

Analysis of two irrigation methods in root canal disinfection against *E. Faecalis* biofilm under the influence of the concentration, volumen, and time in contact of the irrigant

Análise de dois métodos de irrigação na desinfecção do canal radicular contra biofilme de *E. Faecalis* sob a influência da concentração, volume e tempo de contato do irrigante

Ánalisis de dos métodos de riego en la desinfección del conducto radicular frente al biofilm de *E. Faecalis* bajo la influencia de la concentración, el volumen y el tiempo de contacto del irrigante

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Abstract

The purpose of this *ex vivo* study was to evaluate the effectiveness of two irrigation techniques against *E. Faecalis* biofilm: conventional irrigation (CI) and passive ultrasonic irrigation (PUI). Furthermore, to investigate a possible cumulative effect of disinfection in the final irrigation, leaving the hypochlorite in the root canal for 2 or 5 minutes without agitation. A total of 63 premolars were divided into 9 groups ($n = 7$): Groups CI - 2.5 and CI - 5.25: Conventional irrigation with 2.5% and 5.25% NaOCl, respectively, a total of 6 mL for 1 min. Groups CI/PUI - 2.5 and CI/PUI - 5.25: CI + PUI with 2.5% and 5.25% NaOCl respectively, a total of 12 mL for 2 min. Groups CI/PUI - 2.5-2 (total of 14 mL for 4 min) and CI/PUI - 2.5-5 (total of 14 mL for 7 min): CI + PUI, plus final irrigation leaving the NaOCl 2,5% in the root canal for 2 or 5 minutes without agitation, respectively. Groups CI/PUI - 5,25-2 (total of 14 mL for 4 min) and CI/PUI - 5,25-5 (total of 14 mL for 7 minutes): CI + PUI, plus final irrigation leaving the NaOCl 5,25% in the root canal for 2 or 5 minutes without agitation, respectively. Control Group: CI + PUI, final irrigation leaving sterile saline solution for 5 min. The CI/PUI - 5,25 group showed a significantly greater reduction of *E. faecalis* than in the CI/PUI - 2.5 group. All other groups did not differ significantly from each other. The control group showed a less efficient reduction of *E. faecalis*. The CI associated with PUI was sufficiently able to reduce the bacterial load of *E. faecalis*. The final irrigation, leaving the hypochlorite in the root canal for 2 or 5 minutes without agitation did not demonstrate relevance in this study. Successive changes of the irrigating liquid, resulting in greater volume, demonstrated better results in disinfecting the root canal system.

Keywords: Endodontics; Root canal irrigants; Sodium hypochlorite.

Resumo

O objetivo deste estudo *ex vivo* foi avaliar a eficácia de duas técnicas de irrigação contra o biofilme de *E. Faecalis*: irrigação convencional (CI) e irrigação ultrassônica passiva (PUI). Além disso, investigar um possível efeito cumulativo da desinfecção na irrigação final, deixando o hipoclorito no canal radicular por 2 ou 5 minutos sem agitação. Um total de 63 pré-molares foram divididos em 9 grupos ($n = 7$): Grupos CI - 2,5 e CI - 5,25: Irrigação convencional com NaOCl 2,5% e 5,25%, respectivamente, um total de 6 mL por 1 min. Grupos CI / PUI - 2,5 e CI / PUI - 5,25: CI + PUI com 2,5% e 5,25% NaOCl respectivamente, um total de 12 mL por 2 min. Grupos CI / PUI - 2,5-2 (total de 14 mL por 4 min) e CI / PUI - 2,5-5 (total de 14 mL por 7 min): CI + PUI, mais irrigação final deixando o NaOCl 2,5% em canal radicular por 2 ou 5 minutos sem agitação, respectivamente. Grupos CI / PUI - 5,25-2 (total de 14 mL por 4 min) e CI / PUI - 5,25-5 (total de 14 mL por 7 minutos): CI + PUI, mais irrigação final saindo do NaOCl 5,25% no canal radicular por 2 ou 5 minutos sem agitação, respectivamente. Grupo controle: CI + PUI, irrigação final com solução salina estéril por 5 min. O grupo CI / PUI - 5,25 mostrou uma redução significativamente maior de *E. faecalis* do que no grupo CI / PUI - 2,5. Todos os outros grupos não diferiram significativamente uns dos outros. O grupo controle apresentou uma redução menos eficiente de *E. faecalis*. O IC associado ao PUI foi suficientemente capaz de reduzir a carga bacteriana de *E. faecalis*. A irrigação final, deixando o hipoclorito no canal radicular por 2 ou 5 minutos sem agitação, não demonstrou relevância neste estudo. Mudanças sucessivas do líquido irrigante, resultando em maior volume, demonstraram melhores resultados na desinfecção do sistema de canais radiculares.

