PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
FACULDADE DE ODONTOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ODONTOLOGIA
DOUTORADO EM ODONTOLOGIA
ÁREA DE CONCENTRAÇÃO EM ENDODONTIA

ANÁLISE DA AÇÃO ANTIMICROBIANA E DA SUBSTANTIVIDADE DE DIFERENTES FORMULAÇÕES DE CLOREXIDINA COMUMENTE UTILIZADAS DURANTE O TRATAMENTO ENDODÔNTICO

MATHEUS ALBINO SOUZA

PORTO ALEGRE / 2013

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Tese apresentada como parte dos requisitos obrigatórios para a obtenção do título de Doutor em Odontologia, área de concentração em Endodontia.

ORIENTADOR: PROF. DR. JOSÉ ANTÔNIO POLI DE FIGUEIREDO

PORTO ALEGRE / 2013

"Acredite que você pode.

Assim você já estará na

metade do caminho".

(Theodore Roosevelt)

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RESUMO GERAL

O propósito da presente Tese foi analisar, *in vitro*, a ação antimicrobiana e a substantividade de diferentes formulações de clorexidina comumente utilizadas durante o tratamento endodôntico.

Para o presente estudo foram utilizados 85 dentes bovinos e 45 dentes humanos extraídos para o experimento de ação antimicrobiana e substantividade respectivamente. Os 85 dentes bovinos foram inoculados com *Enterococcus faecalis*, permanecendo em cultura por 30 dias para a formação do biofilme. Os dentes foram divididos em dez grupos de acordo com a presença de penetração desinfetante, medicação intracanal utilizada e o local de colocação desta medicação. . Teste microbiológico (contagem de UFCs) e microscopia eletrônica de varredura (MEV) foram realizados para avaliar e ilustrar respectivamente a eficácia dos tratamentos propostos. Os 45 dentes humanos foram divididos em três grupos de acordo com a substância química auxiliar utilizada no preparo do canal radicular. Os canais radiculares foram preparados apicalmente até o instrumento #45. Sulcos longitudinais foram confeccionados na superfície das raízes, proporcionando duas metades de cada raíz e resultando em 30 amostras por grupo. As amostras de cada grupo foram aleatoriamente divididas em três subgrupos e a substantividade foi avaliada após 24 horas, 30 dias e 90 dias de incubação. A quantidade de clorexidina (em μM) foi mensurada através de cromatografia de fase-reversa de alta performance. Análise estatística foi realizada através de ANOVA, seguido pelo post-hoc de Tukey ($\alpha = 0.05$) em ambos experimentos.

Diante da limitação dos estudos da presente tese, foi possível concluir que as formulações de clorexidina, líquida e gel, são efetivas medicações intracanais no que diz respeito ao combate ao *Enterococcus faecalis*, quando associadas à prévia penetração desinfetante com hipoclorito de sódio 2%. Além disso, foi possível concluir que as formulações de clorexidina, líquida e gel, permanecem retidas no interior do canal radicular por até 90 dias após a realização do preparo químico-mecânico com estas substâncias.

Palavras-chave: endodontia, clorexidina, medicação intracanal, substantividade.

GENERAL ABSTRACT

The purpose of this thesis was to evaluate, *in vitro*, antimicrobial activity and substantivity of chlorhexidine formulations which are used during root canal therapy.

Eighty five bovine teeth and forty five human teeth were extracted for antimicrobial and substantivity experiments respectively. The eighty five bovine teeth were inoculated with *Enterococcus faecalis* in order to provide biofilm formation. The teeth were divided into ten groups according to disinfectant penetration, intracanal dressing and medication placement site. Microbiological test (UFCs counting) and scanning electronic microscopy (SEM) were performed to evaluate and illustrate the efficacy of proposed treatments respectively. The forty five human teeth were divided into three groups according to chemical auxiliary substance used during root canal therapy. The root canals were prepared up to #45 file. Longitudinal grooves were made in the root surface, providing two halves of each root and resulting in thirty samples per group. The samples of each group were randomly divided into three subgroups and substantivity was evaluated after 24 hours, 30 days and 90 days of incubation. The amount of CHX (in µM) was measured through reverse-phase high-performance liquid chromatography. Statistical analysis was performed by analysis of variance and the Tukey test for post hoc comparisons ($\alpha = 0.05$) in both experiments.

According to limitation of experiments from this thesis, it was possible to conclude that chlorhexidine formulations, liquid and gel, can be considered effectives as intracanal dressing against *Enterococcus faecalis*, when associated to previous disinfectant penetration with 2% sodium hypochlorite. Furthermore, it was possible to conclude that chlorhexidine formulations, liquid and gel, remained into the root canal up to 90 days after chemo-mechanical preparation.

Key words: endodontics, chlorhexidine, intracanal dressing, substantivity.

ARTIGO 1

RESUMO

Objetivo: o propósito deste estudo foi avaliar, *in vitro*, a eficácia de diferentes protocolos de medicação intracanal em canais radiculares infectados com *Enterococcus faecalis*. **Metodologia:** oitenta e oito incisivos bovinos foram inoculados com *Enterococcus faecalis*, permanecendo em cultura por 30 dias para a formação do biofilme. Os dentes foram divididos em dez grupos de acordo com a presença de penetração desinfetante, medicação intracanal utilizada e o local de colocação desta medicação: G1(CHX gel) – clorexidina gel 2% (terço cervical), G2(CHX liq) – clorexidina líquida 2% (terço cervical), G3(TC) – tricresol formalina (entrada do canal); nestes grupos (n=10) não foi realizada penetração desinfetante com hipoclorito de sódio 2%. Seguindo, G4(DP+CHX gel) – clorexidina gel 2% (todos os terços), G5(DP+CHX liq) – clorexidina líquida 2% (todos os terços), G6(DP+TC) – tricresol formalina (entrada do canal), G7(DP+Ca $(OH)_2$) – pasta de hidróxido de cálcio (todos os terços); nestes grupos (n=10) foi realizada penetração desinfetante com hipoclorito de sódio 2%. Seguindo, G8(DP NaOCl) – penetração desinfetante com hipoclorito de sódio 2%, G9(DP H_2O) – penetração desinfetante com água destilada, G10(sem tratamento); estes grupos (n=6) foram considerados controles. Teste microbiológico (contagem de UFCs) e microscopia eletrônica de varredura (MEV) foram realizados para avaliar e ilustrar respectivamente a eficácia dos tratamentos propostos. Análise estatística foi realizada através de ANOVA, seguido pelo post-hoc de Tukey ($\alpha = 0.05$). **Resultados:** o teste microbiológico demonstrou que os grupos G4(DP+CHX gel), G5(DP+CHX liq), G6(DP+TC) e G7(DP+Ca$(OH)_2$) não apresentaram crescimento bacteriano, sendo estatisticamente diferentes dos demais grupos ($p<0,05$). **Conclusão:** clorexidina gel 2%, clorexidina líquida 2% e pasta de hidróxido de cálcio em todos os terços do canal radicular, bem como tricresol formalina na entrada do canal radicular, podem ser consideradas efetivas medicações intracanais contra *Enterococcus faecalis*, quando associadas à penetração desinfetante prévia com hipoclorito de sódio 2%.

Palavras-chave: biofilme, *Enterococcus faecalis*, dentes bovinos, medicação intracanal

ARTIGO 2

RESUMO

Objetivo: o propósito deste estudo foi avaliar a substantividade das formulações líquida e gel de clorexidina no sistema de canais radiculares nos períodos de 24 horas, 30 dias e 90 dias. **Metodologia:** quarenta e cinco dentes humanos anteriores extraídos foram usados no presente estudo. As amostras foram divididas em três grupos de acordo com a substância química auxiliar utilizada no preparo do canal radicular: G1 – clorexidina líquida 2%, G2 – clorexidina gel 2%, G3 – água destilada (controle). O comprimento de trabalho foi determinado através da inserção de uma lima tipo-K #10 no interior do canal até o momento da sua ponta ser vista no forâmen apical, reduzindo em 1 mm esta medida. Os canais radiculares foram preparados apicalmente até o instrumento #45. Sulcos longitudinais foram confeccionados na superfície das raízes, proporcionando duas metades de cada raíz e resultando em 30 amostras por grupo. As amostras de cada grupo foram aleatoriamente divididas em três subgrupos (n=10) e a substantividade foi avaliada após 24 horas, 30 dias e 90 dias de incubação. A quantidade de clorexidina (em μM) foi mensurada através de cromatografia de fase-reversa de alta performance. Análise estatística foi realizada através de ANOVA, seguido pelo post-hoc de Tukey ($\alpha = 0.05$). **Resultados:** o grupo controle não apresentou substantividade. Quantidade significante de clorexidina líquida e gel permaneceram na superfície dentinária, independentemente do tempo de incubação ($p<0,05$). A clorexidina líquida apresentou maior substantividade que a clorexidina gel, com exceção dos grupos incubados por 90 dias. A quantidade decrescente de clorexidina retida no interior do canal foi: para a clorexidina líquida: 24h > 30 dias > 90 dias; para a clorexidina gel: 24h > 30 dias ≥ 90 dias. **Conclusão:** os resultados deste estudo indicam que as formulações líquida e gel de clorexidina permanecem retidas na dentina radicular por até 90 dias.

Palavras-chave: dentina, clorexidina, substantividade.

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SUMÁRIO

1 INTRODUÇÃO GERAL

A grande maioria das alterações patológicas que acometem a polpa e os tecidos perirradiculares é de natureza inflamatória e de etiologia microbiana. Bactérias e seus produtos exercem um papel significativo na indução e, principalmente, na perpetuação de tais doenças (1,2).

