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GRASIELA SABRINA LONGHI GRÜNDLING

EFEITO DO ULTRASSOM NA LIMPEZA DE CANAIS RADICULARES DE DENTES BOVINOS INFECTADOS *IN VITRO* POR *ENTEROCOCCUS FAECALIS*

Prof. Dr. José Antonio Poli de Figueiredo

Orientador

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SUMÁRIO

RESUMO

Introdução: O propósito deste estudo foi avaliar, *in vitro*, o efeito da agitação do hipoclorito de sódio e do EDTA com o ultrassom, em canais radiculares de dentes bovinos infectados com *Enterococcus faecalis.* **Métodos:** Foram utilizados 84 incisivos bovinos, os quais foram inoculados com *E. faecalis*, permanecendo em cultivo por 50 dias para formação do biofilme. Os dentes foram divididos em quatro grupos: grupo 1, controle, não recebeu tratamento; grupo 2, irrigação ultrassônica passiva com água destilada; grupo 3, irrigação convencional com hipoclorito de sódio + EDTA; grupo 4, irrigação ultrassônica passiva com hipoclorito de sódio + EDTA. Foram realizados testes microbiológicos com contagem de unidades formadoras de colônias e análise em microscópio eletrônico de varredura (MEV). **Resultados:** No teste microbiológico, os grupos que utilizaram hipoclorito de sódio (grupos 3 e 4) não apresentaram crescimento bacteriano. Houve diferença significativa entre os grupos 1 e 2, e entre estes grupos e os grupos 3 e 4. Na análise em MEV, na luz do canal, não houve diferença significativa entre os grupos 3 e 4, porém estes foram diferentes dos grupos 1 e 2. O grupo 1 foi significativamente diferente do grupo 2. Na interface canal/túbulos dentinários não houve diferença significativa entre os grupos. **Conclusões:** O ultrassom pode ser um auxiliar na limpeza do canal radicular, porém, o papel principal na eliminação das bactérias é exercido pela solução irrigadora.

SUMMARY

Introduction: The purpose of this study was to evaluate *in vitro* the effect of the ultrasonic irrigation of sodium hypochlorite and EDTA in root canals of bovine teeth infected with *Enterococcus faecalis*. **Methods**: 84 bovine incisors were inoculated with *E. faecalis*, remaining in culture for 50 days for biofilm formation. The teeth were divided into four groups: group 1, control, received no treatment; group 2, passive ultrasonic irrigation with distilled water; group 3, conventional irrigation with sodium hypochlorite + EDTA; group 4, passive ultrasonic irrigation with sodium hypochlorite + EDTA. Microbiological tests and analysis in scanning electron microscopy (SEM) were performed. **Results:** In microbiological testing, groups utilizing sodium hypochlorite (groups 3 and 4) did not show bacterial growth. There were significant differences between groups 1 and 2, and between these groups and groups 3 and 4. In SEM analysis, at the canal wall area, there was no significant difference between the

groups 3 and 4, but these were different from groups 1 and 2. Group 1 was significantly different from group 2. At the exposed tubule area there was no significant difference between the groups. **Conclusions:** Passive ultrasonic irrigation can be an aid in cleaning the root canal; however, the main role in bacteria elimination is played by the irrigant.

Keywords: biofilm, *Enterococcus faecalis*, passive ultrasonic irrigation, scanning electron microscopy, sodium hypochlorite.

INTRODUÇÃO

Atualmente, são reconhecidas centenas de espécies bacterianas como habitantes normais da cavidade oral. Porém, as bactérias presentes em canais radiculares infectados representam um grupo restrito de espécies quando comparado a este número (SUNDQVIST, 1994). MILLER, há 115 anos, observou que dentes com a cavidade pulpar aberta apresentavam bactérias na câmara diferentes daquelas encontradas no interior do canal radicular. Ele foi o primeiro a descrever a presença de uma microbiota específica do interior do canal radicular.

A importância das bactérias e seus produtos na indução e, principalmente, na perpetuação de lesões periapicais foi descrita no importante estudo de SUNDQVIST (1976) em dentes humanos com polpa necrosada devido a traumatismos dentários. Os resultados deste estudo estão de acordo com os achados de KAKEHASHI et al., em 1965, os quais demonstraram que, após exposições pulpares experimentais, a necrose pulpar e a lesão periapical só ocorreram em ratos convencionais, e não em ratos *germ-free*, evidenciando o forte papel das bactérias no desenvolvimento das patologias periapicais.

A terapia endodôntica visa à eliminação dos microrganismos da cavidade pulpar, com consequente reparo da região periapical. Porém, algumas bactérias, como o *Enterococcus faecalis*, possuem certa resistência aos procedimentos endodônticos.

O *E. faecalis* é um coco Gram-positivo anaeróbio facultativo comumente encontrado em casos de insucesso na terapia endodôntica (HANCOCK et al., 2001; PINHEIRO et al., 2003; SUNDQVIST, 1998). Sua prevalência é maior em infecções persistentes que em infecções primárias (STUART et al., 2006). Isto pode ser explicado pela sua capacidade de suportar prolongados períodos com limitação de nutrientes, permitindo que ele persista como um patógeno no interior do canal radicular (FIGDOR et al., 2003). Alguns fatores de virulência do *E. faecalis* são de extrema importância para a sua patogenicidade, incluindo substâncias de agregação, feromônios, ácido lipoteicóico, produção de superóxido extracelular, enzimas líticas e citolisinas (KAYAOGLU & ØRSTAVIK, 2004). Além disso, possui a capacidade de facilmente invadir os túbulos dentinários (CHIVATXARANUKUL et al., 2008) e formar biofilme (DISTEL et al., 2002; ESTRELA et al., 2009).

Durante o tratamento endodôntico, uma das ferramentas utilizadas para o combate aos microrganismos é a solução irrigadora. Diversas soluções irrigadoras têm

sido recomendadas para o uso no tratamento das infecções do canal radicular. O hipoclorito de sódio tem sido amplamente utilizado desde a sua introdução na Endodontia por Walker em 1936. Além da sua ação alvejante, desodorizante e de dissolução de tecidos, o hipoclorito tem se mostrado um efetivo agente desinfetante (SIQUEIRA et al., 1997).

Segundo alguns estudos, o hipoclorito de sódio é eficaz na eliminação de *E. faecalis* do interior dos túbulos dentinários (SIQUEIRA et al., 1997; GOMES et al., 2001; OLIVEIRA et al., 2007). Outros estudos demonstraram existir uma interação entre tempo de ação e concentração do hipoclorito na eliminação do *E. faecalis* (RADCLIFFE et al., 2004; OLIVEIRA et al., 2007). Por outro lado, estudos de SIQUEIRA et al., 2000, concluíram que um grande volume de solução irrigadora pode manter a efetividade bacteriana do hipoclorito, compensando os efeitos da concentração.

A associação entre hipoclorito de sódio e ácido etilenodiaminotetracético (EDTA) tem demonstrado produzir uma ação bactericida mais forte que o uso do hipoclorito sozinho (BERUTTI et al., 1997), além de resultar em uma maior remoção de *smear layer* (KISHEN et al., 2008). O EDTA complementa a ação do hipoclorito através da quelação de íons cálcio da dentina e facilitando a instrumentação do canal. Por ser um agente quelante, não é dependente de uma alta concentração de íons hidrogênio para efetuar a descalcificação, e é efetivo em pH neutro (GUERISOLI et al., 2002). O uso de um agente quelante é importante para preparar a superfície do canal radicular para o hipoclorito, assim este pode exercer sua ação em profundidade, nos canais acessórios e no interior dos túbulos dentinários (BERUTTI et al., 1997).

Essas soluções, entretanto, devem estar em contato direto com as superfícies radiculares para uma ação efetiva (GUERISOLI et al., 2002). Devido ao pequeno diâmetro do canal e suas ramificações, torna-se difícil o agente irrigante ter ação em toda a região apical.

Diversos estudos têm sugerido a utilização do ultrassom como uma maneira de otimizar a ação da solução irrigadora (HUQUE et al., 1998; PLOTINO et al., 2007). O ultrassom foi introduzido na Endodontia por Richman em 1957, e a irrigação ultrassônica passiva (IUP) foi descrita pela primeira vez por Weller et al., em 1980.

A ativação ultrassônica de limas tem o potencial de preparar e limpar o canal mecanicamente (VAN DER SLUIS et al., 2007). O ultrassom é um ótimo auxiliar na limpeza de áreas anatômicas complexas. Tem sido demonstrado que uma irrigação conjunta com o ultrassom, o qual gera um movimento contínuo da solução irrigadora,

está diretamente associada a uma maior efetividade na limpeza do canal radicular (PLOTINO et al., 2007). Estudos comparando irrigação ultrassônica e irrigação com seringa, concluíram que a primeira foi mais efetiva na remoção de bactérias do interior do canal radicular (SPOLETI et al., 2003; VAN DER SLUIS et al., 2007).

O termo 'irrigação ultrassônica passiva' refere-se a uma ação sem corte da lima (VAN DER SLUIS et al., 2007). Esta pode ser realizada com lima fina (#10 – #20), vibrando livremente no interior do canal radicular, criando os efeitos de microfluxo acústico e cavitação no agente irrigante. O microfluxo acústico é o rápido movimento circular do fluido em torno da lima vibratória no interior do canal radicular. O fenômeno da cavitação pode ser descrito como a formação de cavidades em um líquido através de forças induzidas por correntes de alta velocidade. Estas bolhas expandem e rapidamente colapsam produzindo um foco de energia. (VAN DER SLUIS et al., 2007)

Analisando estes estudos, cabe a inferência de que o ultrassom poderia potencializar a ação bactericida do hipoclorito, e que juntamente com o EDTA, uma maior profundidade de ação da solução irrigadora seria alcançada.

Frente a essas questões, levando-se em consideração que o *E. faecalis* é um dos microrganismos mais comumente encontrado em casos de insucesso na terapia endodôntica, e que sua eliminação é de fundamental importância para o sucesso do tratamento, torna-se justificável a realização deste estudo, com o intuito de avaliar a participação do ultrassom, em acionamento passivo (através de lima de pequeno calibre), na eliminação deste patógeno do canal radicular.