Palavras-chave: Endodontia; Irrigantes do canal radicular; Hipoclorito de sódio.

Resumen

El propósito de este estudio *ex vivo* fue evaluar la efectividad de técnicas de irrigación contra el biofilm de *E. Faecalis*: irrigación convencional (CI) e irrigación ultrasónica pasiva (PUI). Además, investigar un posible efecto acumulativo de la desinfección en el riego final, dejando el hipoclorito en el conducto radicular durante 2 a 5 minutos sin agitación. Un total de 63 premolares se dividieron en 9 grupos ($n = 7$): Grupos CI - 2.5 y CI - 5.25: Irrigación convencional con NaOCl al 2.5% y 5.25%, respectivamente, un total de 6 mL durante 1 min. Grupos CI / PUI - 2.5 y CI / PUI - 5.25: CI + PUI con 2.5% y 5.25% NaOCl respectivamente, un total de 12 mL durante 2 min. Grupos CI / PUI - 2.5-2 (total de 14 mL durante 4 min) y CI / PUI - 2.5-5 (total de 14 mL durante 7 min): CI + PUI, más irrigación final dejando el NaOCl 2,5% en el conducto radicular durante 2 o 5 minutos sin agitación, respectivamente. Grupos CI / PUI - 5,25-2 (total de 14 mL durante 4 min) y CI / PUI - 5,25-5 (total de 14 mL durante 7 minutos): CI + PUI, más irrigación final saliendo del NaOCl 5,25% en el conducto radicular durante 2 o 5 minutos sin agitación, respectivamente. Grupo de control: CI + PUI, irrigación final dejando solución salina estéril durante 5 min. El grupo CI / PUI - 5,25 mostró una reducción significativamente mayor en *E. faecalis* que en el grupo CI / PUI - 2.5. Todos los demás grupos no difirieron significativamente entre sí. El grupo de control mostró una reducción menos eficiente de *E. faecalis*. El IC asociado con PUI fue suficientemente capaz de reducir la carga bacteriana de *E. faecalis*. El riego final, dejando el hipoclorito en el conducto radicular durante 2 o 5 minutos sin agitación, no demostró relevancia en este estudio. Los cambios sucesivos del líquido de irrigación, que dieron como resultado un mayor volumen, demostraron mejores resultados en la desinfección del sistema de conductos radiculares.

Palabras clave: Endodoncia; Irrigantes del conducto radicular; Hipoclorito de sodio.

1. Introduction

Microbial control in endodontics is of utmost importance to achieve successful treatment (Alves et al., 2011). Therefore, failures have been associated with the permanence of microorganisms in the root canal system which, because of the complex anatomy, are difficult to access by endodontic instruments, consequently harboring persistent infections (Vera et al., 2012; Gonçalves et al., 2016).

The use of a chemically active irrigation solution, as a complement to the instrumentation, is indicated, being sodium hypochlorite (NaOCl), the solution traditionally used, due to its tissue dissolution capacity and high antimicrobial spectrum (Moreno et al., 2018). However, regarding the ideal concentration of sodium hypochlorite, there are controversies in the literature. The influence of different concentrations of NaOCl ranging from 0.5% to 5.25% on various aspects, such as dentin erosion, microhardness, antimicrobial action, tissue dissolution, postoperative pain and accidents, obtaining conflicting results and not reaching an ideal concentration (Siqueira et al., 2000; Gernhardt et al., 2004; Rôças e Siqueira, 2010; Kaya et al., 2011Wong e Cheung, 2014; Martin et al., 2014; Verma et al., 2019).