Mais de cem espécies bacterianas diferentes, muitas potencialmente patogênicas, têm sido isoladas de canais radiculares infectados, com grande prevalência de bactérias anaeróbias estritas (3). As espécies aeróbias ou anaeróbias facultativas também têm sido encontradas na microbiota endodôntica, associadas às infecções persistentes ou secundárias. Estas espécies podem comprometer o sucesso da terapia endodôntica, destacando-se entre elas o *Enterococcus faecalis* (4,5).

O *Enterococcus faecalis* é um microorganismo anaeróbio facultativo (6), altamente resistente e desempenha um papel importante na etiologia de lesões perirradiculares persistentes após o tratamento de canais radiculares, sendo freqüentemente encontrado nos casos de insucesso endodôntico (7). Sua prevalência é maior em infecções persistentes que em infecções primárias (8). 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 (9). Alguns fatores de virulência do *Enterococcus. faecalis* são de extrema importância para a sua patogenicidade, incluindo substâncias de agregação, feromônios, ácido lipoteicóico, produção de superóxido extracelular, enzimas líticas e citolisinas (10). Além disso, possui a capacidade de facilmente invadir os túbulos dentinários (11) e formar biofilme microbiano (12,13).

A maioria das bactérias encontradas na microbiota dos canais radiculares pode ser removida, simplesmente, através da ação mecânica dos instrumentos endodônticos. No entanto, devido à complexidade anatômica do sistema de canais radiculares, bactérias e resíduos orgânicos localizados profundamente nos túbulos dentinários, bem como em regiões de istmos e reentrâncias, podem não ser alcançados (14).

Nesse sentido, diferentes substâncias químicas auxiliares têm sido utilizadas durante o preparo dos canais radiculares no intuito de ajudar na neutralização dos microorganismos que não puderam ser eliminados pela instrumentação mecânica (15).

Além disso, a utilização de medicações intracanais pode contribuir para a redução da microbiota endodôntica (16).

O hipoclorito de sódio é a substância química auxiliar mais freqüentemente utilizada na endodontia atualmente (17). Este composto apresenta uma série de propriedades e vantagens, entre as quais se inclui a capacidade de dissolver matéria orgânica (18,19) e o amplo espectro antibacteriano que possibilita a eliminação efetiva de microorganismos do canal radicular (20) e do interior dos túbulos dentinários (21). A ação antimicrobiana ocorre a partir da formação de compostos contendo cloro ativo, como o ácido hipocloroso e o íon hipoclorito, os quais induzem, através de diferentes mecanismos, injúria aos componentes microbianos (22). Entre as suas desvantagens, é instável ao armazenamento (23), extremamente citotóxico quando extravasado no interior dos tecidos perirradiculares (24), diminui a resistência à fratura dos dentes e a resistência de união dos materiais restauradores à dentina (25).

Conforme mencionado anteriormente, a utilização de uma medicação intracanal também pode contribuir para o sucesso da terapia endodôntica. A medicação intracanal mais usualmente utilizada na endodontia é a pasta de hidróxido de cálcio, sendo aceita em razão de apresentar ação antimicrobiana, neutralizar toxinas bacterianas, ser biocompatível com os tecidos periapicais e estimular o processo de mineralização (26-28). A ação antimicrobiana ocorre a partir da liberação de íons hidroxila que se difundem no interior dos túbulos dentinários, atingindo níveis de pH alcalino suficientes para destruição das bactérias. Além disso, funciona como barreira física inibindo o fluxo de nutrientes e a recolonização bacteriana (29). Por outro lado, como desvantagem, apresenta limitada efetividade contra microorganismos como *Enterococcus faecalis* (7) e *Candida albicans* (30), e diminui a resistência à fratura do elemento dentário (31).

O tricresol formalina também tem sido utilizado entre as sessões do tratamento endodôntico, sendo considerado um importante agente antimicrobiano (32) e atuando tanto por contato quanto à distância através da liberação de vapores (33). A atividade antimicrobiana ocorre a partir da ação alquilante do formaldeído sobre proteínas e ácidos nucléicos microbianos, ocorrendo a penetração e, conseqüentemente, injúria da célula bacteriana (34). Por outro lado, pode induzir efeitos mutagênicos (35), sendo considerado potencialmente carcinogênico (36).

Devido a uma série de propriedades, o uso de clorexidina na terapia endodôntica tem sido preconizado como medicação intracanal ou como substância química auxiliar no

preparo químico-mecânico de canais radiculares (37). A clorexidina é usada principalmente na forma de apresentação líquida, mas tem sido preconizado o seu uso na formulação gel dentro dos protocolos de tratamento do sistema de canais radiculares (14,38).

O amplo espectro antimicrobiano é uma das propriedades desejáveis presentes nas formulações de clorexidina (39,40). A sua eficácia contra um grande número de microorganismos se deve ao fato da interação entre a carga positiva de sua molécula com a parede celular bacteriana carregada negativamente, alterando por esse mecanismo o equilíbrio celular osmótico (41). Com isso aumenta a permeabilidade da parede celular, o que permite a penetração das moléculas de clorexidina no interior das bactérias e, conseqüentemente, a eliminação destes microorganismos (42).

Alem da ação antimicrobiana de amplo espectro, a clorexidina apresenta substantividade (43), isto é, se liga por adsorção à hidroxiapatita do esmalte ou dentina e a grupos aniônicos ácidos de glicoproteínas, sendo lentamente liberada à medida que a sua concentração no meio decresce, permitindo, desse modo, um tempo de atuação prolongado (44,45).

Em conjunto com estas propriedades, a clorexidina retarda a recontaminação do sistema de canais radiculares via coronária quando utilizada como medicação intracanal (42), não interfere na estabilidade de união entre o material restaurador e a dentina (46), apresenta ausência de citotoxicidade (47) e promove efetiva ação lubrificante (14). No entanto, como desvantagem, não possui a capacidade de dissolver tecido orgânico (19).

Levando em consideração a necessidade de inativação dos microorganismos para que ocorra o sucesso da terapia endodôntica, julga-se necessário o uso de uma substância química auxiliar que promova adequada descontaminação e possua um adequado tempo de atuação no interior do sistema de canais radiculares. Nesse sentido, torna-se justificável a realização deste estudo no intuito de avaliar a ação antimicrobiana e a substantividade de diferentes formulações de clorexidina comumente utilizadas durante o tratamento endodôntico.

A presente tese compreende dois estudos apresentados sob a forma de artigo científico, atendendo aos requisitos necessários do Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Rio Grande do Sul, para a obtenção do título de Doutor em Odontologia – Área de Concentração em Endodontia.

2 ARTIGO 1

O artigo a seguir intitula-se "**Effectiveness of intracanal dressing protocols on *Enterococcus faecalis* biofilm in a bovine tooth model – an *in vitro* study**" e foi formatado de acordo com as normas para publicação do periódico *Australian Endodontic Journal*.

Effectiveness of intracanal dressing protocols on *Enterococcus faecalis* biofilm in a bovine tooth model – an *in vitro* study

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ABSTRACT

Aim: the purpose of study was to evaluate, *in vitro*, the effectiveness of intracanal dressing protocols in root canals infected with *E.faecalis*. **Methodology:** eighty eight bovine incisors were inoculated with *E.faecalis*, remaining in culture for 30 days for biofilm formation. The teeth were divided into ten groups according to presence of disinfectant penetration (DP), intracanal dressing and medication placement site: G1 (CHX gel) – 2% chlorhexidine (CHX) gel (cervical third), G2 (CHX liq) – 2% CHX liquid (cervical third), G3 (TC) – tricresol formalin (canal entrance). In these groups (n=10), DP was not performed. G4 (DP+CHX gel) – 2% CHX gel (all thirds), G5 (DP+CHX liq) – 2% CHX liquid (all thirds), G6 (DP+TC) – tricresol formalin (canal entrance), G7 (DP+$Ca(OH)_2$) – calcium hydroxide paste (all thirds). In these groups (n=10) DP with 2% NaOCl was performed. Groups G8 (DP NaOCl) – DP with 2% NaOCl, G9 (DP H_2O) – DP with distilled water, and G10 – (no treatment) were considered controls (n=6). Microbiological test (CFUs counting) and scanning electron microscopy (SEM) were performed to evaluate and illustrate respectively the effectiveness of proposed treatments. **Results:** microbiological test demonstrated that groups G4 (DP+CHX gel), G5 (DP+CHX liq), G6 (DP+TC) and G7 (DP+$Ca(OH)_2$) showed no bacterial growth, being statistically different from all other groups ($p < 0.05$). **Conclusion:** 2% chlorhexidine gel, 2% chlorhexidine liquid and calcium hydroxide paste in all root canal thirds, as well as tricresol formalin on root canal entrance, are effective intracanal dressings against *E.faecalis*, when associated to previous DP with 2% NaOCl.

Key-words: biofilm, *Enterococcus faecalis*, bovine teeth, intracanal dressing.

INTRODUCTION

Most pathological changes affecting pulp and periradicular tissues have microbial etiology. Bacteria and their products play essential role in the pathogenesis and progression of such conditions (1,2)

Studies have reported that bacteria found in endodontic microbiota can be removed using sodium hypochlorite in chemomechanical preparation (3,4). However, some bacteria such as *Enterococcus faecalis* possess resistance to endodontic treatment and remain viable into dentinal tubules even after root canal preparation (5).