Artigo

Effect of Ultrasonics on *Enterococcus faecalis* Biofilm in a Bovine Tooth Model

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SUMMARY

Introduction: The purpose of this study was to evaluate *in vitro* the effect of the ultrasonic irrigation of sodium hypochlorite and EDTA in root canals of bovine teeth infected with *Enterococcus faecalis*. **Methods**: 84 bovine incisors were inoculated with *E. faecalis*, remaining in culture for 50 days for biofilm formation. The teeth were divided into four groups: group 1, control, received no treatment; group 2, passive ultrasonic irrigation with distilled water; group 3, conventional irrigation with sodium hypochlorite + EDTA; group 4, passive ultrasonic irrigation with sodium hypochlorite + EDTA. Microbiological tests and analysis in scanning electron microscopy (SEM) were performed. **Results:** In microbiological testing, groups utilizing sodium hypochlorite (groups 3 and 4) did not show bacterial growth. There were significant differences between groups 1 and 2, and between these groups and groups 3 and 4. In SEM analysis, at the canal wall area, there was no significant difference between the groups 3 and 4, but these were different from groups 1 and 2. Group 1 was significantly different from group 2. At the exposed tubule area there was no significant difference between the groups. **Conclusions:** Passive ultrasonic irrigation can be an aid in cleaning the root canal; however, the main role in bacteria elimination is played by the irrigant.

Keywords: biofilm, *Enterococcus faecalis*, passive ultrasonic irrigation, scanning electron microscopy, sodium hypochlorite.

INTRODUCTION

Bacteria and their products are considered the main etiological agents of periapical lesions (1, 2). Endodontic therapy aims the elimination of these microorganisms from the root canal, with consequent repair in cases with periapical periodontitis. However, some bacteria such as *E. faecalis* possess some resistance to endodontic treatment.

E. faecalis is a Gram-positive anaerobic facultative coccus commonly found in cases of failure in endodontic therapy (3-5). Its prevalence is higher in persistent infections than in primary infections (6). This can be explained by its ability to withstand prolonged periods of nutrient limitation, allowing it to persist as a pathogen within the root canal (7).

Several irrigants have been recommended for use in the treatment of root canal infections. Sodium hypochlorite has been widely used since its introduction in endodontics by Walker in 1936 (8). Besides its bleaching, deodorant, tissue dissolution effect and low surface tension, sodium hypochlorite has been proven to be an effective disinfectant (9).

The association between sodium hypochlorite and ethylenediaminetetraacetic acid (EDTA) has shown greater bactericidal action than sodium hypochlorite alone (10), besides resulting in a greater removal of smear layer (11). These solutions, however, must be in direct contact with the surface of the root canal for effective action (12).

Due to the small diameter of the canal and its ramifications, it becomes difficult for the irrigant to reach the whole apical region. Several studies have suggested the use of ultrasonics as a means to enhance the action of the irrigant (13, 14), being a great help in cleaning complex anatomical areas.

The purpose of this study was to evaluate *in vitro* the effect of the agitation of the sodium hypochlorite and of the EDTA with ultrasonic in root canals of bovine teeth infected with *E. faecalis*.

MATERIALS AND METHODS

This study was submitted to the Science and Ethics Commission of the Dentistry Faculty of the Pontifical Catholic University of Rio Grande do Sul – PUCRS.

Obtaining and Preparation of the Teeth

Eighty-four bovine incisors, from animals killed for commercial reasons, were used. They were removed from the jaws immediately after the death of the animals and stored in vials containing sodium hypochlorite at 1% (Biodinâmica-Ibiporã, Brazil), for a period not exceeding 48 hours. Dental crowns and 1 mm from the apices were cut so that all the roots remained with 15 mm in length. With the objective of removing the pulp tissue, and to standardize the diameter of the canals, the roots were prepared up to the instrument #60 (Dentsply, Maillefer - Ballaigues, Switzerland), using irrigation with sodium hypochlorite at 2% (VirexPlus 2%, Johnson Diversey Brasil Ltda – São Paulo, SP, Brazil). Then, the roots remained immersed in EDTA 17% (Iodontosul – Porto Alegre, RS, Brazil) for 5 minutes under agitation for the removal of smear layer.

Each tooth was fixed in a plastic micro-tube (GenuineAxygenQuality – CA, USA) with cyanoacrylate (SuperBonder plastic glue - SP, Brazil), so that it remained upright, with the cervical portion facing upward. A hole was opened in the side of the micro-tube for exchange of the culture medium. The teeth were divided into four groups and each group was placed in a polypropylene box (Heathrow Scientific–Vernon hills, IL, USA). The samples were sterilized in autoclave (Dabi Atlante – Ribeirão Preto, SP, Brazil) for a period of 30 minutes.

Control of sterilization

A tooth from each group was subjected to the sterilization control. After sterilization of each polypropylene box containing the teeth, a sterile paper cone was put inside the root canal of one of the teeth in the box, and this cone was immediately inserted in a tube containing sterile saline solution at 0.85%. The material was homogenized and, after five minutes, an aliquot of 100 µL of the saline solution was cultivated, in duplicate, on blood agar and incubated for 18 to 24 hours at 37°C. The tooth utilized to perform the sterilization control was removed from the box, and 20 teeth remained in each box.

Cultivation and Preparation of the Inoculum

The strain used, *E. faecalis* (American Type Culture Collection - ATCC 29212), was obtained and cultivated in the Laboratory of Immunology and Microbiology of the Faculty of Biosciences of the Pontifical Catholic University of Rio Grande do Sul, Brazil.

The bacteria were cultivated in BHI (Brain Heart Infusion) broth for 18 to 24 hours, at 37°C, in bacteriological incubator.

The number of colony forming units (CFU/mL) of the inoculum was determined by counting the colonies on blood agar. For this, the culture of *E. faecalis* was diluted serially up to 10^{-8}, in saline solution at 0.85%, and 100 µL of the dilutions 10^{-6}, 10^{-7} and 10^{-8} were cultivated on blood agar with the aid of a Drigalski handle in duplicate. The plates were incubated at 37ºC for 24 hours, and after that period, a count of the CFU/mL of the plates that grew from 15 to 150 colonies was performed. The results varied from 3.9 to 6.3 x 10^{7}.

In each of the 80 samples previously sterilized, 100 µL of the culture of *E. faecalis* were inoculated inside the root canal. After this procedure, the sterile BHI was added into the micro-tube, so that it was completely filled with the culture medium. The culture of *E. faecalis* was maintained for 50 days for the formation of biofilm, with the renewal of one third of the BHI every 2 days. All manipulation of the teeth was performed under aseptic conditions in a laminar flow hood. Once a week, an aliquot of BHI from the teeth was submitted to Gram staining and cultured on blood agar followed by catalase and esculin tests to verify the absence of contamination.

Classification of the Groups

The working length was established at 14 mm. The roots were mounted on a basis of utility wax (Wilson, Polidental – Cotia, SP) in order to avoid extravasation of the irrigant. Group distribution was made as follows:

- Group 1 (positive control): the root canal was filling with sterile saline solution, agitation with hand file for 15 seconds and collected the material. Counting of CFU's of this group served as basis for comparison with other groups, being considered the pre-treatment counting.
- Group 2: the treatment was performed according to the flowchart in Figure 1 (taking as example group 4), but the irrigants were replaced by distilled water in order to evaluate only the action of the ultrasonics. The irrigation was done with disposable sterile syringe (Descarpack – São Paulo, SP, Brazil). The manual agitation was performed with a K #40 file (Dentsply, Maillefer - Ballaigues, Switzerland) at the working length, for 15 seconds. The passive ultrasonic irrigation (PUI) was performed with Nac Plus ultrasonics (Adiel – Ribeirão Preto, SP, Brazil), using the scale power 2 for endodontics. A K #40 file with cut handle and coupled with the ultrasound via an adapter (QuickEndHolder, Adiel - Ribeirão Preto, SP, Brazil) was utilized.
- Group 3: the treatment was performed according to the flowchart of Figure 1. Sodium hypochlorite (NaOCl) at 2% (Virex Plus 2%, JohnsonDiversey Brazil Ltda – São Paulo, SP, Brazil) was used, with sterile disposable syringe. EDTA solution at 17% (Iodontosul – Porto Alegre, RS, Brazil) was

used. The manual agitation was done with a K #40 file, at the working length for 15 seconds.

- Group 4: the treatment was performed according to the flowchart in Figure 1. Sodium hypochlorite (NaOCl) at 2% was used, with sterile disposable syringe. EDTA solution at 17% was used. The manual agitation was done with a K #40 file, at the working length for 15 seconds. The passive ultrasonic irrigation (PUI) was performed following the same specifications described for group 2.

The flowchart shown below (Figure 1) illustrates the sequence of procedures performed, using as example the test groups 3 and 4.

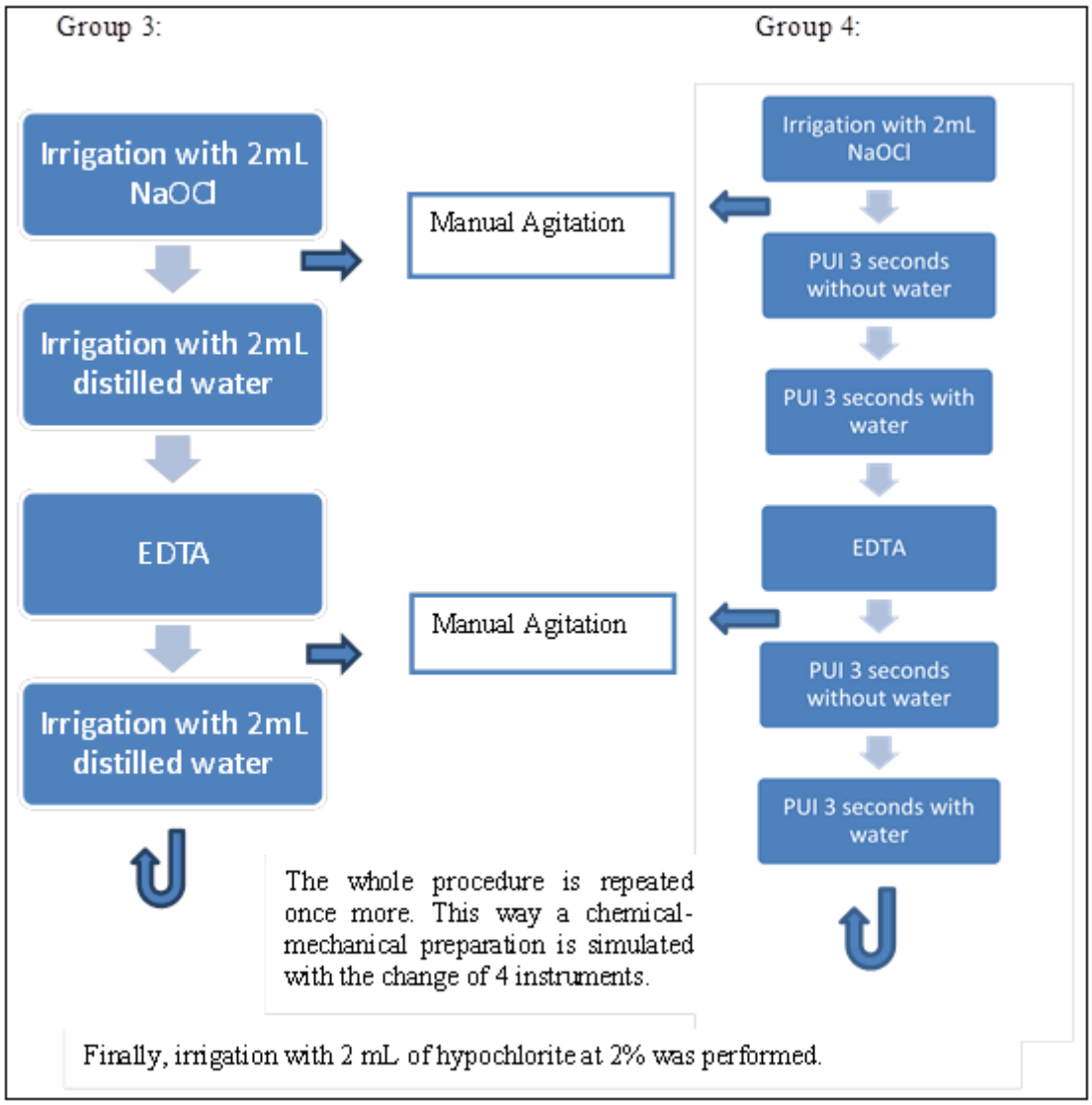

Figure 1: flowchart of the treatments performed in the groups 3 and 4.

