameter of the point equivalent a one instrument like K #20, in the 30 kHz frequency, placed 1mm less than the working length for 20 seconds. The activation by Easy Clean was done with a rotational motor placed 1 mm of distance of the working length, in other words a 16 mm for 20 seconds. When finalized the irrigation, a final collection was made (A), repeating the same procedure as in initial collection (B).

Statistics Analyses: The Biostat program 4.0 analyzed the results. A test of D'Agostino normality was made. The sample presented abnormal behavior. The results were submitted to a not parametric test of Wilcoxon and Friedman with the significance of 5%.

RESULTS

All the protocols of final irrigation resulted in a significant reduction of the microbial present in the root canal system $p<0.05$). There was a major microbial reduction in the final irrigation protocols that have used hypochlorite as the irrigation solution, independently of the utilization form: convectional, ultrasonic and passive or Easy Clean ($p<0.0001$). There was no significant difference among the microbial initial count of the different groups of samples ($p>0.05$, Tabela 2).

DISCUSSION

In the study presented, root canal disinfection promoted by different irrigation protocols was evaluated, observing the effectiveness of agitation of three instruments used in endodontics. Mesiobuccal canals were selected because of their morphological variations. Based on the results obtained in the research (Silveira et al., 2005), the variation in the morphology of the lower molar mesial root canals is relatively high. These anatomical variations are considered a challenge because it hinders root canal cleaning and disinfection, as well as clinical practice Siqueira Jr et al., 2013). Due to the emergence of new instrumentation methods, the modeling step is performed in a shorter time, thus allowing the irrigating solution to act briefly within the channel. The contact of the solution within the canal is important due to the anatomical variations found in the molars, so it is important to obtain a final irrigation protocol that provides better cleaning and antimicrobial effect. There are anatomical regions of the dental element that the mechanical instrument cannot reach or touch, which makes the irrigating substance important to achieve this goal (Cerqueira et al., 2007). The irrigant of choice in this research was NaOCl at a concentration of 2.5%. Assessing the effectiveness of different NaOCl concentrations, it was observed that the 2.5% concentration causes a complete inhibition of E. Faecalis biofilm growth at all stages of development (Reyhani et al., 2017). Although there is extensive literature on the use of sodium hypochlorite solutions as an irrigant in endodontics, little information is available on the effect of these solutions on mature biofilm. Thus, the present study simulated an infection of the root canal system using a mature biofilm with Enterococcus faecalis, because in the roots bacteria prevail in a biofilm configuration. Therefore, the objective is to give a better insight into the behavior of these specimens to disinfectant agents than planktonic culture-based studies for example (Van der Wall et al., 2015). A group with saline solution was established as study control, considering that this substance has no dissolution capacity and no chelating action. EDTA was then used in all groups because of its high chelation power because, despite the possibility of microorganism reduction only with ultrasonic or manual agitation, the association of chelators with this agitation promotes better quality (Câmara et al., 2010). The three techniques were performed in three 20-second cycles totaling one minute of agitation. This protocol shows that this combination promotes a cumulative effect, becoming efficient in removing microoganisms (Van der Sluis et al., 2010). The amount of irrigant used was defined to avoid irrigation volume interference in the techniques used. The results obtained in this research showed that there was no significant difference in the microbiota present in the root canal system between the protocols used, and the reduction was better observed when associated with the use of sodium hypochlorite, thus confirming the null hypothesis. It can be observed in the research result that due to the use of NaOCl the number of microorganisms decreased, and when the saline was used in the same irrigation protocols and with the same instruments the bacterial count did not significantly decrease. NaOCl is a potent antimicrobial agent, eliminating most bacteria instantly in direct contact, and is considered the gold standard in endodontic treatment (Mohmmed et al., 2017). The mesial root of the mandibular molars presents a great degree of complexity, making it difficult to obtain an excellent antibacterial effect. It is, therefore, reasonable to suppose that some areas not touched by mechanical instrumentation before final irrigation were successfully disinfected by NaOCl, which has high antibacterial activity. Studies are reporting better antimicrobial efficacy using PUI. This improvement may be related to the use of microorganisms more susceptible to disinfection procedures, shorter incubation periods, or due to the analysis of the samples performed soon after treatment (Guerreiro-Tanomaru et al., 2015).

CONCLUSION

The irrigating solution influenced the reduction of the root canal system biofilm, regardless of the protocols used for final irrigation.

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