On this way, intracanal dressing is advocated to prevent multiplication of microorganisms remaining even after careful cleaning and shaping of root canal (6).

Calcium hydroxide has been recommended as intracanal dressing due to some properties such as antibacterial activity, endotoxin neutralization and inducement of hard tissue formation (7,8). However, microorganisms such as *Enterococcus faecalis* may persist (9) and its antimicrobial activity may vary depending on the location into root canal (10).

Tricresol formalin has been used as an alternative to intracanal dressing, especially in situations which the root canal is not enlarged enough to allow calcium hydroxide placement. It is considered a strong disinfectant and effective bactericide (11).

Chlorhexidine gluconate has been used because of its broad antimicrobial spectrum (12-14) and substantivity (15). However, the actual impact of these substances on teeth with a biofilm along the canal space and dentinal tubules needs further investigations.

The purpose of this study was to evaluate, *in vitro*, the effectiveness of intracanal dressing protocols in root canals of bovine teeth infected with *Enterococcus faecalis*. For that, the influence of previous disinfectant penetration with 2% sodium hypochlorite and the site of medication placement were assessed.

MATERIALS AND METHODS

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

Sample obtaining and preparation

Eighty eight bovine incisors were extracted from animals killed for commercial reasons. The dental crowns were sectioned so that all the roots remained with 18 mm in length. The pulp tissue was removed by irrigation with 2% sodium hypochlorite (NaOCl) (Virex Plus – JohnsonDiversey, São Paulo, Brazil) and instrumentation with #60 k-file (Dentsply-Maillefer, Ballaigues, Switzerland) calibrated in 17 mm. Then, a final rinse with 17% EDTA (Iodontosul, Porto Alegre, Brazil) was performed for *smear layer* removal.

Each root was fixed in a plastic micro-tube (GenuineAxygenQuality, CA, USA), so that it remained upright with the cervical portion facing upward. A hole was opened in the side of micro-tube for culture medium exchange. The samples were randomly divided into seven experimental groups (n=10) and three control groups (n=6). The samples were sterilized in autoclave (Dabi Atlante – Ribeirão Preto, SP, Brazil) for a period of 30 minutes.

Culture and inoculum preparation

The culture and inoculum preparation were performed according to previous study (16). The reference strain used was *Enterococcus faecalis* (ATCC 19433). The bacteria were cultivated in BHI (Brain Heart Infusion) broth for 18 to 24 hours, at 37°C, in bacteriological incubator.

100 µL of *Enterococcus faecalis* culture were inoculated inside the root canal of the 88 samples previously sterilized. Following, the sterile BHI was added into the micro-tube so that it was completely filled with the culture medium. The culture of *Enterococcus faecalis* was maintained for 30 days in order to obtain the biofilm formation, with the renewal of one third of the BHI every 2 days. 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 with other microorganisms.

Classification of the groups

The roots were mounted on utility wax basis (Wilson, Cotia, Brazil) to avoid substance extravasation. The group distribution is demonstrated in Table 1, according to presence of disinfectant penetration, intracanal dressing and medication placement site.

Firstly, the root canal was filled with 2% NaOCl in all groups, except 9 (DP H_2O) and 10 (no treatment). A size 2 LA Axxess (Sybron-Endo,Orange,USA) was used to prepare the cervical third, followed by irrigation with 2 ml of 2% NaOCl and concomitant aspiration. The same procedure was performed in group 9 (DP H_2O), replacing the chemical substance with distilled water.

The disinfectant penetration (DP) was simulated through root canal filling with 2% NaOCl or distilled water according to Table 1, and agitation of the solution with a K #25 file (Dentsply-Maillefer,Ballaigues,Switzerland) for 60 seconds.

Irrigation with 2 ml of distilled water followed by aspiration was performed before the intracanal dressing placement, in order to neutralize de NaOCl.

Both formulations of 2% chlorhexidine (CHX) (Essencial Pharma, Itapetininga, Brazil) were introduced into root canal using a disposable sterile syringe (Descarpack, São Paulo, Brazil) and Ultradent needle (Ultradent, Indaiatuba, Brazil). Tricresol formalin (TC) (Essencial Pharma, Itapetininga, Brazil) was impregnated in a sterilized cotton pellet which was positioned in the root canal entrance. The calcium hydroxide paste ($Ca(OH)_2$) (Calen – SS White, Rio de Janeiro, Brazil) was introduced into root canal with ML endodontic syringe (SS White, Rio de Janeiro, Brazil) attached to a Septojet XL needle (Septodont, Barueri, Brazil) The roots were sealed with sterilized cotton pellet and Cavit (3M, Sumaré, Brazil). In the group 10, no procedure was performed.

The samples were stored in bacteriological incubator at 37^{o}C for 7 days.

Microbiological analysis

After storage period, the intracanal dressing was removed by irrigation with 5 ml of distilled water. Then, 5 teeth in the experimental groups and 3 teeth in the control groups were immediately immersed in the fixation solution and were used for analysis in scanning electron microscopy (SEM). The remaining teeth in each group were used for microbiological test. Following treatment, the canal was immediately filled with sterile saline solution, which was stirred with a file number 60 (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, the counting of number of colony-forming units of the plates was performed.

SEM preparation and analysis

The roots were fixed for 7 days in 2% glutaraldehyde and washed three times for 30 minutes in a 1:1 ratio of 0.2M phosphate buffer and distilled water. After dehydration, the roots were longitudinally sectioned, providing two halves of each sample. The samples were coated with gold and the image acquisition was made under SEM (Philips XL 30, Eindhoven, Netherlands), using the backscattering resource (BSE). The image records were made at 5000x in the canal wall, into all thirds of root canal, in order to illustrate the effectiveness of proposed treatments.

Data analysis

One-way ANOVA was applied in the microbiologic evaluation, followed by Tukey´s post hoc procedure, at 5% of significance level. Descriptive analysis was performed over SEM illustrations.

RESULTS

The results are expressed in Figure 1. Groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) showed no bacterial growth, being statistically different from all other groups ($p<0.05$). Group 2 (CHX liq) showed a lower median of CFU/ml than groups 1 (CHX gel), 3 (TC), 8 (DP NaOCl), 9 (DP H_2O) and 10 (no treatment), being statistically different from them ($p<0.05$). Groups 1 (CHX gel), 3 (TC), 8 (DP NaOCl) showed a lower median of CFU/ml than groups 9 (DP H_2O) and 10 (no treatment), being statistically different ($p<0.05$). However, there were no significant differences between groups 1 (CHX gel), 3 (TC) and 8 (DP NaOCl).

SEM revealed that root canal walls of samples from group 10 (no treatment) were densely colonized by *Enterococcus faecalis* (Fig 2 – A and B). In several areas, cells were organized in biofilm and were seen penetrating the dentinal tubules. At the same time, root canal walls of samples from groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) (Fig 2 – C,D,E,F,G,H,I and J) showed absence of bacteria.

DISCUSSION

One of the most important factors of endodontic success is an effective decontamination of root canal system. Chemical substances and intracanal dressings are available to perform this role concurrently with mechanical action of endodontic instruments.

The model of biofilm formation used in this study simulates the clinical conditions which are found in infected root canals. *Enterococcus faecalis* was chosen because of its ability to successfully colonize the root canal system in the biofilm form (5,17).

However, there is no consensus in literature about time of biofilm formation, varying from 24 hours (18) to 21 (19) and 50 days (16). In the present study, 30 days of biofilm formation was adopted, believing that the biofilm would be better structured and mimicking the clinical situation. Then, decontamination protocols were effectively tested.

Bovine teeth were used to perform the model of biofilm formation in the present study, as in previous studies (11,20). These teeth are used because of the anatomical and physical similarities with human teeth, plus the ease of obtaining (21).

The counting of colony forming units (CFUs) was used to evaluate the effect of the proposed treatments in the present study. This method was chosen based in previous studies (11,20) and because it allows bacteria quantification per milligram of dentin (22).

The groups where DP with 2% NaOCl was performed, previously to intracanal dressing placement in the descripted regimens, showed better results in the *Enterococcus faecalis* elimination when compared to groups where intracanal dressing was used alone. These findings testify that previous neutralization of microbial content is necessary to promote an appropriate cleaning of root canal system, as well as the antimicrobial activity of NaOCl showed in previous studies (23,24).

According to present study, 2% CHX liquid, when used only in the cervical third, showed a lower median in the counting of CFUs when compared to gel formulation in the same regimen and concentration. It can be explained by the lower superficial tension of liquid formulation, which provides a higher diffusion into root canal system and dentinal tubules.

Studies have suggested that CHX gluconate is an effective intracanal medication due its antimicrobial activity (11-13), which is in agreement of the findings of the present

study. The present results showed that both CHX formulations, when placed in all extension of root canal, after previous DP with NaOCl, promoted complete elimination of *Enterococcus faecalis*. CHX efficacy is explained by interaction between positive charge of the molecule and negatively charged phosphate groups on microbial cell walls, altering the cells' osmotic equilibrium. This increases the permeability of cell wall, which allows the CHX molecule penetration, resulting in bacteria cell death (11).

The procedures performed in groups 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) promoted complete elimination of *Enterococcus faecalis*. Both regimens were associated to previous DP with 2% NaOCl. These findings are in accordance with previous studies which showed the antimicrobial activity of tricresol formalin (15) and calcium hydroxide (25,26). The tricresol formalin antimicrobial activity occurs from formaldehyde action over microorganism components, making penetration and inducing injury in bacterial cell (27). In other hand, the mechanism of action of calcium hydroxide is dependent on dissociation of the calcium and hydroxyl ions, followed by its diffusion through the dentinal tubules and ramifications of the root canal (8). Furthermore, it promotes the inhibition of bacterial LPS (28).