The volume recommended for root canal treatment is also another important factor. It is known that larger volumes (2 and 5 mL) between each file change, instead of smaller ones (0.5 and 1 mL), result in greater sanitizing, also exercising the mechanical influence on bacterial elimination (Brito et al., 2009; Morago et al., 2016; Moreno et al., 2018). A frequent renewal and larger volumes of the irrigating solution improves antibacterial efficiency (Brito et al., 2009; Moreno et al. 2018).

Antibacterial irrigants, such as NaOCl, are used to act in the canal for a short time, being generally insufficient to eliminate bacteria located in anatomical complexities, being this irrigant time dependent (Siqueira, 2001). The time needed for NaOCl to eliminate bacteria from the root canal system, it ranged from 2 to 30 minutes (Retamozo et al., 2010). The disinfection is significantly better with a high volume and an extended exposure to NaOCl (Gazzaneo et al., 2019).

There is evidence that the antibacterial efficacy of NaOCl depends on the concentration, temperature, volume, and time in contact in the root canal (Siqueira et al., 2000; Sirtes et al., 2005; Retamozo et al., 2010; Del Carpio-Perochena et al., 2011). Strategies directed to the use of effective irrigation techniques that can maximize the disinfection of the root canal system must be adopted. The conventional technique of irrigation using of syringe and needle, despite the possibility of controlling the amplitude of penetration and the volume of the irrigant, has not shown satisfactory results, as this method, consider simple, is ineffective in cleaning remote areas (Gu et al., 2009; Căpută et al., 2019). Technological advances in root canal system disinfection allowed the idealization of some complementary irrigation methods. The passive ultrasonic irrigation (PUI) enabled a better canal cleaning, due to the irrigating liquid agitation, microacoustic current, and cavitation, allowing the reach of anatomical complexities with ease (Van der Sluis et al., 2010).

Based on these assumptions, the objective of this *ex vivo* study was to evaluate the disinfection efficacy of two irrigation techniques against *E. Faecalis*: conventional irrigation with syringe and needle and the PUI technique. Also, to investigate a possible cumulative effect of disinfection in the final irrigation, leaving the hypochlorite in the root canal for 2 or 5 minutes without agitation. The null hypotheses tested were that (I) the irrigation techniques would not show any influence on the disinfection capacity against *E. Faecalis*, (II) that the concentration, volume, and the final irrigation, leaving the hypochlorite in the root canal for 2 or 5 minutes without agitation would not influence the disinfection against *E. Faecalis*.

2. Methodology

This study was submitted and approved by the institutional Research Ethics committee (Protocol number: 3.564.900). The sample calculation was performed in the G*Power 3.1.5 software, adopting the analysis of variance model. For the effect size of 0.57, obtained from the results presented by Sasanakul et al., 2019, 5% significance level and 80% power.

Sample selection and standardization

A total of 63 lower permanent premolars, extracted for reasons unrelated to the study were select, following the inclusion criteria: teeth with fully formed single roots, with a single canal, without calcifications, absence of resorptions, root caries, or fractures, without previous endodontic treatment and teeth with the oval-shaped canal (vestibule-lingual diameter 2 or more times larger than the mesio-distal diameter, 5mm from the root apex) (Metzger et al., 2010; Lacerda et al., 2017), root curvature between 0° and 10° (Schneider, 1971) and apical diameter corresponding to a manual file type K nº 15 (Kendo, Bayerwaldstr, Munich, Germany). Each tooth was previously analyzed through an operating microscope (Alliance Comércio Ltda, São Carlos, SP, Brazil), under 8X magnification and radiographic analysis. These teeth were cleaned with and stored in 0.1% thymol until the time of the experiment.