Microbiological analysis

After the respective treatments, 10 teeth were immediately immersed in the fixation solution (described below) and were utilized for analysis in electron microscopy scan. The 10 remaining teeth were used for microbiological analysis. After closing the treatment, the canal was immediately filled with sterile saline solution, which was stirred with a file number 40 (Dentsply, Maillefer - Ballaigues, Switzerland) for 15 seconds. An aliquot of 50 µL of the solution was removed from the canal and transferred to a tube containing 450 µL of sterile saline solution at 0.85%. The material was homogenized and diluted to 10^{-3}. Aliquots of 100 µL of the solution and the dilutions were cultivated on the surface of the blood agar, in duplicate, with the aid of a Drigalsky handle, being incubated for 18 to 24 hours at 37°C. After the incubation period, was performed the counting of the number of colony-forming units of the plates that had between 15 and 150 colonies.

Preparation for SEM

The scanning electron microscopy (SEM) was performed at the Center for Electron Microscopy and Microanalysis at the Pontifical Catholic University of Rio Grande do Sul.

The roots were fixed for 7 days in 2.5% glutaraldehyde and then washed three times for 30 minutes each in 0.2 M phosphate buffer and distilled water in a ratio of 1:1. Then the samples were dehydrated by immersion in acetone 30, 50, 70, 90 and 100%. Longitudinal grooves were carved on the free surfaces of the roots with a diamond saw (Dhpro, Rhadartrade – Paranaguá, PR), taking care not to invade the inner part of the root canal. The complete fracture was made with chisel and hammer, providing two halves of each sample, which were placed on stubs with the portion of the root canal positioned upward. Then the samples were coated with gold-palladium for conducting electrons.

The evaluation was made in a scanning electron microscope (Philips XL 30, Eindhoven, Netherlands) with an increase from 500 to 20000x, dividing the root into thirds. Beginning in a smaller increase, selecting the areas with the highest concentration of bacterial biofilm, the recordings were made at 5000x.

Image acquisition was performed through emission of secondary electrons (SE) and backscattering (BSE) for the presence of bacteria.

In possession of images, a single observer blinded as to the experimental groups, classified them by the presence of bacteria through a position rank. Adopting the PowerPoint program, each image occupied a slide, which was transferred to the computer screen in the form of presentation. Following, the images were modified in position according to the level of contamination found, so that number 1 was the most contaminated and number 40 the least contaminated. This classification by rank was performed on each third (coronal, middle and apical) e by the location of the image (canal wall and exposed tubule area). Then, for each third and for each image location, the average position of the group was calculated.

Data analysis

To detect a difference of at least 1.5 standard deviation units between the mean scores observed in the groups, reaching a statistical power of 90% with a significance level of 5%, a need for 10 experimental units by group was estimated.

Data on levels of contamination measured with scanning electron microscope were ranked within thirds. In the microbiology evaluation all data were log transformed. One-way ANOVA was applied on these data followed by Tukey´s post hoc procedure. The level of significance was set to $\alpha=0.05$. Data were analyzed using SPSS version 17.0.

RESULTS

Microbiological analysis

Group 1 showed an average count of 6.35 $\log_{10}$ CFU/mL, and group 2 of 4.68 $\log_{10}$ CFU/mL, being statistically different from each other and different from groups 3 and 4. Groups 3 and 4 showed no bacterial growth.

Analysis in SEM

Table 1 shows the results according to the position ranks.

The different methods of image acquisition (SE and BSE) showed very similar results. In the canal wall, there were no significant differences between groups 3

(conventional irrigation) and 4 (PUI – Figure 4) in any of the thirds observed. However, there were significant differences between these groups and the control group (Figure 2) in all thirds. There was also a significant difference between group 2 (PUI + distilled water – Figure 3) and the control group in the apical and middle thirds and between group 2 and groups 3 and 4 in the cervical and middle thirds.

There were no significant differences between groups at the exposed tubule area.

DISCUSSION

The model of biofilm formation used in this study had already been reported in several studies that had as purpose the study of antimicrobial strategies. However there is still no consensus in the literature regarding the time of formation of this biofilm. Some studies used 24 hours (15-18), others 48 hours (19), 72 hours (20), 21 days (21), and even 6 weeks (22). In the latter, the times of 3 and 6 weeks were used, although with a greater time for biofilm formation, the elimination of bacteria was lower. For this reason, in the present study, the option was made for a time of 50 days of biofilm formation, believing that, this way, the biofilm would be better structured and more aggregated, better mimicking the clinical situation.

If there are no differences between the physical properties of bovine dentin and dentin of human permanent teeth (23), the option for the use of bovine teeth was made in order to facilitate the viewing by SEM. Moreover, it is possible to obtain samples of similar age and dentin characteristics, making it possible to distribute the teeth from the same animal in several experimental groups, which reduces the variables that happen in clinical research.

By allowing the display of the amount and distribution of bacteria on the surface of the biofilm, the use of SEM in the observation of this bacterial aggregate is usual in the literature (13, 18-20, 24). However, it is very debatable for not demonstrating the viability of these bacteria. In order to complement the data obtained in Scanning Electron Microscopy, the microbiological examination was performed, enabling the counting of the colony-forming units.

The use of ultrasonics as an aid in root canal irrigation has been suggested as an alternative to increase cleaning and disinfection of the root canal system (14, 25-27). However, in this study, no significant differences were found between the groups that used sodium hypochlorite with or without ultrasonic agitation. Nevertheless, at the

canal wall, these groups were different from the control group. At the canal wall, there was also a significant difference between the group that used PUI + distilled water and the control group, demonstrating the cleaning effect of the ultrasonics without the aid of disinfectant solutions. There were also differences between the group receiving PUI + distilled water and the groups using NaOCl (with or without ultrasound) in the middle and cervical thirds, largely due to the action of hypochlorite. Although group 2 did not use hypochlorite, the apical third displayed the same results as the groups where NaOCl was utilized, probably because of the cleaning action performed by the ultrasonics in that third. At the exposure tubule area, no differences were found between groups, which points to the need of using an intra-canal medication, whereas none of the methods was able to eliminate the bacteria present there.

A size 40 file was used to perform ultrasonic irrigation. It was considered passive, since the root canals of the bovine teeth are very large, and this instrument would not touch the canal wall, similarly to a size 15 instrument in human teeth. It could be speculated that active ultrasonic irrigation could have performed differently, but this was not within the scope of the present study.

The microbiological test confirmed the findings of the electron microscopy in the case of the canal wall. However, as the protocol used for this test the microbiological collection was performed immediately after treatment, the collected material came only from the main canal, not being possible to assess the presence of bacteria in the dentinal tubules. In the results of the microbiological tests, sodium hypochlorite with or without ultrasonic irrigation, has shown to be totally effective in eliminating the bacteria from the main canal. Ultrasonic irrigation with distilled water showed statistically significant decrease in bacterial count, when compared with the control group, demonstrating that ultrasonics has cleansing effect, but it has to be enhanced by a disinfectant.

These results are consistent with the findings of SIQUEIRA (9) and BHUVA (20), who also found no difference between conventional irrigation with sodium hypochlorite and the ultrasonic passive irrigation using this irrigant. Together with that, ultrasonic irrigation is not able to avoid typical problems present in root canal instrumentation, such as extrusion of debris (28).

The efficacy of ultrasonic passive irrigation in cleaning areas unreachable by endodontic instruments has been tested in other studies using simulated lateral canals (27, 29) and irregularities (30) created in human teeth. The artificial production of these

inaccessible areas may help to explain the superiority of ultrasonic irrigation found in these studies, since irregularities or artificially created lateral canal are larger than dentinal tubules, which favors the action of the irrigating solution and of the ultrasonics. Moreover, these studies did not assess the bacterial elimination, only the penetration of the irrigant (27, 29) and the removal of debris (30), by low magnification microscope. In the present study, the proposed treatments were not effective in eliminating bacteria from inside the dentinal tubules.

Considering such results, we believe that ultrasonics can be an aid in root canal cleaning, but the main role in the elimination of bacteria is carried by the irrigant. More studies are needed to assess the removal of bacteria from dentinal tubules.

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Table 1– Comparison of contamination levels between different cleaning treatments applied on incisive bovine root canal

Variable	Control n=10	US+W n=10	Hy n=10	US+Hy n=10	P
Canal Wall BSE					
Apical third	6.80±5.33[a]	19.90±8.65[b]	26.70±9.68[b]	28.60±8.41[b]	<0.001
Medium third	6.50±4.81[a]	16.50±6.21[b]	29.50±7.61[c]	29.50±7.56[c]	<0.001
Coronal third	8.30±6.53[a]	15.70±9.12[a]	31.10±9.05[b]	26.90±4.82[b]	<0.001
Canal Wall SE					
Apical third	6.80±5.33[a]	19.60±7.65[b]	26.60±3.34[b]	29.0±8.21[b]	<0.001
Medium third	6.50±4.81[a]	16.50±6.21[b]	29.50±7.61[c]	29.50±7.56[c]	<0.001
Coronal third	8.30±6.53[a]	15.70±9.12[a]	31.10±9.05[b]	26.90±4.82[b]	<0.001
Exposed Tubules BSE					
Apical third	16.22±10.91[*]	20.70±10.56	22.80±10.83	19.90±13.81	0.669
Medium third	12.70±10.11	22.00±8.77	23.50±12.39	23.80±12.93	0.103
Coronal third	15.00±9.68	19.60±12.14	26.10±12.65	21.30±11.00	0.204
Exposed Tubules SE					
Apical third	15.78±10.41[*]	20.67±10.55[*]	22.00±10.54	19.30±13.38	0.673
Medium third	12.20±11.02	21.70±8.73	24.00±12.01	24.10±12.00	0.066
Coronal third	14.40±10.27	19.70±11.98	26.20±12.52	21.70±10.33	0.153

Data are presented as mean ranks ± standard deviation within thirds. US+W: Ultrasonics and distilled water; Hy: Sodium hypochlorite; US+Hy: Ultrasonics and sodium hypochlorite. BSE: Backscattering scanning electron microscopy, SE: Secondary electrons scanning electron microscopy. [*]: sample size reduced to n=9. P: significance using ANOVA on ranks. Different index letters represent statistical significant different at the post-hoc procedure (Tukey test).