Groups 4 (DP+CHX gel), 5 (DP+CHX liq), 6 (DP+TC) and 7 (DP+$Ca(OH)_2$) have not showed statistical difference between them. These findings suggest that 2% CHX gel, 2% CHX liquid, tricresol formalin and calcium hydroxide paste can be used as intracanal dressing, helping with the elimination of *Enterococcus faecalis*, when associated with previous DP with 2% NaOCl. However, components of tricresol formalin, especially formaldehyde, may cause mutagenic effects (29), being considered potentially carcinogenic (30).

The present study suggests the use of CHX gluconate as an alternative of intracanal dressing, from the moment that is effective against *Enterococcus faecalis* and doesn't have the disadvantages of the other tested substances. In addition, chlorhexidine promotes substantivity, ensuring its activity for long period of time in dentin (14), absence of citotoxicity (31) and beneficial effects in the bond strength between restorative material and dentin (32,33).

Under the limitation of this study, it can be concluded that 2% CHX gel, 2% CHX liquid and calcium hydroxide paste in all root canal thirds, as well as tricresol formalin on root canal entrance, are effective intracanal dressings against *Enterococcus faecalis*, when associated to previous DP with 2% NaOCl.

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Table 1: group distribution according to presence of disinfectant penetration, intracanal dressing and medication placement site.

Group	N	Disinfectant penetration (substance)	Intracanal Dressing	Medication placement site
1.CHX gel	*10*	*No*	*2% Chlorhexidine gel*	*Cervical third*
2.CHX liq	*10*	*No*	*2% Chlorhexidine liquid*	*Cervical third*
3.TC	*10*	*No*	*Tricresol formalin*	*Canal entrance*
4.DP+CHX gel	*10*	*Yes (2% NaOCl)*	*2% Chlorhexidine gel*	*All thirds*
5.DP+CHX liq	*10*	*Yes (2% NaOCl)*	*2% Chlorhexidine liquid*	*All thirds*
6.DP+TC	*10*	*Yes (2% NaOCl)*	*Tricresol formalin*	*Canal entrance*
7.DP+$Ca(OH)_2$	*10*	*Yes (2% NaOCl)*	*Calcium hydroxide paste*	*All thirds*
8. DP NaOCl	*6*	*Yes (2% NaOCl)*	*No*	-
9. DP H_2O	*6*	*Yes (H_2O)*	*No*	-
10. no treatment.	*6*	*No*	*No*	-

** CHX gel: chlorhexidine gel; CHX liq: chlorhexidine liquid; TC: tricresol formalin; DP: disinfectant penetration; $Ca(OH)_2$: calcium hydroxide; NaOCl: sodium hypochlorite; H_2O: distilled water.

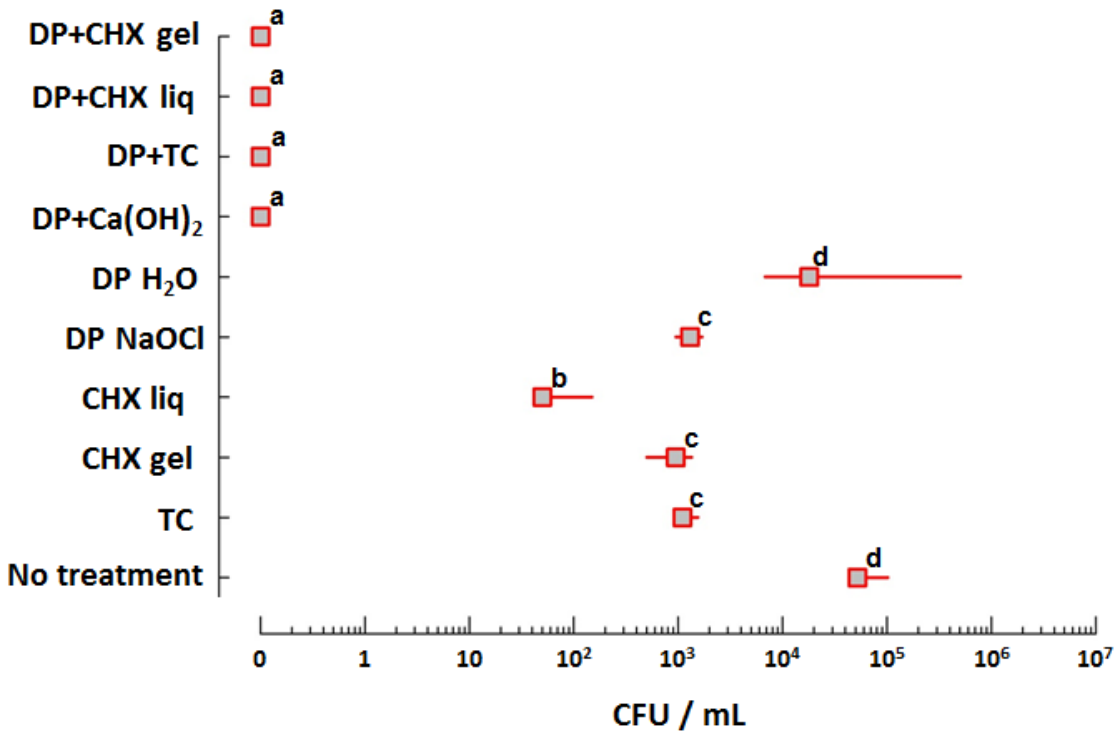

Figure 1: Graph of microbiological test results, depicting the median of CFU/ml observed for all groups. DP+CHX gel: disinfectant penetration with 2% NaOCl + 2% chlorhexidine gel (all thirds); DP+CHX liq: disinfectant penetration with 2% NaOCl + 2% chlorhexidine liquid (all thirds); DP+TC: disinfectant penetration with 2% NaOCl + tricresol formalin (canal entrance); DP+$Ca(OH)_2$: disinfectant penetration with 2% NaOCl + calcium hydroxide paste (all thirds); DP H_2O: disinfectant penetration with distilled water; DP NaOCl: disinfectant penetration with 2% NaOCl; CHX liq: 2% chlorhexidine liquid (cervical third); CHX gel: 2% chlorhexidine gel (cervical third); TC: tricresol formalin (canal entrance); No treatment: no treatment was performed. Different letters indicate a statistically significant difference at the 5% level.

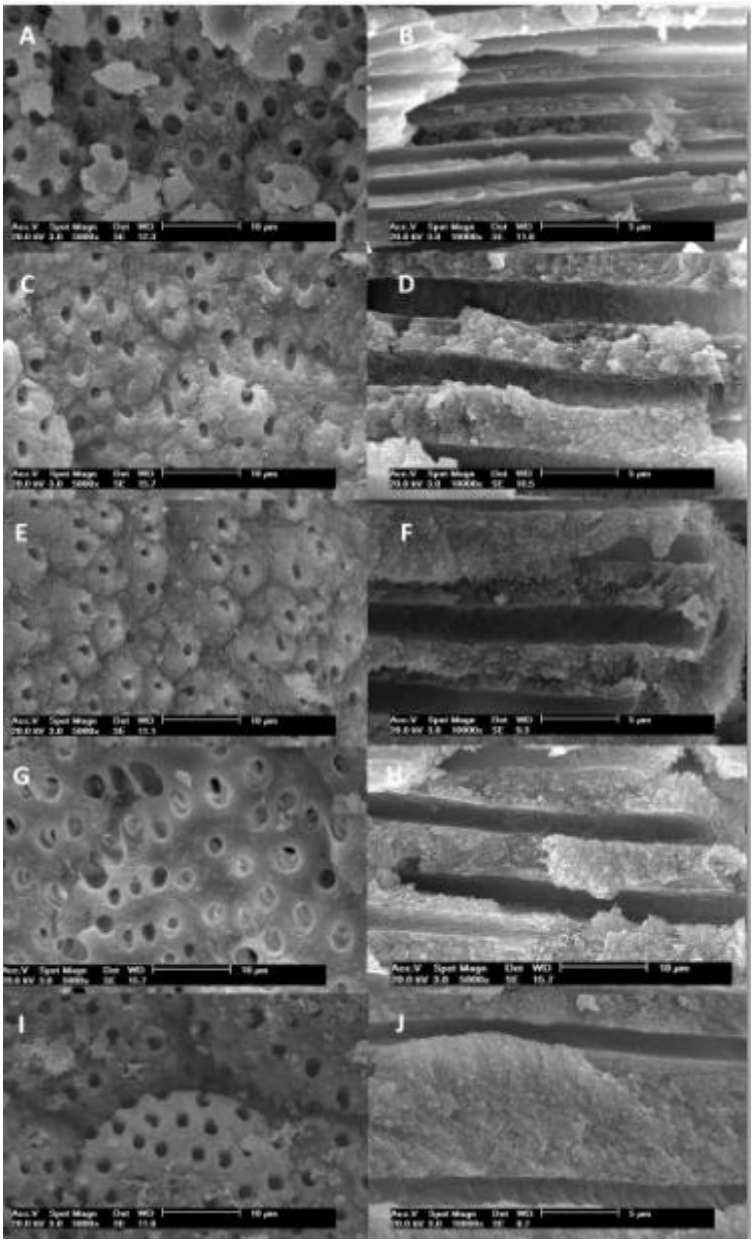

Figure 2: SEM illustrating the pattern of colonization by *Enterococcus faecalis* and the effectiveness of proposed treatments - A and B: G10(no treatment) in the canal wall and exposed tubule area respectively; C and D: G4(DP+CHX gel) in the canal wall and exposed tubule area respectively; E and F: G5(DP+CHX liq) in the canal wall and exposed tubule area respectively; G and H: G6(DP+TC) in the canal wall and exposed tubule area respectively; I and J: G7(DP+$Ca(OH)_2$) in the canal wall and exposed tubule area respectively.