Subsequently, these roots were washed in running water for one hour, to remove any residue from the 0.1% thymol solution, and then dried with an air jet and gauze. The teeth were standardized at 16mm by cutting the coronary portion, with a diamond disc (Horico Dental Hopf, Ringleb & Co. GmbH & Cie, Berlin, Germany, and the working length (WL) was set at

15mm. Then, the apical foramen was sealed with light-curing resin Filtek™ Z350 XT (3M, Maplewood, Minnesota, USA), and each specimen inserted in Condensation Silicone (Precise SX, Dentsply, Argentina SACI) to prevent the leakage of the irrigating solution through the apical foramen throughout the cleaning and shaping process. Thereafter, all samples had their root canals enlarged up to the apical foramen, with manual K file up to no. 20, under irrigation with saline solution, to allow contamination of the canal by *E. faecalis* later, (Gomes et al., 2009). Subsequently, all samples were previously sterilized in an autoclave, numbered, and randomly assigned to each experimental group using the Random.org program (Available on <https://www.random.org/>).

Specimen inoculation with *E. Faecalis*

For inoculation, a suspension of *E. faecalis* was prepared. 100 µL *E. Faecalis* stock was transferred to 10 mL of BHI broth (Difco, Detroit, MI), being kept in an oven at 37°C for five days (McFarland scale 10). Each canal was filled to the cervical level of the root hole with the suspension of *E. faecalis*, using sterile syringes of 1 mL insulin, with a 30-gauge needle. Then, all samples were immersed in a glass at 37°C for 28 days in the oven at 100% humidity, to allow the bacteria colonization on the canal walls and dentinal tubules. The viability of the microorganisms was checked daily, and a 5.0 mL aliquot of the culture medium was added, certifying the effectiveness of the contamination.

Previous collection (PC): sample contamination assessment

The contamination of the samples was checked before starting the cleaning and shaping procedures for each sample group. In each specimen, an absorbent paper cone no. 20 was inserted, up to the WL, to collect the material inside the canals, and thus obtain the PC samples. This absorbent paper cone remained in the root canal for 1 minute and then transferred to an Eppendorf tube containing 1 mL of sterile saline solution (0.9% NaCl) and agitated in Vortex (AD 56, Phoenix, Araraquara, SP/Brazil) for 30 seconds. With the biological material collected, dilutions were made, an aliquot of 25 µL (0.025 mL) of each dilution was transferred to the Petri dish in BHI, incubated at 37° for 48 hours, and the grown colony-forming units (CFU) were counted, where it was possible to verify the contamination of the samples by *E. Faecalis* before starting any intervention.

Cleaning and shaping procedures

The following sample groups were performed (Chart 1):

Chart 1 - Schematic of the experimental procedure.

	Concentration	Conventional instrumentation and irrigation	PUI NaOCl + EDTA 17% + NaOCl	Irrigation in rest of the NaOCl (Time) Filling the entire canal	Total Volume	Total Time
Group CI -2,5	NaOCl 2,5%	3 syringes of 2 mL for 20'' = 6 mL	No PUI	No irrigation	6 mL	1 min
Group CI - 5,25	NaOCl 5,25%	3 syringes of 2 mL for 20'' = 6 mL	No PUI	No irrigation	6 mL	1 min
Group CI/PUI - 2,5	NaOCl 2,5%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL = 6 mL	No irrigationn	12 mL	2 min
Group CI/PUI - 5,25	NaOCl 5,25%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL = 6 mL	No irrigation	12 mL	2 min
Group CI/PUI - 2,5-2	NaOCl 2,5%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL= 6 mL	1 syringe with 2 mL for 2 min	14 mL	4 min
Group CI/PUI - 2,5-5	NaOCl 2,5%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL= 6 mL	1 syringe with 2 mL for 5 min	14 mL	7 min
Group CI/PUI - 5,25-2	NaOCl 5,25%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL = 6 mL	1 syringe with 2 mL for 2 min	14 mL	4 min
Group CI/PUI - 5,25-5	NaOCl 5,25%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL = 6 mL	1 syringe with 2 mL for 5 min	14 mL	7 min
Control Group C	Sterile Saline Solution 0,9%	3 syringes of 2 mL for 20'' = 6 mL	3 x 20'' with 2 mL = 6 mL	1 syringe with 2 mL for 5 min	14 mL	7 min

Source: Authors.