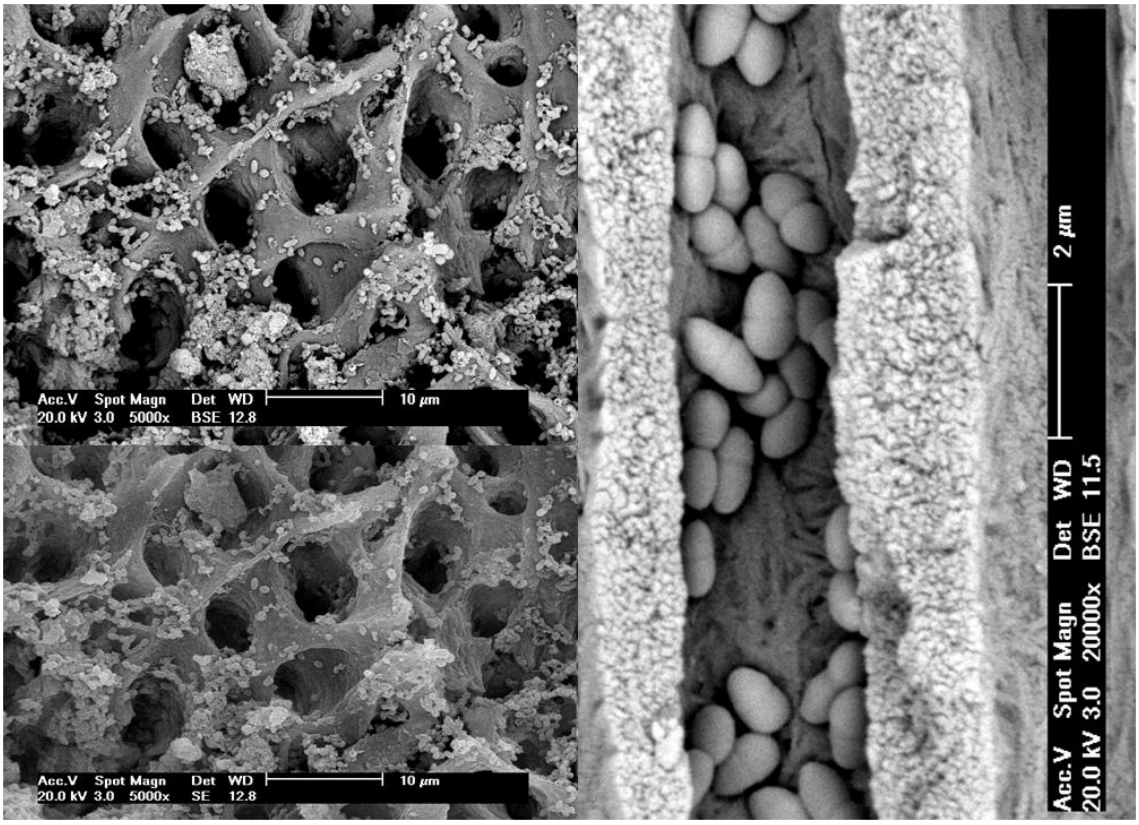

Figure 2 – Control group displaying biofilm of *E faecalis* in SEM views in the apical third (canal wall top left 5000x Basckattering - BSE; bottom left 5000x Secondary electrons – SE; right 20000x exposed tubule area BSE). Please note that identification of bacteria x debris becomes easier in BSE view.

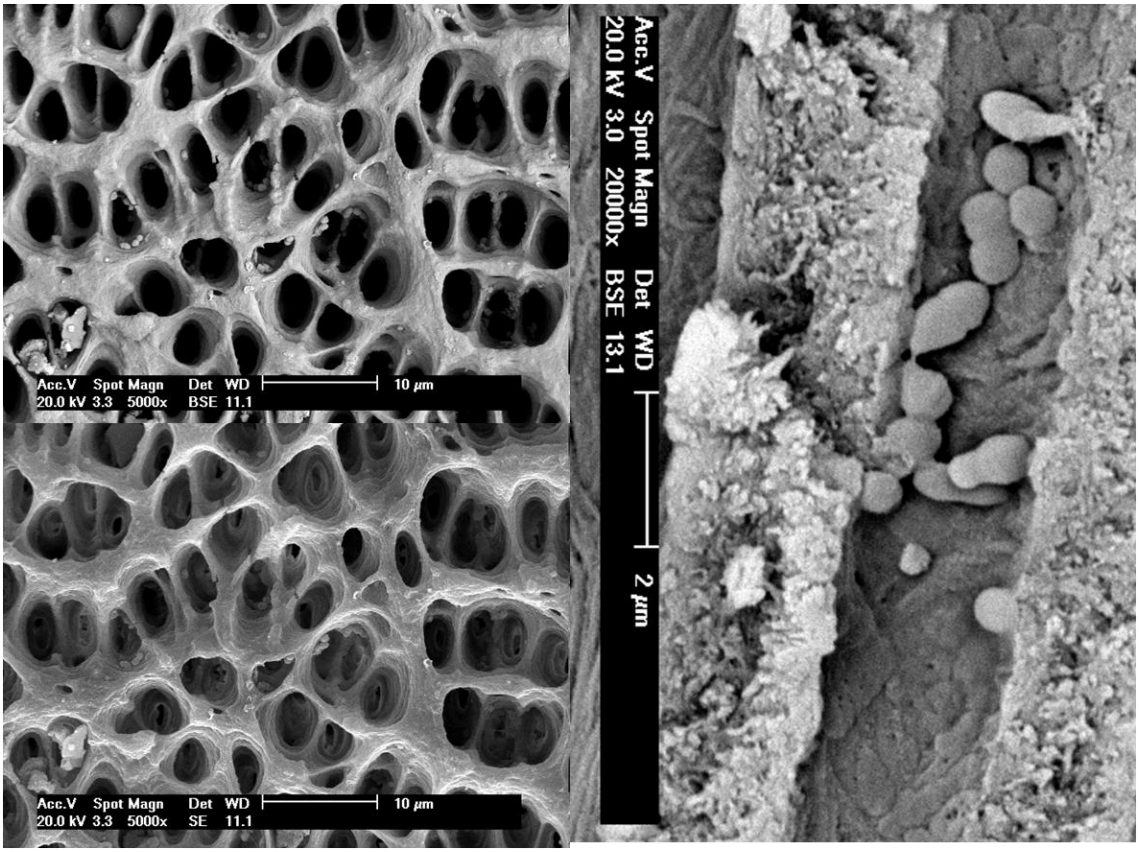

Figure 3 – US + distilled water group displaying disperse *E faecalis* in SEM views in the apical third (canal wall top left 5000x Basckattering - BSE; bottom left 5000x Secondary electrons – SE; right 20000x exposed tubule area BSE). Please note that identification of bacteria x debris becomes easier in BSE view.

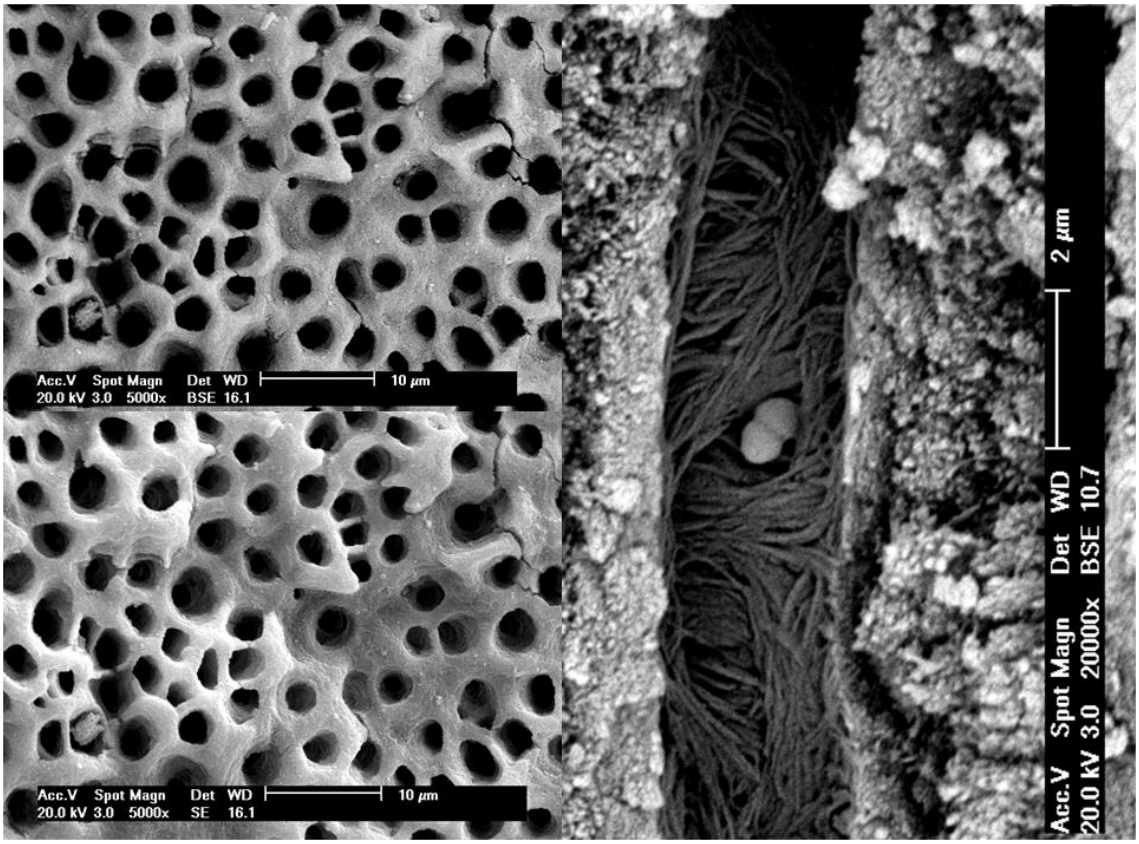

Figure 4 – US + NaOCl group displaying clean canal walls but *E faecalis* still present in tubule area in SEM views in the apical third (canal wall top left 5000x Basckattering - BSE; bottom left 5000x Secondary electrons – SE; right 20000x exposed tubule area BSE).