3 ARTIGO 2

O artigo a seguir intitula-se "**Evaluation of chlorhexidine substantivity on human dentin – a chemical analysis**" e foi formatado de acordo com as normas para publicação do periódico *Journal of Endodontics*.

Evaluation of chlorhexidine substantivity on human dentin – a chemical analysis

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ABSTRACT

Aim: to evaluate the substantivity of solution and gel chlorhexidine (CHX) within a root canal system for 24 hours, 30 days and 90 days. **Methodology**: forty-five extracted human anterior teeth were used for the present study. The samples were divided into three groups according to the chemical auxiliary substance used to perform the root canal preparation: G1, 2% liquid CHX; G2, 2% gel CHX; and G3, distilled water (control). The working length (WL) was determined by inserting a #10 K-file into the canal up to the moment its tip was seen in the apex foramen, then withdrawing it 1 mm. The roots were prepared up to the instrument #45. Longitudinal grooves were carved on the free surfaces of the roots providing two halves of each root and resulting in 30 samples per group. Each group was randomly divided into three subgroups (n=10) and substantivity was evaluated after 24 h, 30 days and 90 days of incubation. The amount of CHX (in μM) was measured through reverse-phase high-performance liquid chromatography. Statistical analysis was performed by analysis of variance and the Tukey test for post hoc comparisons ($\alpha = 0.05$). **Results**: the control group showed no substantivity. Significant amounts of solution and gel CHX remained retained in dentin substrates, independent of the time of incubation ($P < .05$). The solution CHX showed a higher substantivity than gel CHX, with the exception of groups incubated for 90 days. The decreasing amounts of retained CHX inside the canal were: for the solution CHX: 24h > 30 days > 90 days; for the gel CHX: 24h > 30 days ≥ 90 days. **Conclusion**: the results of this study indicate that solution and gel CHX are retained in root canal dentin for up to 90 days.

Keywords: dentin, chlorhexidine, substantivity

INTRODUCTION

A major goal of endodontic therapy is to eliminate bacteria from the root canal system to create an environment that is most favorable for healing (1). This is achieved through mechanical cleaning and shaping, as well as irrigation with antibacterial agents (2,3). Sodium hypochlorite (NaOCl) in a concentration range from 0.5% to 5.25% has traditionally been used for irrigation during root canal treatment because of its antimicrobial activity and the ability to dissolve organic matter (2). Its antimicrobial property is proportional to the chemical concentration. In low concentrations, it is ineffective against specific microorganisms (4,5), and severe irritations have been reported when such concentrated solutions were forced into the periapical tissues (6,7).

Chlorhexidine digluconate (CHX) has been suggested as an auxiliary irrigant substance in endodontic treatment because of its antimicrobial activity (8, 9) and substantivity (10-13). Studies comparing the antimicrobial effectiveness of NaOCl and CHX have reported that CHX is more effective (11, 14), and others observed no significant difference between them (15-17). Furthermore, CHX does not affect the bond strength of resin composite restorations to the coronal dentin (18) or the root canal sealer to dentin (19). According to Moreira et al. (20), CHX is an auxiliary chemical substance that does not interfere with collagen present in the organic matrix of root dentin. In this way, it maintains the quality of the dentin substrate for posterior filling or restoration of the tooth with resin-based materials. Recent studies showed that CHX improves the longevity of composite adhesive bonding to dentin by inhibiting hybrid layer collagen-degrading enzymes called of matrix metalloproteinases (MMPs) (21–23), thereby offering a valuable alternative to clinicians who seek to delay the degradation process of adhesive restorations.

Unlike sodium hypochlorite, the chlorhexidine is capable of remaining onto dentin. This remaining imparts long-lasting effects on dentin, termed substantivity (10-13). Dametto et al. (11) showed that the 2% CHX (gel and liquid) keeping low colony-forming units (CFU) of E. faecalis for 7 days after the biomechanical preparation. In an in vivo study, Leonardo et al. (10) evaluated the antimicrobial substantivity of 2% CHX in teeth with pulp necrosis and radiographically visible chronic periapical lesions. They showed that CHX prevented microbial activity with residual effects in the root canal system for up to 48 h after application.

Thus, the aim of this study was to investigate the substantivity of gel and solution CHX within a root canal system for 24 hours, 30 days and 90 days, by chemical analysis. The tested null hypotheses were that: (1) chlorhexidine gel and solution have the substantivity; (2) the substantivity is time-dependent.

MATERIAL AND METHODS

Specimen Preparation

This study was submitted to the Science and Ethics Commission. Teeth were stored in 0.02% thymol solution, prepared within 1 month of extraction and autoclaved before use. Forty-five freshly human extracted anterior teeth with similar root segments and fully developed apices were selected. The root surfaces were examined for the absence of fracture lines or anatomical irregularities and were discarded if any of these features were found. Each tooth was decoronated below the cementoenamel junction perpendicular to the longitudinal axis using a slow-speed, water-cooled diamond disc (Isomet 2000; Buehler Ltd., Lake Bluff, IL). The roots were cut to a uniform length of 15 mm from the apical end. Following the procedures, the root canals were irrigated with distilled water and the pulp tissue was removed with a #15 K-file (Maillefer, Ballaigues, Switzerland). The working length (WL) was determined by inserting a #10 K-file (Maillefer) into the canal up to the moment its tip was seen in the apex foramen, then withdrawing it 1 mm. None they had the initial endodontic treatment and the apical foramen was sealed with composite resin (B0.5, Z250, 3M ESPE St Paul, MN).

Chemo-mechanical preparation

All teeth were instrumented with the crown down technique using rotary nickel-titanium K3 instruments (SybronEndo, Glendora, CA, USA) at a constant speed of 350 rpm up to a #45.02 file to the WL. The apical stop was established using files up to a size 45, followed by a step-back instrumentation, which ended after the use of 3 files larger than the last file used for the apical preparation. Stepping-back ended after the use of three files larger (K-file 60) than the file that prepared the apical stop. This technique was described previously by Berber et al. (24).

The following regimen was used: Group 1: prior to a new instrument, the canal was filled with 2% solution CHX (Natufarma, Passo Fundo, RS, Brazil). The root canal was

filled with CHX using 3 mL syringe with 19-ga needle. The needles were centered within the canal, 3 mm short of the working length. Each instrument was used for 3 minutes about in the root canal. After the use of each instrument, 5.0 mL of distilled water was used as irrigating solution with 5 mL syringe and 30-ga needle 3 mm short of the working length. In Group 2 the same protocol was used as in G1, however, 2% gel CHX (Natufarma) was used as auxiliary chemical substance. In Group 3 (control), the same protocol as in G1 was used, however, distilled water was used as an auxiliary chemical substance. Final irrigation with 2 ml of 17% EDTA for 3 min followed by irrigation with 5 ml of distilled water was performed in order to remove the smear layer (25). After that, all canals were dried with sterile paper points to conclude the protocol.

The CHX, in the group 1 and 2, was the chemical auxiliary used with the endodontic instrument for root canal preparation. Distilled water was irrigating solutions used to remove the CHX and material originated from instrumentation of the root canal.

Longitudinal grooves were carved on the free surfaces of the roots with a diamond disk, 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 root and resulting in 30 samples per group. The samples were stored at 37ºC, under 100% of relative humidity. Each group was randomly divided into three subgroups (n=10) and substantivity was evaluated after 24 h, 30 days and 90 days of incubation.

Quantification of chlorhexidine

The method for chlorhexidine determination was adapted from Rasimick et al. (13). The samples were place in tubes of 5 mL and 1 mL of extraction solution (acetonitrile: formic acid 1%, 20:80) was added. The tubes were heated in water bath at 80°C for 20 minutes and sonicated for 10 minutes. Subsequently, the liquid contents were transferred to 1.5 mL tubes and centrifuged at 6000 rpm for 15 minutes. The supernatants were diluted 10 times and 20 uL were injected into the HPLC system.

Chlorhexidine was assayed by using an isocratic separation with methanol:water (63:37, v/v). Triethylamine (0,4%) was added to mobile phase and the pH was adjusted to 3.7 with chloridric acid. A 1.0 mL/min flow rate was maintained with the DAD set at 260 nm, producing a total run time of 8 min. Twenty microliters of samples were injected in a high-performance liquid chromatograph equipped with an isocratic pump,

DAD detector, degasser, and manual injection system (all HPLC components and software ChemStation were from Agilent Technologies Inc., Santa Clara, CA, USA).

Chromatographic separations were performed using a reverse-phase column (250 mm x 4 mm, 5 mm LiChrospher 100 RP-18). The column was protected by a guard column (4 x 4 mm, 5 mm LiChrospher 100 RP-18) and was maintained at a temperature of 22±2°C.

The means and standard deviations of substantivity of solution and gel CHX were calculated in μM, and the data were analyzed using two-way ANOVA and Tukey's test for post-hoc comparisons ($\alpha = 0.05$).