Conventional canal instrumentation and irrigation

The entire experimental part was performed inside a laminar flow chamber (Veco, Piracicaba - Brazil). The cleaning and shaping procedures for each sample were always performed by the same operator.

The specimens (63) were randomly divided into nine sample groups ($n = 7$) were removed from the inoculation container to start the experiment. All samples had a standardized WL of 15 mm, and the instrumentation system selected for this study was the WaveOne Gold Medium 35/6 reciprocating system (Dentsply Maillefer, Ballaigues - Switzerland). The manual glide path to the file type K No. 20 (Kendo, Bayerwaldstr, Munich, Germany), was performed previously to cause the contamination of the samples. This way, the instrumentation of each sample was divided into 3 thirds: cervical, middle, and apical, always with 3 in and out movements each third. Starting with the cervical third, with a 5 mm length towards the apex, instrumentation of the first third of the root was performed, followed by irrigation with 2 mL of NaOCl in the concentration of each sample group (2.5% or 5.25%) for 20 seconds, or sterile 0.9% saline in the control group, with the nedlle 2 mm short of the WL to avoid locking the needle in the canal. Following the instrumentation of the middle third of the root, also with 3 in and out movements and 5 mm in length towards the apex and new irrigation in the same way. And finally, the apical third, up to the WL and new irrigation with the needle 2 mm below the WL, in the same way.

Passive Ultrassonic Irrigation (PUI)

After the completion of the instrumentation and conventional irrigation with syringe and needle, in the groups that were complemented with PUI, in each specimen, 3 ultrasonic activations of 20 seconds each were made, based on the protocol

by Van der Sluis et al., 2010. These 20 seconds were measured at each activation, using a professional timer (Mormaii, Santa Catarina, Brazil), activated at the beginning and paused at the end, restarting at the beginning of another ultrasonic activation and so on. Through a 5mL syringe (Ultradent Products Inc., South Jordan, UT), with Endo-Eze 27 Gauge needle positioned at 2 mm below of the WL. 2 mL of the NaOCl solution was first dispensed with the concentration assigned to each sample group (2.5% or 5.25%) filling the entire length of the canal or sterile 0,9% saline in the control group. The irrigating solution ultrasonic activation was performed with the 20/ .01 E1-Irrisonic insert (Helse Indústria e Comércio Ltda, Santa Rosa de Viterbo, SP, Brazil), powered by Gnatus Jet Sonic (Alliage S / A Indústrias Médico Odontológica, Ribeirão Preto, SP, Brazil) in the power adjustment of 30. In the second activation cycle, it was performed the same way, but with the 17% EDTA solution or sterile 0,9% saline in the control group. And in the third activation cycle, again with NaOCl, renewing the irrigant, with the concentration of each sample group (2.5% or 5.25%) or sterile 0,9% saline in the control group.

Final irrigation of NaOCl

After performing a step of PUI, in the groups which the final irrigation with NaOCl was performed, in each sample, using a 5 mL syringe with Endo-Eze 27 Gauge needle positioned 2 mm below the WL, 2 mL of NaOCl solution was dispensed, with the concentration of each sample group (2.5% or 5.25%), filling the entire length of the canal, which remained for 2 min or 5 min without agitation, depending on the sample group, measured using a professional timer (Mormaii, Santa Catarina, Brazil). Sterile 0,9% saline was used in the control group, in the same way, for 5 min.

Post procedures collection (PPC) - Disinfection assessment

After all the cleaning and shaping procedures of the sample group, a new collection, the PPC, was carried out to assess disinfection. The root canal of each specimen was rinsed with 1 mL of 10% Sodium Thiosulfate to neutralize the NaOCl, then rinsed with 1 mL of sterile 0.9% saline solution. The material inside each canal was collected with an absorbent paper #30, which remained in the canal for 1 minute. Then, it was transferred to a polypropylene flask (Eppendorf) containing 1 mL of sterile saline solution (0.9% NaCl), which was stirred for 30 seconds in a tube shaker (Vortex AD 56, Phoenix, Araraquara, SP / Brazil). With the biological material collected, the dilutions were made, an aliquot of 25 µL (0.025 mL) of each dilution was transferred to the Petri dish in BHI, incubated at 37°C for 48 hours, and the grown colony-forming units (CFU) were counted to assess the disinfection of each sample.