DISCUSSÃO GERAL

O principal objetivo do tratamento endodôntico é a eliminação dos microrganismos presentes na cavidade pulpar. Dentre estes microrganismos, o *E. faecalis* é o mais comumente encontrado em casos de insucesso da terapia endodôntica (PINHEIRO et al., 2003). É uma bactéria altamente resistente aos procedimentos endodônticos, podendo permanecer viável mesmo após prolongados períodos com limitação de nutrientes (FIGDOR et al., 2003). Sua eliminação é de fundamental importância para o sucesso do tratamento. Diversos estudos já utilizaram o modelo de formação de biofilme de *E. faecalis* (LIU et al., 2010; BHUVA et al., 2010; SOARES et al., 2010; PRABHAKAR et al., 2010). No presente trabalho, optou-se por um tempo de formação de biofilme de 50 dias, por se acreditar que, desta maneira, o biofilme estaria melhor estruturado e mais agregado, mimetizando melhor a situação clínica.

Não havendo diferenças de propriedades físicas entre a dentina bovina e a dentina humana de dentes permanentes (SCHILKE et al., 1999), optou-se pelo uso de dentes bovinos, a fim de facilitar a visualização em microscopia eletrônica de varredura (MEV). Além disso, torna-se possível a obtenção de amostras similares em idade e propriedade de dentina, sendo possível distribuir elementos dentários de um mesmo animal para vários grupos experimentais, o que reduz as variáveis existentes em pesquisas clínicas.

A utilização da MEV é comum na literatura, visto que possibilita uma avaliação qualitativa e quantitativa dos tratamentos propostos. Apesar de não ser possível demonstrar a viabilidade das bactérias, a quantidade e distribuição destas na superfície do biofilme podem ser comparadas entre os tratamentos propostos. Buscando uma melhor qualidade das imagens, estas foram captadas através da emissão de elétrons secundários e *backscattering*, não apresentando diferença nos resultados entre os métodos. Com a finalidade de complementar os dados obtidos na MEV, realizou-se o exame microbiológico, possibilitando a contagem de unidades formadoras de colônias.

O uso do ultrassom como auxiliar na irrigação do canal radicular vem sendo sugerido como alternativa para uma maior limpeza e desinfecção do sistema de canais radiculares (PLOTINO et al., 2007; VAN DER SLUIS et al., 2007; GREGORIO et al., 2009). Seus efeitos de microfluxo acústico e cavitação sobre o agente irrigante exercem um papel importante na sua eficácia. Porém, neste estudo, não encontramos diferenças

significativas entre os grupos que utilizaram hipoclorito de sódio com ou sem agitação ultrassônica (grupos 3 e 4). Estes resultados podem ser devido ao modelo de dentes bovinos utilizado, os quais apresentam túbulos dentinários maiores (TAGAMI et al., 1990), o que facilita a ação do agente irrigante. Outro fator que pode ter influenciado nos resultados é o protocolo de irrigação com seringa utilizado, o qual foi realizado de maneira abundante (2 mL por ciclo de irrigação) e com movimentos de vai-e-vem, o que também favorece a ação do irrigante.

Na luz do canal, os grupos 3 e 4 foram diferentes do grupo controle. Além disso, esses grupos foram diferentes do grupo que utilizou IUP + água destilada (grupo 2) nos terços médio e cervical, certamente devido à ação do hipoclorito. Apesar do grupo 2 não ter utilizado hipoclorito, no terço apical este grupo apresentou os mesmos resultados dos grupos em que o irrigante foi utilizado, provavelmente devido à ação de limpeza exercida pelo ultrassom neste terço. Também houve diferença significativa entre o grupo 2 e o grupo controle, demonstrando o efeito de limpeza do ultrassom sem o auxílio de uma substância irrigadora desinfetante.

O teste microbiológico confirmou os achados da microscopia em se tratando da luz do canal. Porém, como no protocolo utilizado para este teste, a coleta microbiológica foi realizada imediatamente após o tratamento, não foi possível avaliar a presença de bactérias nos túbulos dentinários. Nos resultados dos testes microbiológicos, o hipoclorito, com ou sem agitação ultrassônica, demonstrou ser totalmente eficaz na eliminação bacteriana da luz do canal. A agitação ultrassônica com água destilada apresentou redução bacteriana estatisticamente significativa em comparação com o grupo controle, demonstrando que o ultrassom apresenta efeito de limpeza, porém, este deve ser potencializado pela solução irrigadora.

Os resultados encontrados para a interface canal/túbulos dentinários foram discrepantes. Estes achados podem ser resultado do tamanho dos túbulos dentinários, que favorecem a penetração do biofilme bacteriano. Porém, também nos alerta para a necessidade da utilização de uma medicação intra-canal, visto que nenhum dos métodos foi capaz de eliminar as bactérias ali presentes.

Os resultados deste estudo estão de acordo com outros estudos já relatados na literatura (SIQUEIRA et al., 1997; BHUVA et al., 2010), os quais também não encontraram diferença entre a irrigação convencional com hipoclorito de sódio e a irrigação ultrassônica passiva com o irrigante. Outros estudos demonstraram superioridade da irrigação ultrassônica (GREGORIO et al., 2009 e 2010; RÖDIG et al.,

2010), porém nestes estudos não foi avaliada a penetração em túbulos dentinários, e sim, em irregularidades criadas artificialmente, as quais são maiores, favorecendo a ação da solução irrigadora e do ultrassom. Além disso, estes estudos não avaliaram a eliminação bacteriana, apenas a penetração do irrigante (GREGORIO et al., 2009 e 2010) e a remoção de debris (RÖDIG et al., 2010), através de microscópio de pequeno aumento.

Spoleti et al., em 2003, apesar de terem observado uma redução de colônias sobreviventes quando o ultrassom foi utilizado, nenhuma técnica foi capaz de promover uma completa desinfecção do canal radicular sem o uso de uma solução antimicrobiana, concordando com os achados deste estudo.

Frente a esses resultados, acreditamos que o ultrassom pode ser um auxiliar na limpeza do canal radicular, porém, o papel principal na eliminação das bactérias é exercido pela ação antimicrobiana da solução irrigadora. Mais estudos são necessários para avaliar a remoção de bactérias do interior dos túbulos dentinários.

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ANEXO A: FIGURAS

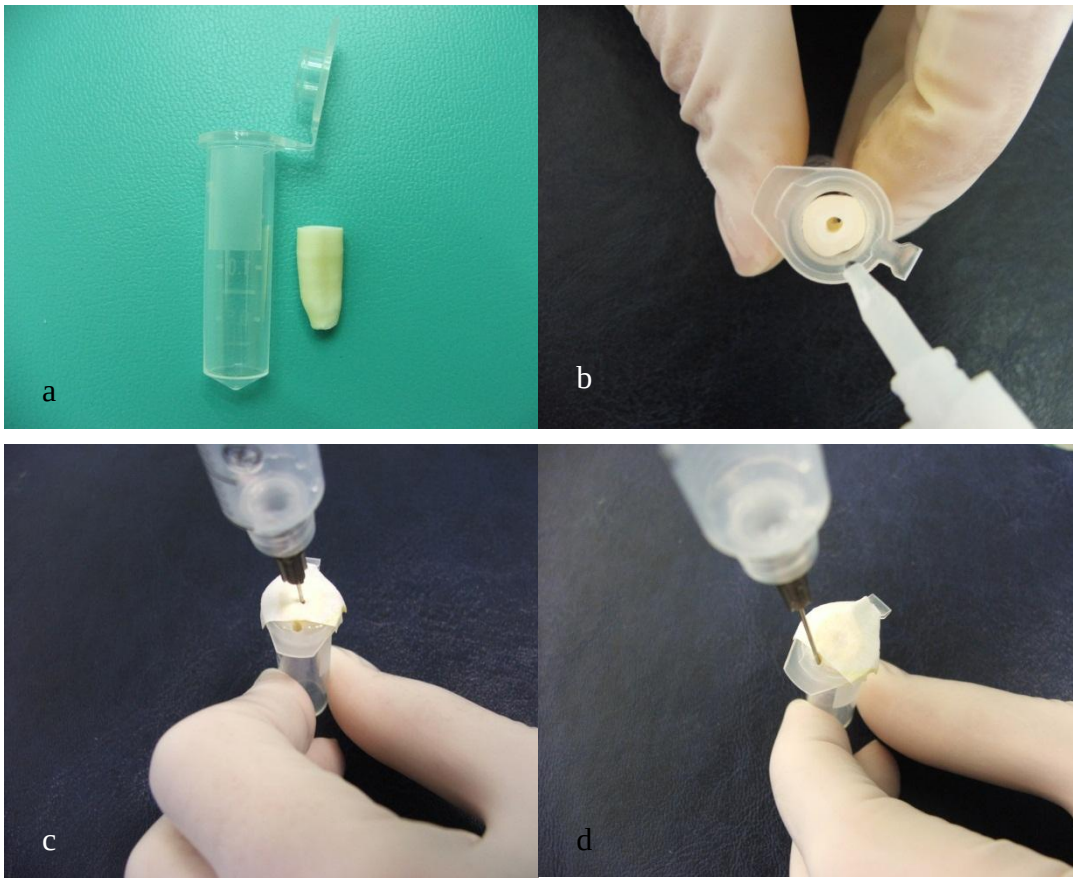

Figura 1: a e b) sequência de montagem do dente no microtubo. c) simulação para demonstrar a inoculação do *E. faecalis*. d) simulação para demonstrar a troca do meio de cultura.