RESULTS

The means and standard deviations are presented in Table 1. The control group showed no substantivity. Significant amounts of solution and gel CHX remained retained in dentin substrates, independent of the time of incubation ($P < .05$). The solution CHX showed a higher substantivity than gel CHX, with the exception of groups incubated for 90 days. The decreasing amounts of retained CHX inside the canal were, for the solution CHX, 24h > 30 days > 90 days and for the gel CHX 24h > 30 days ≥ 90 days.

DISCUSSION

Although chemo-mechanical preparation reduces the bacterial load, complete disinfection is almost impossible to achieve as a result of the complex anatomy of the root canal system (26, 27). In this regard, Peters et al. (28) have reported that mechanical instrumentation alone left more than 35% of the root canal surface untouched. Sodium hypochlorite, owing to its powerful germicidal and bactericidal properties, is still the most frequently used root canal irrigant (2). However, NaOCl acts only during the instrumentation procedures, but it does not exert any residual antimicrobial activity (11) so that the recolonization of persistent microorganisms would not be prevented. On initial exposure to chlorhexidine, Dametto et al. (11) showed that the antimicrobial activity of CHX is at least as effective as NaOCl. In addition, as revealed in this study, its substantive antimicrobial activity offers potential protection of the canal tissues for as many as 7 days after instrumentation. Although NaOCl may be equally effective on initial exposure, it is not a substantive antimicrobial

agent. As antimicrobial effectiveness is surely the most important property required for an irrigant solution to be used during treatment of teeth with apical periodontitis (17), some investigators have suggested the use of CHX as an auxiliary antimicrobial agent during the biomechanical procedures (9,11,14).

CHX is characterized by being a strong base with cationic properties (29). Its efficacy is because of the interaction of the positive charge of the molecule and the negatively charged phosphate groups on microbial cell walls, thereby altering the cells' osmotic equilibrium. This increases the permeability of the cell wall, which allows CHX molecule to penetrate into the bacteria (30). At low concentration (0.2%), low molecular weight substances, specifically potassium and phosphorous, will leak out of the cell. On the other hand, at higher concentration (2%), CHX is bactericidal as precipitation of the cytoplasmic contents occurs, which results in cell death (30). Furthermore, chlorhexidine adsorbs to surfaces covered with acidic proteins, such as hydroxyapatite, and is gradually released in the form of an active cation (substantivity), justifying its clinical use (10-13). This substantivity was confirmed in this study.

The results of this study indicate that CHX (solution or gel) is retained in root canal dentin amounts for at least 90 days. Therefore, the first hypothesis tested in this study was confirmed. Previous studies that have investigated the substantive properties of CHX have tested for its presence for up to 48 hours (10), 7 days (11), and 8 weeks (12). In addition, according to Rasimick et al. (13), groups monitoring decomposition of chlorhexidine in water had half-lives of 40 weeks. The half-life of the antimicrobials on dentin is suspected to be largely due to diffusion of the antimicrobials. These previous studies only analyzed the substantivity of solution CHX. In the current study, the substantivity of solution or gel CHX were measured through reverse-phase high-performance liquid chromatography used to estimate the amount of CHX that is retained in the dentin of the root canal wall. We observed that solution CHX has greater substantivity than gel CHX. This is possibly due to the higher capacity of the solution to penetrate the dentinal tubules than gel.

Moreover, in our study we observed that CHX substantivity is time-dependent. Thus, over time, the amount that remains in the chlorhexidine on dentin reduces. These results are consistent with other studies (12, 13) and confirm the (2) hypothesis in the study. There are several factors that might limit the substantivity of chlorhexidine. In addition to dentin, other molecules present in the root canal can alter the efficacy of irrigants.

Proteins such as serum albumin and collagen as well as killed microbes tend to reduce efficacy. Bacteria undergoing rapid growth tend to be sensitive to irrigants, whereas stressed microbes are usually resistant. These mitigating factors might shorten the life span of CHX (13).

Chemical substances used during biomechanical preparation of root canals may alter the structure of dentin, mainly collagen. This may interfere with the penetration of monomers to within the demineralized dentin structure, consequently putting the quality and durability of direct restorations and fiber post-cementation at risk (31, 32). Details of this information are important due of the necessity of sealing endodontically treated teeth using resin-based materials or when using a resin sealer for root canal obturation. Moreira et al. (20) showed that when bovine root dentin was exposed to 5.25% NaOCl for 30 minutes, whether it was associated or not to 17% EDTA, a morphologic disorganization and structure loss of the dentin organic matrix was observed closer to the root canal. Sodium hypochlorite causes dentin degeneration because of the dissolution of collagen by breaking down bonds between carbon atoms and disorganizing the proteic primary structure (32). On the other hand, the 2% CHX, whether associated or not associated with 17% EDTA, did not promote morphologic structure alterations of the dentin organic matrix. Hence, these results indicate that 2% CHX is an auxiliary chemical substance that does not interfere with the collagen present in the organic matrix of root dentin; thus, it maintains the quality of the dentin substrate for posterior obturation or restoration of the tooth with resin-based materials.

Furthermore, CHX also has potent anti-MMP-2, -8 and -9 activity, resulting in beneficial effects on the preservation of resin–dentin bonds (21). MMP-2, -8, and -9 have been detected in human crown dentin (33, 34) and radicular dentin (35) and their release and activation contribute to the organic matrix degradation along resin-dentin–bonded interfaces (36, 37), compromising the durability of adhesive restorations over time. Cecchin et al. (22) and Cecchin et al. (23) showed that the pretreatment of root dentin with CHX kept the adhesive longevity for 12 months because the bond strength of the anatomic post to the root dentin remained high and unchanged in relation to the immediate control groups. Carrilho et al. (36) and Ricci et al. (37) showed in vivo that the protection of CHX application against the degradation of the coronal adhesive interface lasted for up to 14 and 12 months, respectively, after the establishment of resin-dentin bonds. According to Carrilho et al. (21), the long-term action of CHX can

be explained by its confinement to the adhesive interface because it is possible that the removal by the dentinal fluid outflow is minimized by the formation of resin tags that obliterate the tubules. In addition, the adhesive monomers that envelop the collagen fibrils treated with CHX as well as the presence of an adhesive layer on the hybrid layer can contribute to the preservation of CHX at the interface and prolong its inhibitory action.

CHX has been suggested as an endodontic intracanal irrigant by a number of authors due to its cleansing ability (8-11), antimicrobial activity (11, 14-17) and substantivity (10-13). Furthermore, Tanomaru Filho (38) evaluated the apical and periapical repair after endodontic treatment of teeth with pulp necrosis and a chronic periapical lesion in dogs using 5.25% NaOCl or 2% CHX as the irrigating solution. These authors observed that the irrigation with chlorhexidine solution resulted in better repair than sodium hypochlorite. Moreover, the recent research indicates that the substantivity of CHX to dentin may play a paramount role in the inhibition of collagen-bound proteases and, consequently, in the stability of CHX-treated resin-bonded interfaces (22, 23, 36, 37). While these substantive and antimicrobial properties of CHX found here are promising, it does not have the tissue-dissolving properties of NaOCl (39). Furthermore, the association between substances should be better investigated. Although the association between NaOCl and CHX is not indicated by the possibility of formation of a precipitate and color change of dental structure (40), Baca et al. (42) suggested an association between CHX and Cetrimide. These authors showed that the combination of CHX and cetrimide would be an effective alternative final irrigation regimen given its antimicrobial action over time. Therefore, the impact of the use of CHX as an endodontic irrigant associated with mechanical instrumentation should be evaluated by clinical trials.

Under the limitation of this study, it can be concluded that solution and gel CHX are retained in root canal dentin for up to 90 days.

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Table 1 The amount of CHX (in μM) remaining (i.e. substantivity) over time as a function of the liquid and gel.

	24 hours	*30 days*	*90 days*
2% CHX Liquid	48.97 ± 7.85^{a}	13.95 ± 4.42^{c}	3.38 ± 1.59^{d}
2% CHX gel	22.22 ± 5.19^{b}	5.40 ± 1.03^{d}	2.03 ± 1.14^{d}
Distilled water (control)	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}	0.00 ± 0.00^{e}

Different letters indicate a statistically significant difference at the 5% level.

4 DISCUSSÃO GERAL

Um dos mais importantes fatores para alcançar o sucesso do tratamento endodôntico é uma efetiva descontaminação do sistema de canais radiculares. Apesar da significativa evolução das técnias e instrumentos endodônticos nos últimos anos, existe uma grande dificuldade de alcançar uma completa desinfecção devido às complexidades anatômicas do sistema de canais radiculares (48,49). Peters et al. (50) relataram que a instrumentação mecânica isoladamente deixou mais de 35% da superfície do canal radicular intacta, o que contribuiria significantemente para a proliferação de microorganismos no interior do canal radicular e progressão em direção aos tecidos periapicais. Nesse sentido, substâncias químicas auxiliares e medicações intracanais estão disponíveis para realizar o processo de descontaminação concomitantemente à ação mecânica dos instrumentos endodônticos.

Estudos têm sugerido que as formulações de clorexidina podem ser utilizadas na terapia endodôntica, devido sua ação antimicrobiana de amplo espectro (42,51,52), o que está em acordância com os achados do primeiro estudo da presente tese. Os resultados deste primeiro estudo demonstraram que ambas as formulações de clorexidina, quando colocadas em toda a extensão do canal radicular, após prévia penetração desinfetante com hipoclorito de sódio, promoveram completa eliminação de *Enterococcus faecalis*. A eficácia antimicrobiana desta substância é explicada pela interação entre cargas positivas da molécula de clorexidina e a parede celular bacteriana carregada negativamente, alterando o equilíbrio osmótico das células microbianas. Esta alteração aumenta a permeabilidade da parede celular bacteriana, permitindo a penetração das moléculas de clorexidina no seu interior e resultando na morte celular bacteriana (42).