Statistical analysis

From the data collected in the pre (PC) and post procedures (PPC) moments, the absolute and percentage reductions of *E. faecalis* were calculated. The absolute reduction data for normal distribution and homoscedasticity was checked, using the Shapiro-Wilk and Levene tests, respectively. With the normality and homoscedasticity assumptions, the comparison between groups was performed by applying the analysis of variance to one criterion and the Tukey test. As for the percentage reduction data, which did not show normal distribution and homoscedasticity, the comparison between the groups was made using the Kruskal-Wallis and Dunn tests. The statistical calculations were performed using the SPSS 23 software (SPSS Inc., Chicago, IL, USA), adopting a significance level of 5%.

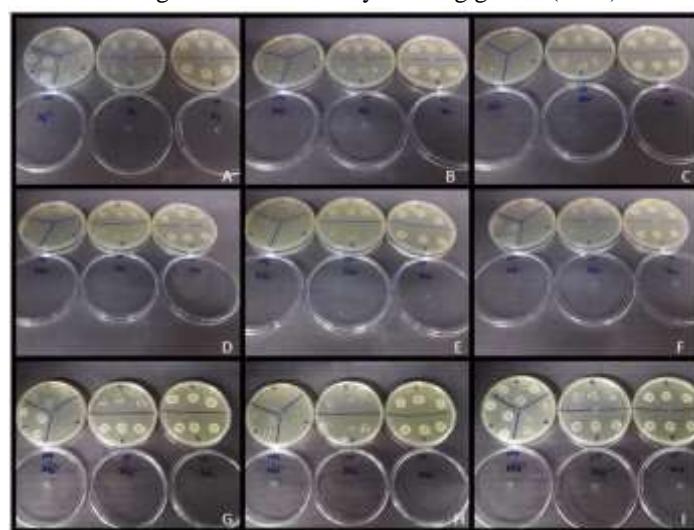
3. Results

When analyzing the absolute reduction data, it was found that all groups adhered to the normal distribution by the Shapiro-Wilk tests ($p>0.05$) and that there was homoscedasticity by the Levene test ($p = 0.065$).

One-way analysis of variance indicated that complementary irrigation at different concentrations influenced the *E. faecalis* count statistically significantly ($p = 0.007$; with 92.8% test power). Investigating the differences using the Tukey test, it was identified that the instrumented and irrigated group in a conventional manner associated to PUI with 5.25% NaOCl (CI/PUI 5,25) showed a significantly greater reduction in *E. faecalis* than the group that received only conventional instrumentation and irrigation associated to PUI with 2.5% NaOCl (CI/PUI 2.5). All other groups did not differ significantly from each other. (Table 1, Figure 1).

However, considering the data on the percentage reduction of *E. faecalis* count, the only group that showed less efficient reduction was a positive control ($p < 0.001$).

Figure 1 - Petri dishes for counting the units of colony forming grown (CFU) before and after procedures.



Caption: Image A = Group CI 2.5; Image B = Group CI 5.25; Image C = Group CI/PUI 2,5; Image D = Group CI/PUI 5,25; Image E = Group CI/PUI 2,5-2; Image F = Group CI/PUI 2,5-5; Image G = Group CI/PUI 5,25-2; Image H = Group CI/PUI 5,25-5; Image I = Control Group.

Source: Authors.

Table 1 - Means and standard deviation of *E. faecalis* count, before and after procedures.