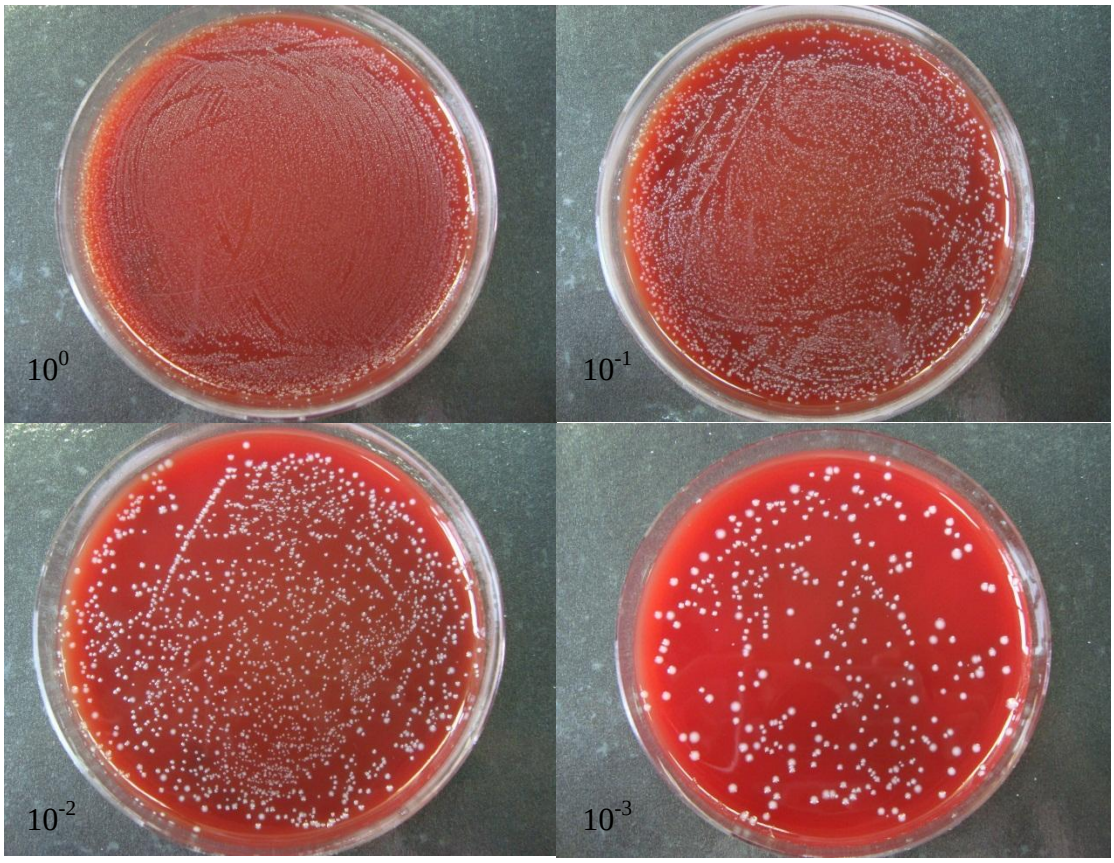

Figura 2: sequência de diluição do grupo controle.

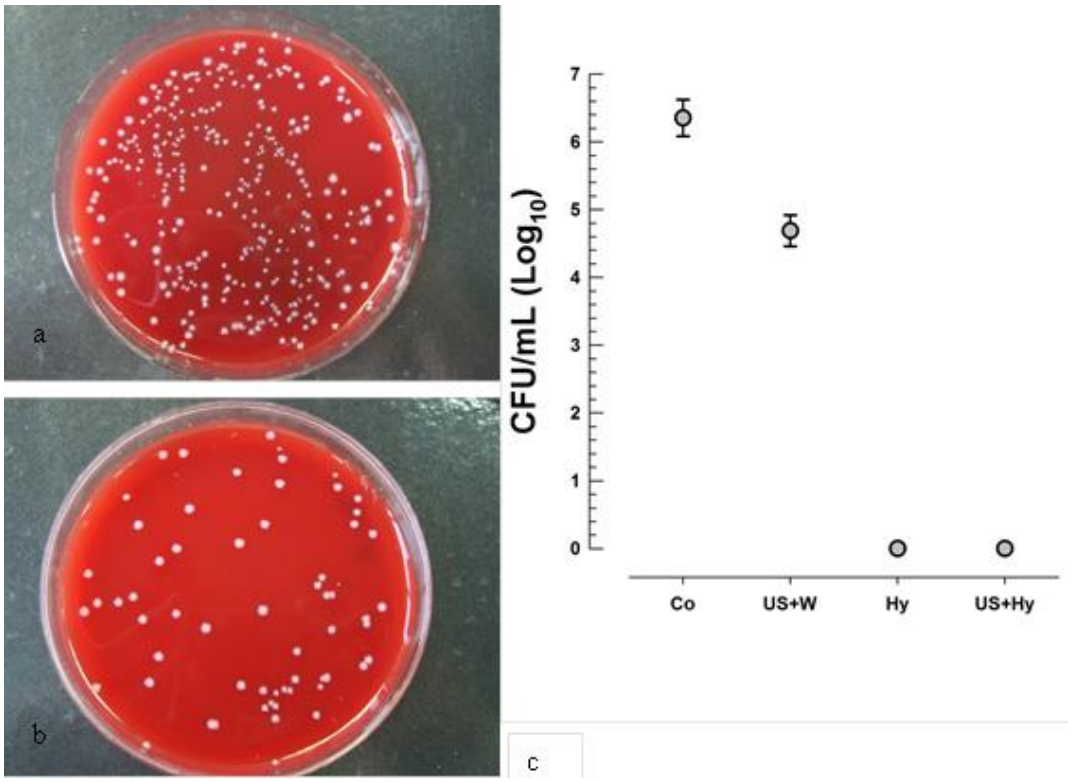

Figura 3: resultados dos testes microbiológicos. a) Grupo 1 (controle), diluição 10^{-3}. b) Grupo 2 (US + água destilada), diluição 10^{-2}. c) Gráfico dos resultados dos testes microbiológicos. Co: Control; US+W: Ultrasonics and distilled water; Hy: Sodium hypochlorite; US+Hy: Ultrasonics and sodium hypochlorite.

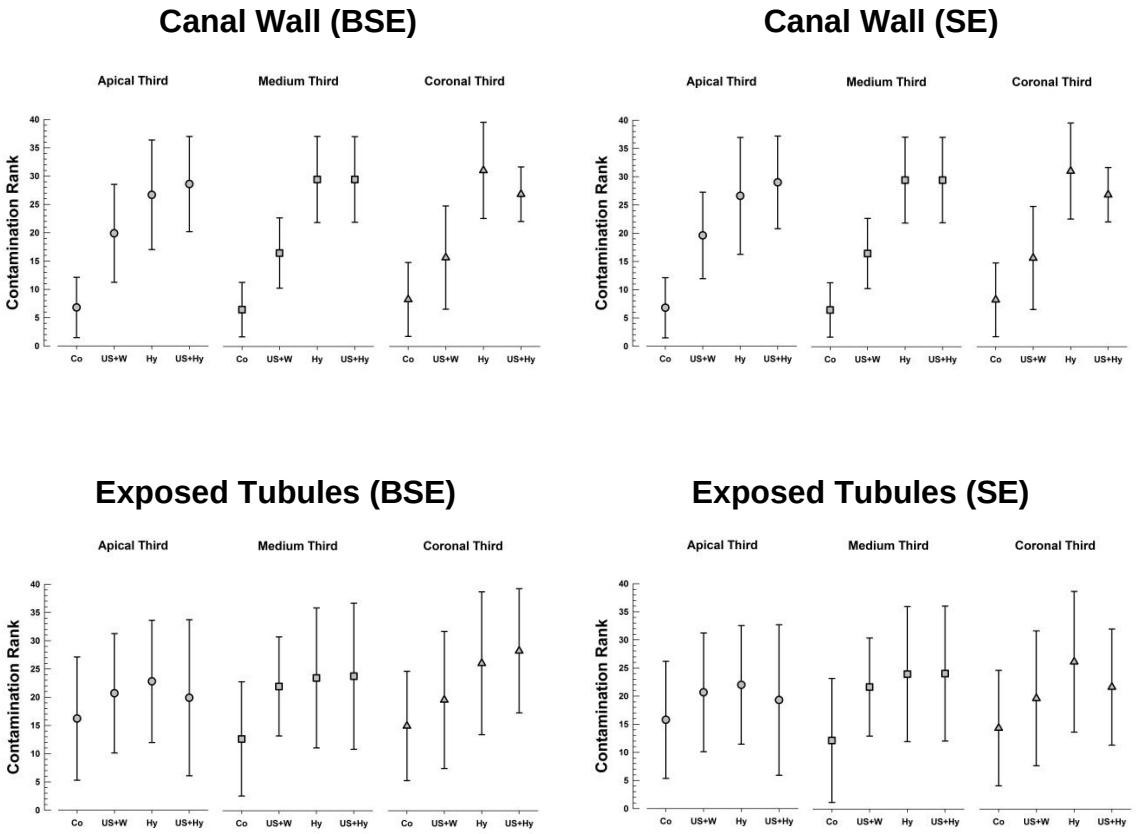

Figura 4: níveis de contaminação. Co: Control; US+W: Ultrasonics and distilled water; Hy: Sodium hypochlorite; US+Hy: Ultrasonics and sodium hypochlorite. BSE: Backscattering scanning electron microscopy, SE: Secondary electrons scanning electron microscopy.

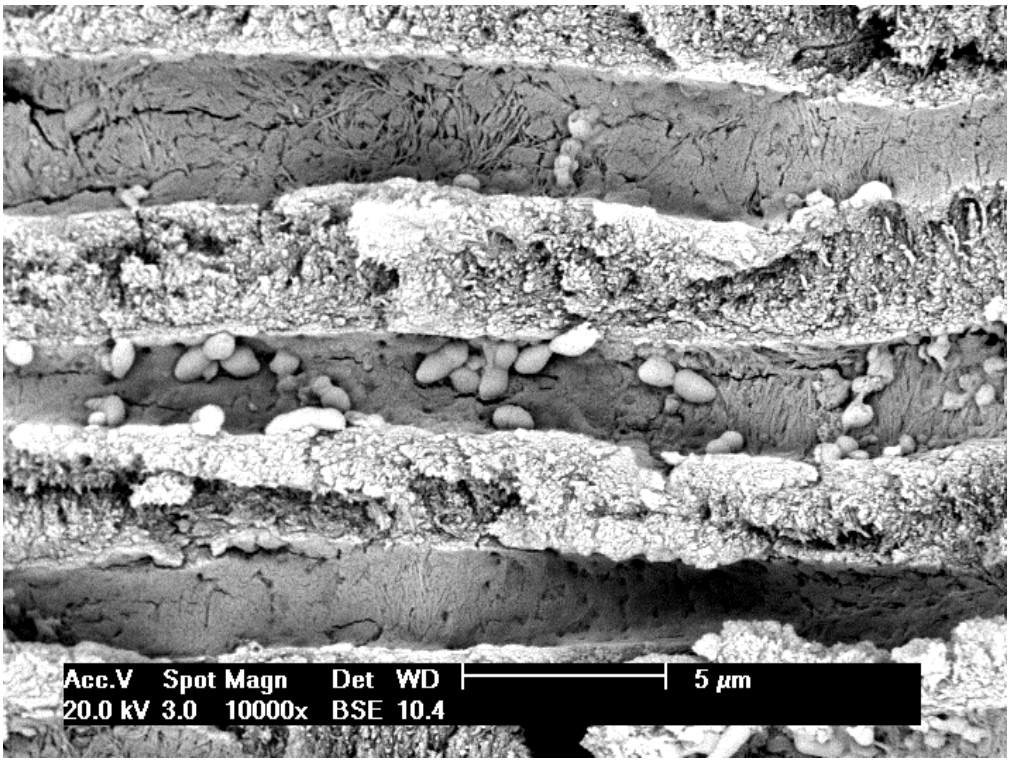

Figura 5: interior do túbulo dentinário contendo *E. faecalis* (grupo controle).