Os procedimentos realizados nos grupos 6(DP+TC) e 7(DP+$Ca(OH)_2$) também promoveram completa eliminação de *Enterococcus faecalis*, sem diferença estatisticamente significante quando comparados aos grupos onde as formulações de clorexidina foram utilizadas como medicação intracanal. Ambos os regimes foram associados com penetração desinfetante prévia com hipoclorito de sódio 2%. A ação antimicrobiana do tricresol formalina ocorre a partir da ação do formaldeído sobre componentes microbianos, ocorrendo penetração e injúria da célula bacteriana (34). Por outro lado, o mecanismo de ação do hidróxido de cálcio é dependente da dissociação

dos íons cálcio e hidroxila, seguido pela sua difusão através dos túbulos dentinários e ramificações do canal radicular (28). Além disso, promove a inibição do LPS bacteriano (53). No entanto, componentes do tricresol formalina, principalmente o formaldeído, podem causar efeitos mutagênicos (35), sendo considerados potencialmente carcinogênicos (36). Ao mesmo tempo, o uso prolongado do hidróxido de cálcio pode diminuir a resistência à fratura dos dentes (31).

Nesse sentido, o primeiro estudo da presente tese sugere o uso das formulações de clorexidina como uma alternativa de medicação intracanal, a partir do momento que se mostra efetiva no combate ao *Enterococcus faecalis* e não apresenta as desvantagens das demais substâncias testadas.

A clorexidina é caracterizada por ser uma base forte com propriedades catiônicas (54). Além da ação antimicrobiana de amplo espectro, apresenta substantividade, ou seja, por adsorção, esta substância se liga à superfícies cobertas com proteínas ácidas, como hidroxiapatita, e é gradualmente liberada na forma de um cátion ativo, propiciando um tempo de atuação prolongado (40,55,56,57), o que também pode justificar o seu uso clínico durante a realização da terapia endodôntica.

Estudos prévios investigaram a propriedade de substantividade da clorexidina em períodos de observação de 48 horas (56), 7 dias (40) e 56 dias (55). Estes estudos analisaram somente a substantividade da clorexidina líquida. No segundo estudo da presente tese, a substantividade das formulações gel e líquida de clorexidina foi avaliada. Esta avaliação ocorreu através de cromatografia líquida de fase-reversa de alta performance, a qual estimou a quantidade de clorexidina que permaneceu retida na superfície dentinária do canal radicular. Os resultados deste segundo estudo indicam que a clorexidina, gel e líquida, permanece retida na dentina do canal radicular pelo período de até 90 dias. Ao mesmo tempo, os resultados demonstraram que a clorexidina líquida apresentou maior substantividade que a clorexidina gel. Isso pode ser explicado, supostamente, pela maior capacidade da formulação líquida de penetrar os túbulos dentinários, comparada à formulação gel. A partir destes resultados, é possível hipotetizar que a clorexidina, quando utilizada na terapia endodôntica, assegura um tempo de atuação prolongado no interior do canal radicular, auxiliando significativamente no processo de descontaminação.

Além disso, neste segundo estudo, foi possível observar que a substantividade da clorexidina é tempo-dependente. Ou seja, a quantidade de clorexidina que permanece

retida na superfície dentinária reduz ao longo do tempo. Estes resultados estão em acordância com estudos prévios (55,57), onde também foi possível observar a relação de tempo-dependência no que diz respeito à retenção de clorexidina na superfície dentinária radicular. Existem diversos fatores que podem limitar a substantividade da clorexidina. Além da estrutura dentinária, proteínas presentes no canal radicular, como, por exemplo, serum albumina e colágeno, e bactérias submetidas a algum tipo de stress podem encurtar a vida útil da clorexidina (57).

Além da efetiva ação antimicrobiana e substantividade demonstradas nos estudos da presente tese, a literatura apresenta outras propriedades em relação as formulações de clorexidina, que tornam esta substância uma alternativa considerável dentro da terapia endodôntica.

As substâncias químicas utilizadas durante o tratamento dos canais radiculares podem alterar componentes da estrutura dentinária, especialmente o colágeno. Isso pode interferir na penetração de monômeros dentro da estrutura dentinária desmineralizada, colocando em risco a qualidade e durabilidade de restaurações diretas, bem como a cimentação de pinos de fibra (58,59)

Detalhes desta informação são importantes devido à necessidade de selamento dos dentes tratados endodonticamente utilizando materiais resinosos ou diante da utilização de cimento endodôntico resinoso para a obturação do canal radicular. Moreira et al. (60) constaram que a dentina radicular bovina, quando exposta ao hipoclorito de sódio 5,25% por 30 minutos, associado ou não ao EDTA 17%, demonstrou uma desorganização morfológica e uma perda estrutural da matriz orgânica dentinária próxima ao canal radicular. O hipoclorito de sódio promove degradação dentinária através da dissolução de colágeno, ocorrendo a quebra das ligações entre os átomos de carbono e a desorganização da estrutura protéica primária (59). Por outro lado, a clorexidina 2%, associada ou não ao EDTA 17%, não promoveu alteração morfológica e estrutural da matriz orgânica dentinária, mantendo a qualidade do substrato dentinário para posterior obturação do canal radicular ou restauração do dente com materiais resinosos.

A clorexidina também apresenta efetiva atividade anti-MMP-2, MMP-8 e MMP-9, resultando em efeitos benéficos na preservação da adesão entre material restaurador e dentina (61). MMP-2, MMP-8 e MMP-9 têm sido detectadas na dentina humana coronária (62,63) e radicular (64) e sua liberação e ativação contribuem para a

degradação da matriz orgânica ao longo da interface dentina/material restaurador (46,65), comprometendo a durabilidade de restaurações adesivas ao longo do tempo. Cecchin et al. (66,67) demonstraram que o pré-tratamento da dentina radicular com clorexidina manteve a longevidade da restauração adesiva por 12 meses, devido ao fato de que a força de adesão do pino anatômico à dentina radicular permaneceu inalterada neste período, comparado aos grupos controle onde alterações foram constatadas.

Em conjunto com estas propriedades, a clorexidina retarda a recontaminação do sistema de canais radiculares via coronária quando utilizada como medicação intracanal (42), apresenta ausência de citotoxicidade (47) e promove efetiva ação lubrificante (14). Além disso, promove a indução de reparo periapical quando utilizada na terapia endodôntica. Tanomaru Filho et al. (68) avaliaram o reparo periapical após o tratamento endodôntico de dentes com necrose pulpar e lesão periapical crônica em cães utilizando hipoclorito de sódio 5,25% ou clorexidina líquida 2% como substância química auxiliar. Estes autores constataram que a irrigação com clorexidina líquida 2% resultou em melhor reparo comparado aos casos onde foi realizada irrigação com hipoclorito de sódio 5,25%.

Diante da limitação dos estudos da presente tese, foi possível concluir que as formulações de clorexidina, líquida e gel, são efetivas medicações intracanais no que diz respeito ao combate ao *Enterococcus faecalis*, quando associadas à prévia penetração desinfetante com hipoclorito de sódio 2%. Além disso, foi possível concluir que as formulações de clorexidina, líquida e gel, permanecem retidas no interior do canal radicular por até 90 dias após a realização do preparo químico-mecânico com estas substâncias.

Frente aos resultados encontrados nos presentes estudos e a partir das propriedades descritas na literatura, a presente tese sugere a utilização das formulações de clorexidina na terapia endodôntica, como medicação intracanal ou substância química auxiliar na realização do preparo químico-mecânico, a partir do momento em que elenca uma série de características desejáveis ao processo de descontaminação do sistema de canais radiculares.

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6 ANEXOS

6.1 Anexo A – Carta de submissão ao Australian Endodontic Journal

Australian Endodontic Journal - Manuscript ID AEJ-2012-254

De: "aej.eo@wiley.com" <aej.eo@wiley.com>
Para: matheus292@yahoo.com.br

10-Dec-2012

Dear Dr. Souza:

Your manuscript entitled "Effectiveness of intracanal dressings on Enterococcus faecalis biofilm in a bovine tooth model – an in vitro study" has been successfully submitted online and is presently being given full consideration for publication in the Australian Endodontic Journal.

Your manuscript ID is AEJ-2012-254.

Please fill-out and return the attached Exclusive License form via post or fax or by emailing a scanned copy. Returning the form promptly assists in the speed of the production process. Should your manuscript not be accepted for publication, all forms will be destroyed and you retain full copyright of your manuscript.

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You can also view the status of your manuscript at any time by checking your Author Centre after logging in to http://mc.manuscriptcentral.com/aej .

Thank you for submitting your manuscript to the Australian Endodontic Journal.

Yours sincerely,
Editor-in-Chief
Australian Endodontic Journal

Wiley Blackwell
John Wiley & Sons
155 Cremorne Street, Richmond, Vic, 3121, Australia.
Ph: +61 3 9274 3133
Fax:+61 3 9274 3390

6.2 Anexo B – Carta de aceitação do Journal of Endodontics

----- Mensagem encaminhada -----
De: The Journal of Endodontics <JEndodontics@uthscsa.edu>
Para: matheus292@yahoo.com.br
Enviadas: Segunda-feira, 4 de Junho de 2012 15:41
Assunto: Acceptance of JOE Manuscript

Ref.: Ms. No. JOE 12-198R1
Evaluation of chlorhexidine substantivity on human dentin - a chemical analysis

Dear Dr. Souza,

I am pleased to inform you that your manuscript has now been accepted for publication in Journal of Endodontics.