	Evaluation time		Absolute reduction	Percentage reduction
	Previous Collection (PC)	Post Procedures Collection (PPC)		
CI 2,5	$1,20 \times 10^6$ ($8,15 \times 10^5$)	$3,33 \times 10^3$ ($7,97 \times 10^3$)	$-1,19 \times 10^6$ AB ($8,10 \times 10^5$)	99,7% a (0,4%)
CI 5,25	$2,18 \times 10^6$ ($1,08 \times 10^6$)	$3,85 \times 10^2$ ($5,88 \times 10^2$)	$-2,18 \times 10^6$ AB ($1,08 \times 10^6$)	100,0% a (0,0%)
CI/PUI 2,5	$6,96 \times 10^5$ ($5,69 \times 10^5$)	$1,10 \times 10^1$ ($1,56 \times 10^1$)	$-6,96 \times 10^5$ B ($5,69 \times 10^5$)	100,0% a (0,0%)
CI/PUI 5,25	$3,48 \times 10^6$ ($1,61 \times 10^6$)	$3,66 \times 10^2$ ($6,20 \times 10^2$)	$-3,48 \times 10^6$ A ($1,61 \times 10^6$)	100,0% a (0,0%)
CI/PUI 2,5-2	$1,42 \times 10^6$ ($1,02 \times 10^6$)	$4,21 \times 10^2$ ($1,02 \times 10^3$)	$-1,42 \times 10^6$ AB ($1,02 \times 10^6$)	100,0% a (0,0%)
CI/PUI 2,5-5	$2,48 \times 10^6$ ($2,18 \times 10^6$)	$3,92 \times 10^2$ ($6,37 \times 10^2$)	$-2,48 \times 10^6$ AB ($2,18 \times 10^6$)	100,0% a (0,0%)
CI/PUI 5,25-2	$3,14 \times 10^6$ ($1,94 \times 10^6$)	$6,63 \times 10^2$ ($1,03 \times 10^3$)	$-3,14 \times 10^6$ AB ($1,94 \times 10^6$)	100,0% a (0,0%)
CI/PUI 5,25-5	$1,27 \times 10^6$ ($9,57 \times 10^5$)	$2,47 \times 10^1$ ($6,54 \times 10^1$)	$-1,27 \times 10^6$ AB ($9,57 \times 10^5$)	100,0% a (0,0%)
C*	$2,56 \times 10^6$ ($1,48 \times 10^6$)	$6,06 \times 10^4$ ($7,61 \times 10^4$)	$-2,50 \times 10^6$ AB ($1,48 \times 10^6$)	97,6% b (3,1%)

Caption: Reduction = average of the differences between pre and post values calculated individually for each sample. CI = conventional irrigation. PUI = passive ultrasonic irrigation. C* = control group. Standard deviation in parentheses. Means followed by different capital letters indicate a statistically significant difference between groups.

Source: Own authorship.

4. Discussion

In the present study, the null hypothesis (I) that irrigation techniques would not show an influence on the disinfection capacity against *E. Faecalis*, was rejected, since conventional irrigation with syringe and needle associated with PUI, were sufficiently capable of reducing the bacterial load of *E. faecalis*. The null hypothesis (II) in which the concentration, volume, and final irrigation would not influence the disinfection against *E. Faecalis*, was partially accepted since the higher concentration of NaOCl demonstrated influence (CI/PUI 5,25). The successive changes of the irrigating liquid, during conventional irrigation with syringe and needle and PUI, were carried out, resulting in greater irrigating volume. However, the time of the final irrigation, at the end of the protocols, was not relevant to this study, it did not enhance the disinfection of the root canal system.

The increase in endodontic infections by bacterial colonies and also fungal pathogens, resistant to mechanical cleaning and the disinfection drugs of the root canal system, underlines the immediate need for new techniques that maximize disinfection. Microorganisms organized in biofilm, adhered to the root canal system walls and in anatomical complexities, acquire greater resistance, leading to unsuccessful endodontic treatment (Iandolo et al., 2019). Therefore, it is necessary to increase the effectiveness of disinfection protocols in endodontic therapy, to reduce the bacterial load as much as possible to provide repair conditions and consequent treatment success. Based on these assumptions, there was a need to test associated irrigation protocols, envisioning greater disinfection in endodontic treatments, as was done in the present study.

The bacterial species *E. faecalis* is the most common isolated in the root canals in cases of failure. This microorganism is a gram-positive coccus, which can tolerate adverse or extreme environmental conditions, and can adapt to

this environment, including alkaline pH (such as calcium hydroxide-based medication), dietary restrictions, adherence to canal walls, invasion of dentinal tubules, alteration of host defenses, and the ability to form solid biofilms. All of which allows this microorganism to be resistant against disinfection solutions and mechanical action of endodontic instruments (Reyhani et al., 2017).