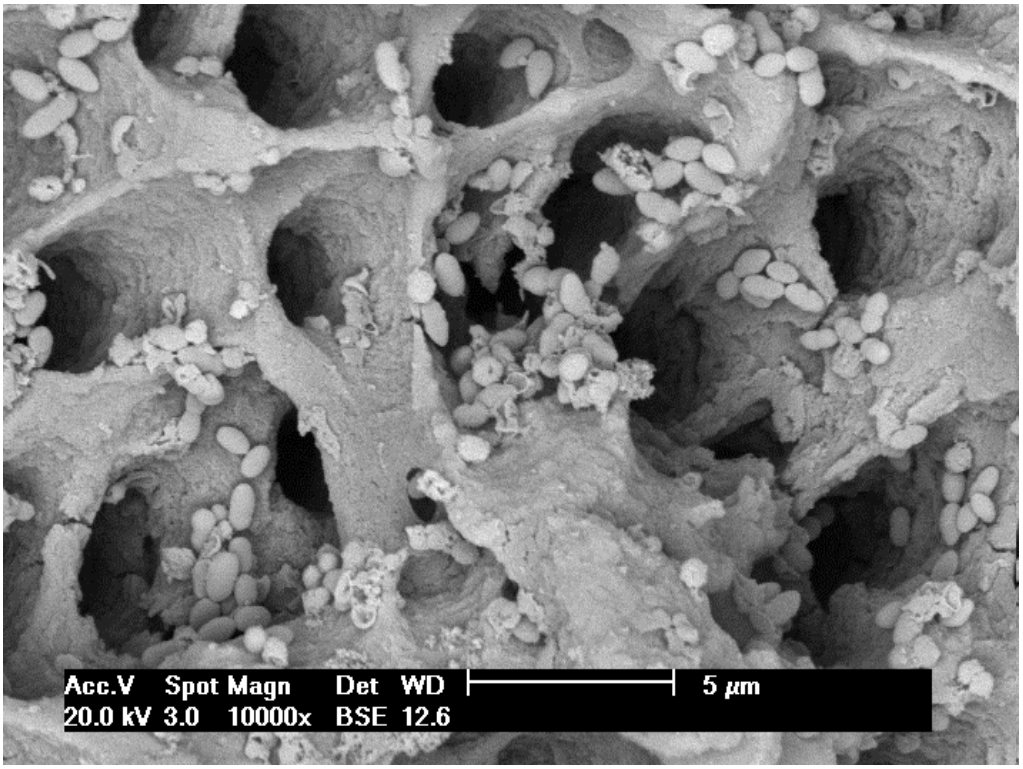

Figura 6: imagem da luz do canal contendo *E. faecalis* (grupo controle).

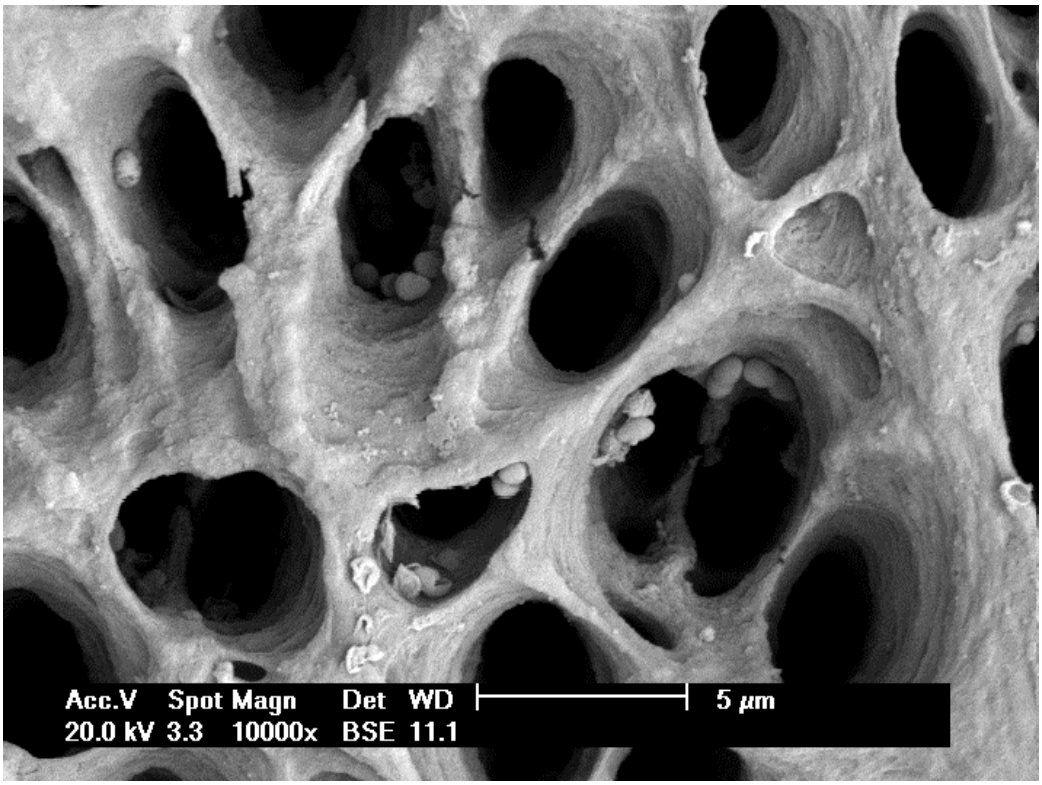

Figura 7: imagem da luz do canal (ultrassom + água destilada).

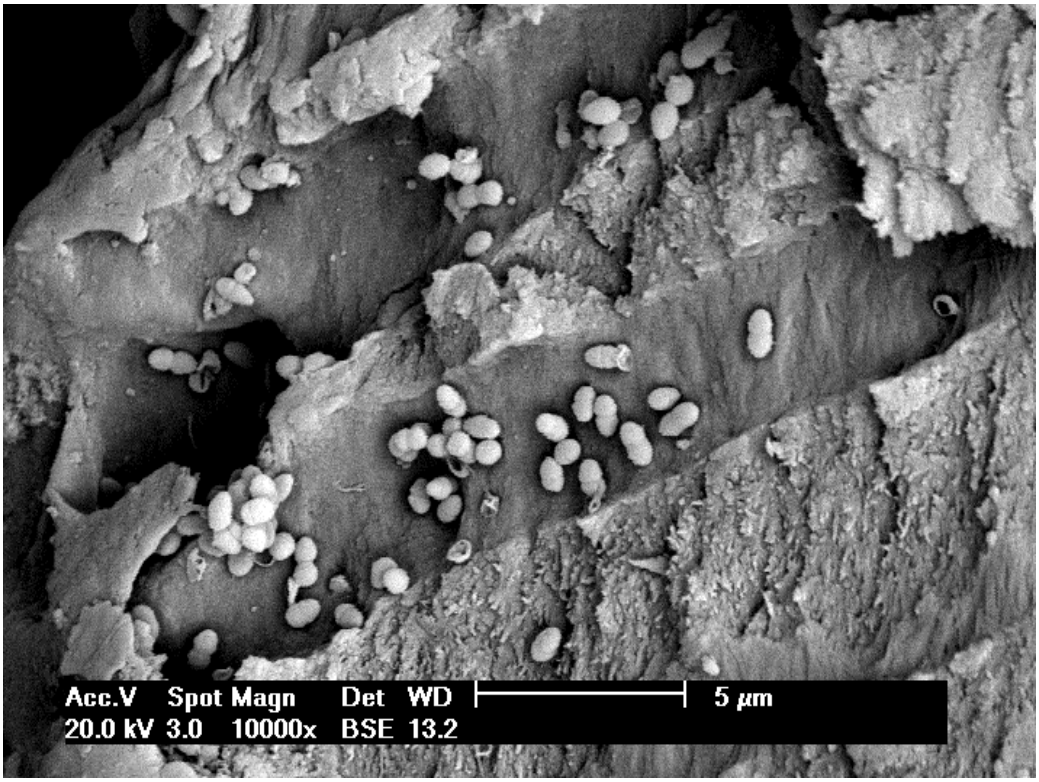

Figura 8: túbulos dentinários (ultrassom + água destilada).

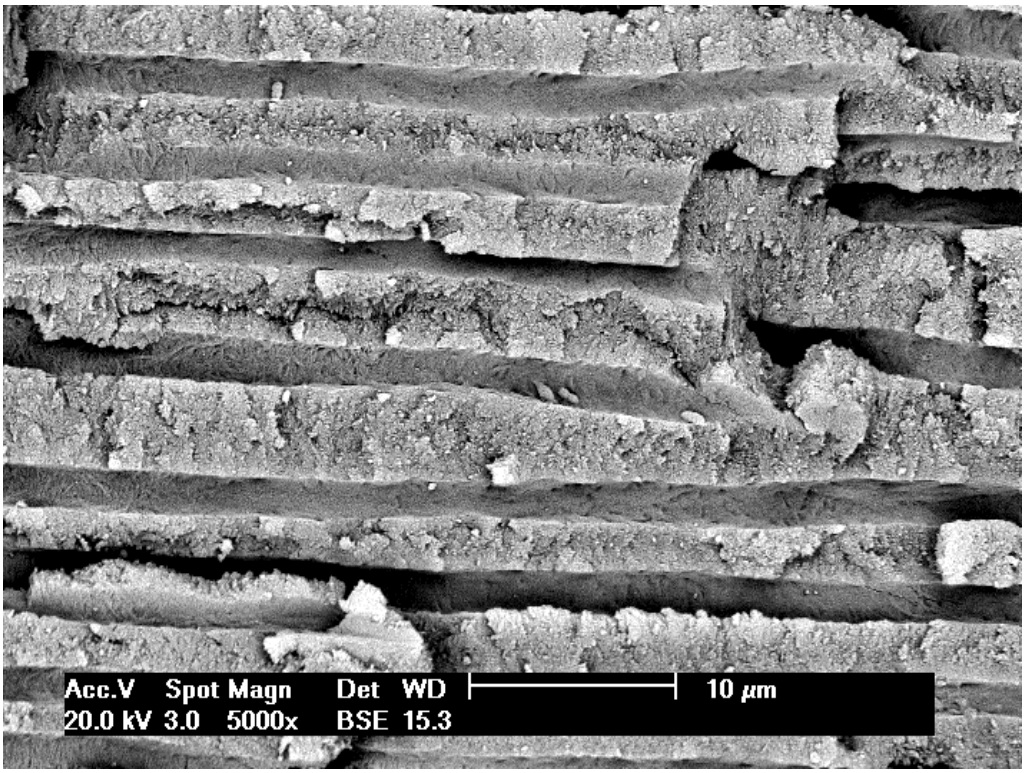

Figura 9: túbulos dentinários (irrigação convencional + hipoclorito).