Boutsioukis et al., 2010 demonstrated a better result in mechanical cleaning when the insertion of the needle with a blind end and lateral ventilation was inserted as close as possible to the WL. For additional safety, against extrusion of irrigants, it was agreed that 2 mm below the WL would be a safety margin and that it would still guarantee an adequate exchange of irrigants, which is consistent with what was done in the present study. The greater the volume of the irrigant, over a prolonged period, better result against adherent biofilms, which is also consistent with the present study, because in the sample groups that used the conventional irrigation technique associated with PUI, frequent replenishment of the irrigating liquid was carried out, thus using a larger volume, for an extended period (Boutsioukis et al., 2010).

Increasing the time and the volume of exposure of NaOCl to 2%, the interruption and dissolution of the biofilm increased significantly (Petridis, et al., 2019). The time and volume of NaOCl application must be considered to maximize the anti-biofilm irrigant efficiency and plan disinfection regimes directed against the remaining biofilms. The importance of applying a larger volume over a long period is consistent with the results shown in the present study, however, the concentration that obtained the best result in this study was NaOCl at 5.25%, which partially differs from the findings by Petridis et al., 2019.

The instrumented and irrigated group in a conventional way associated with PUI with 5.25% NaOCl (CI/PUI - 5,25) showed a significantly greater reduction in *E. faecalis* than in the group that received only CI and irrigation associated with PUI with 2.5% NaOCl (CI/PUI - 2.5), demonstrating that the higher concentration of NaOCl was more effective in reducing bacterial load. A high concentration and prolonged exposure to NaOCl are necessary for the elimination of *E. Faecalis* from the contaminated dentin (Retamozo, et al., 2010).

A statistical difference in the bacterial load reduction *ex vivo*, using NaOCl 5.25% compared to NaOCl 2.5%, was demonstrated in this study, showing that the concentration influences the greater efficacy. However, Verma et al., 2019 concluded that the use of NaOCl in lower concentrations (1%), is adequate, and higher concentrations may not provide any additional benefit. Currently, several concentrations of NaOCl are being used, ranging from 1% to 6%. The discrepancy in the results, together with the fact that the precise relationship between the amount of bacterial reduction, the periapical healing and the subsequent damage caused by NaOCl, referring to its antibacterial activity, physicochemical properties of dentin and cytotoxicity, still need to be elucidated, indicating that the role of NaOCl concentration in the endodontic result is still conflicting.

A final irrigation without agitation at the end of the protocols (2 or 5 min depending on the sample group) was not relevant in this study, it did not offer additional benefits or maximized the disinfection of the root canal systems. As a result, it was also possible to infer that both associated irrigation techniques were sufficiently capable of bacterial load reducing. The ultrasonic activation of the irrigant combined with the conventional irrigation method produces a cumulative effect over the cycles of ultrasonic activation (Van der Sluis, et al., 2010).

Considering the current knowledge concerning the factors: time, volume, and concentration of the irrigant it is plausible to report that successive replacements of the irrigating liquid, in high volumes, and high concentrations, over a prolonged period (Retamozo, et al., 2010; Del Carpio-Perochena, et al., 2011; Gazzaneo et al., 2019), demonstrate favorable results, which is consistent with the results of the present study. Future research is suggested to better elucidate such factors and final irrigation systems that can maximize root canal system disinfection.

5. Conclusion

Within the limitations of this *ex vivo* study, it was concluded that: conventional irrigation with syringe and needle associated with PUI, were sufficiently able to reduce the bacterial load of *E. faecalis*. The final irrigation time without agitation of the solution did not demonstrate relevance in this study. Successive replacement of the irrigating liquid, resulting in greater volume, demonstrated better results in disinfecting the root canal system. NaOCl 5.25% (CI/PUI - 5.25) showed a significantly greater reduction in *E. faecalis* than NaOCl 2.5% (CI/PUI - 2.5).

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