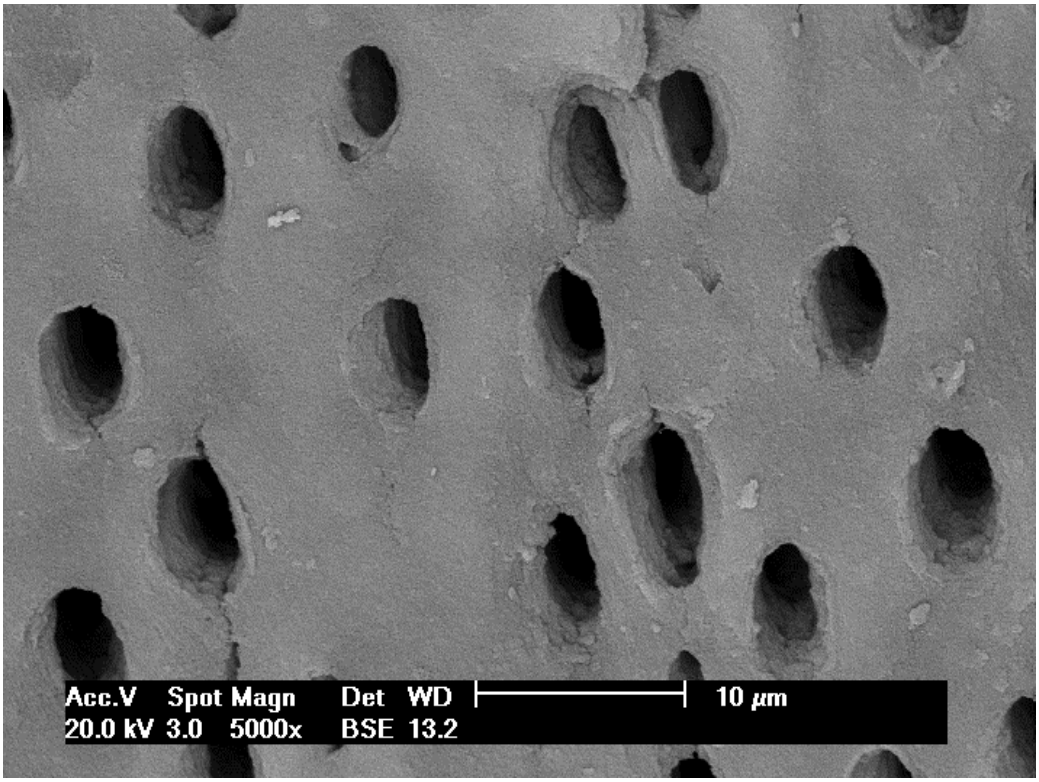

Figura 10: imagem da luz do canal (irrigação convencional + hipoclorito).

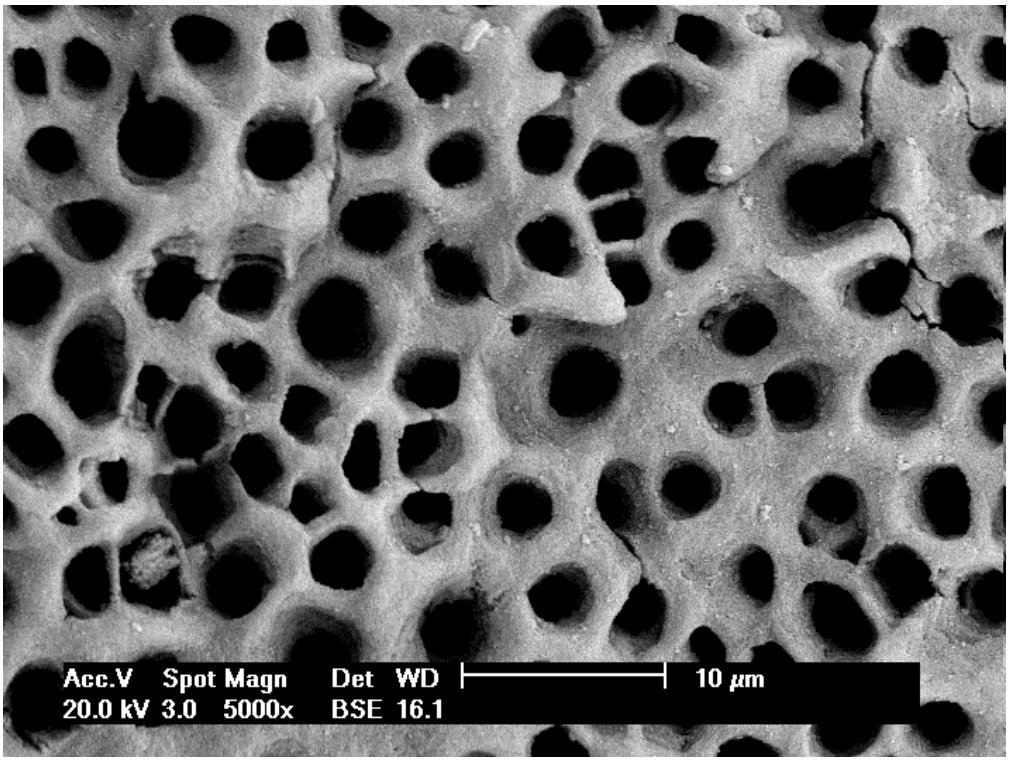

Figura 11: imagem da luz do canal (ultrassom + hipoclorito).

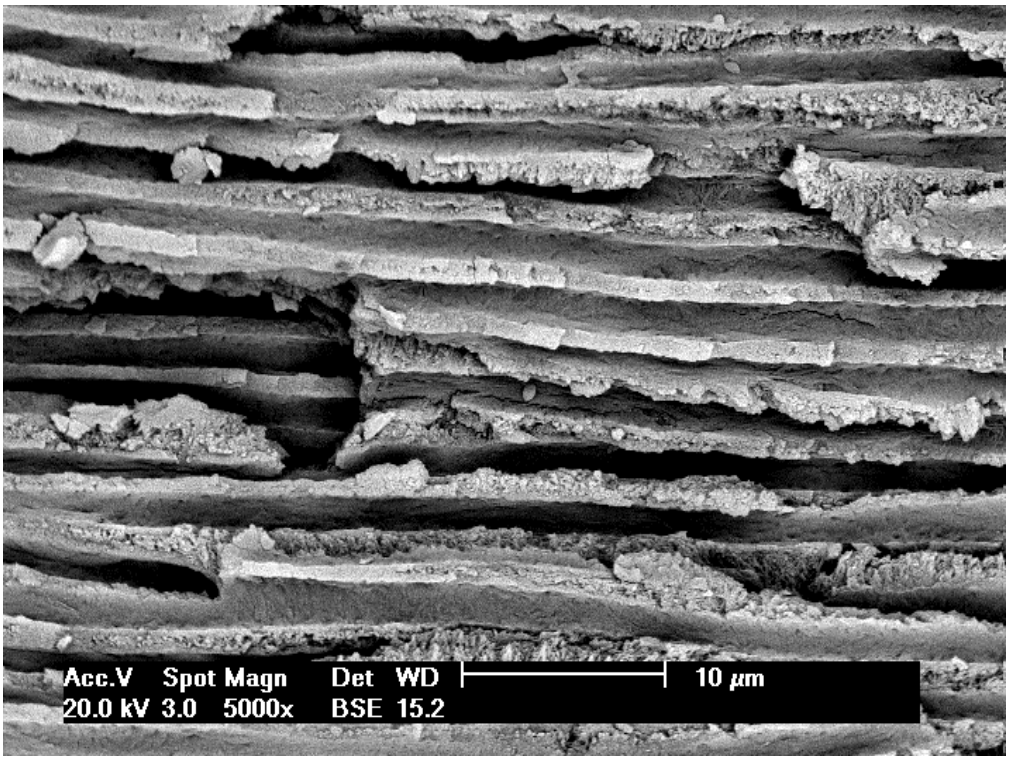

Figura 12: túbulos dentinários (ultrassom + hipoclorito).

ANEXO B: CARTA DE SUBMISSÃO

Jose Antonio P de Figueiredo

De: ees.joe.0.ebfc1.e7c1dcf5@eesmail.elsevier.com em nome de The Journal of Endodontics [JEndodontics@uthscsa.edu]
Enviado em: terça-feira, 25 de janeiro de 2011 16:38
Para: Jose Antonio P de Figueiredo
Assunto: Submission Confirmation for Effect of Ultrasonics on Enterococcus faecalis Biofilm in a Bovine Tooth Model

Dear Dr. Figueiredo,

Your submission entitled "Effect of Ultrasonics on Enterococcus faecalis Biofilm in a Bovine Tooth Model" has been received by the Journal of Endodontics.

You will be able to check on the progress of your paper by logging on to the Journal of Endodontics web site as an author.

The URL is http://ees.elsevier.com/joe/

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http://ees.elsevier.com/joe/automail_query.asp

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to the Journal of Endodontics.

Kind regards,

Journal of Endodontics

ANEXO C: APROVAÇÃO DO CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA PARA O USO DE ANIMAIS

Ofício 057/10 – CEUA

Porto Alegre, 22 de abril de 2010.

Senhor Pesquisador:

O Comitê de Ética para o Uso de Animais apreciou e aprovou seu protocolo de pesquisa, registro CEUA 10/00158, intitulado: **"Efeito do ultrasom na limpeza de canais radiculares de dentes bovinos infectados *in vitro* por *Enterococcus faecalis*".**

Sua investigação está autorizada a partir da presente data.

Atenciosamente,

Profa. Dra. Anamaria Gonçalves Feijó
Coordenadora do CEUA – PUCRS

Ilmo. Sr.
Prof. José Antonio P. Figueiredo
N/Universidade

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