You will soon be contacted by our publisher to review the galley proofs.

Thank you for submitting this manuscript. I look forward to seeing it published soon.

With kind regards,

Ken Hargreaves
Editor
Journal of Endodontics

7 APÊNDICES

7.1 Imagens MEV – grupo 10 (nenhum procedimento)

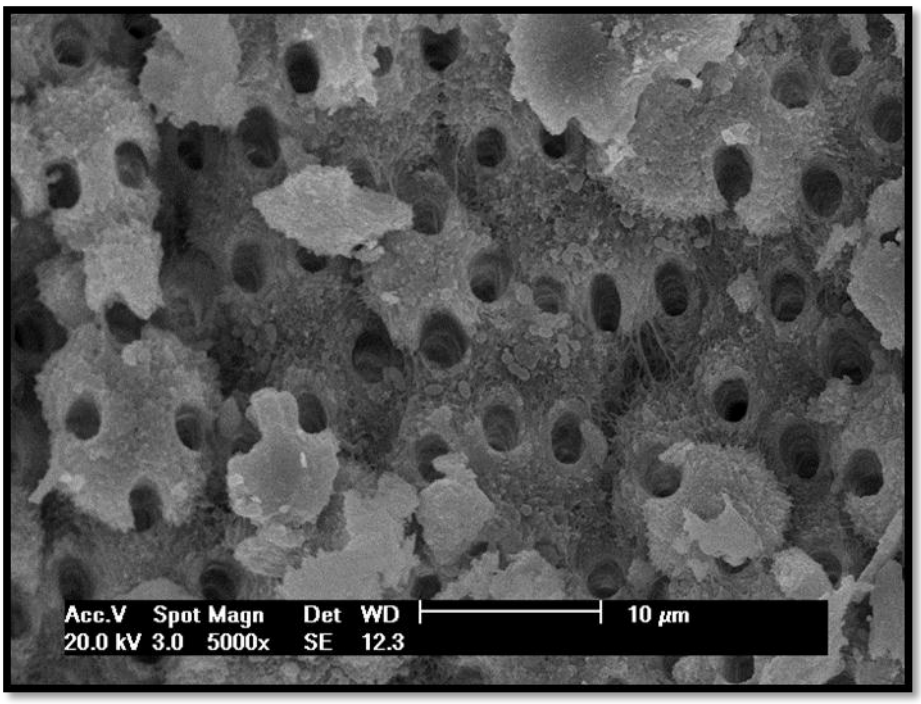


Figura 1 – magnificação de 5.000x – luz do canal.

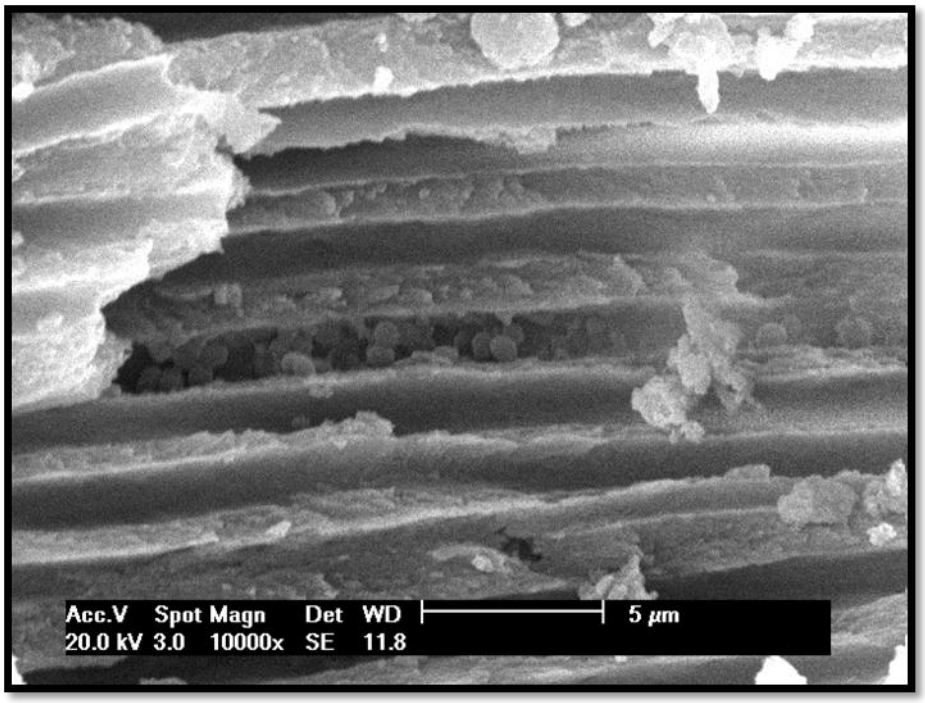
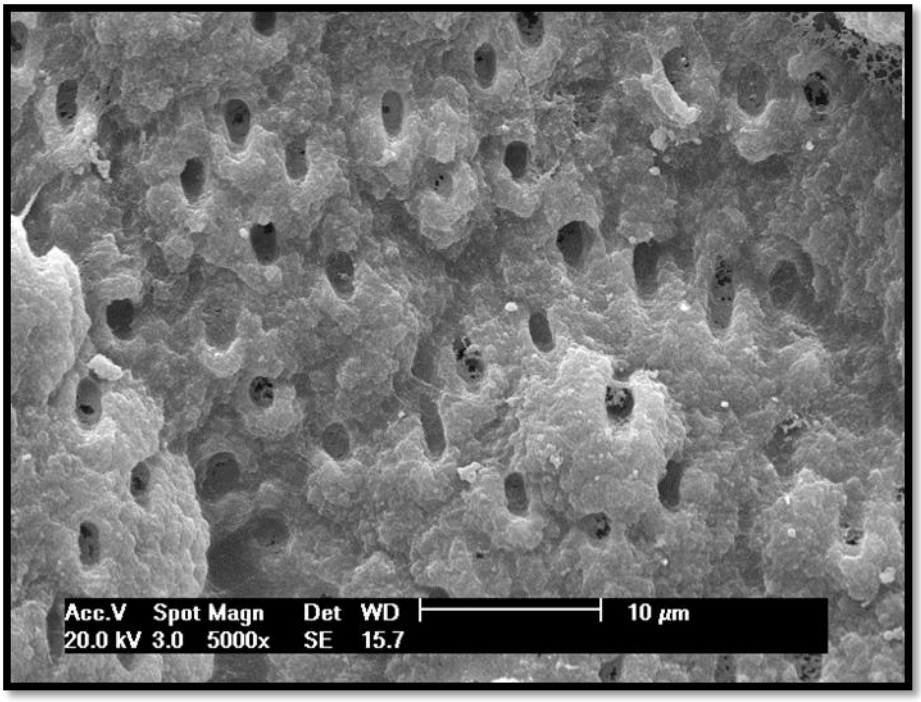


Figura 2 – magnificação de 10.000x – interface dentinária.

7.2 Imagens MEV – grupo 4 (DP+CHX gel)



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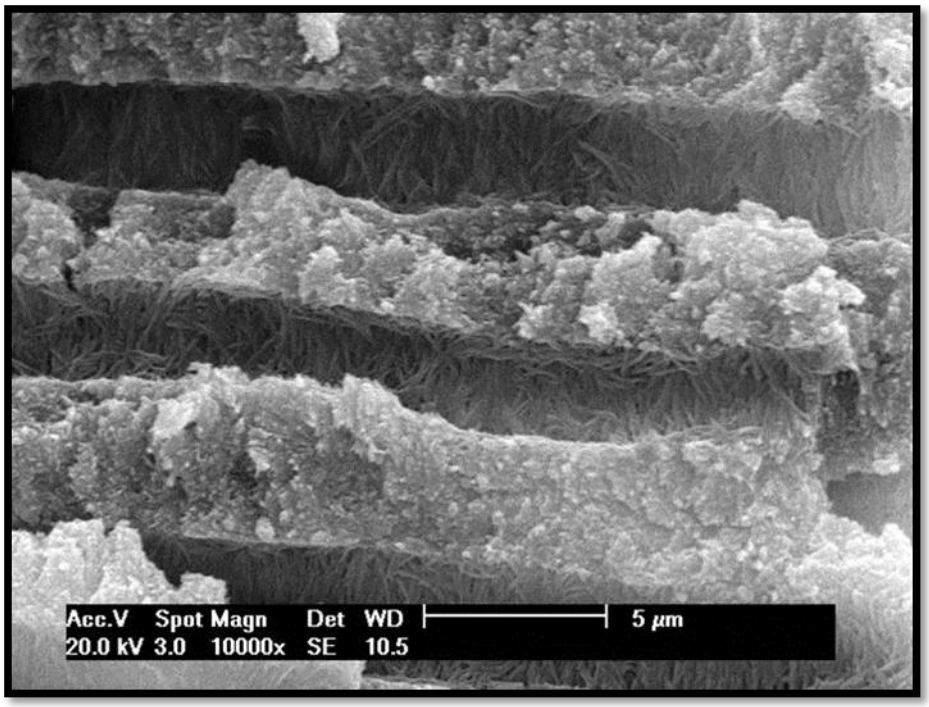
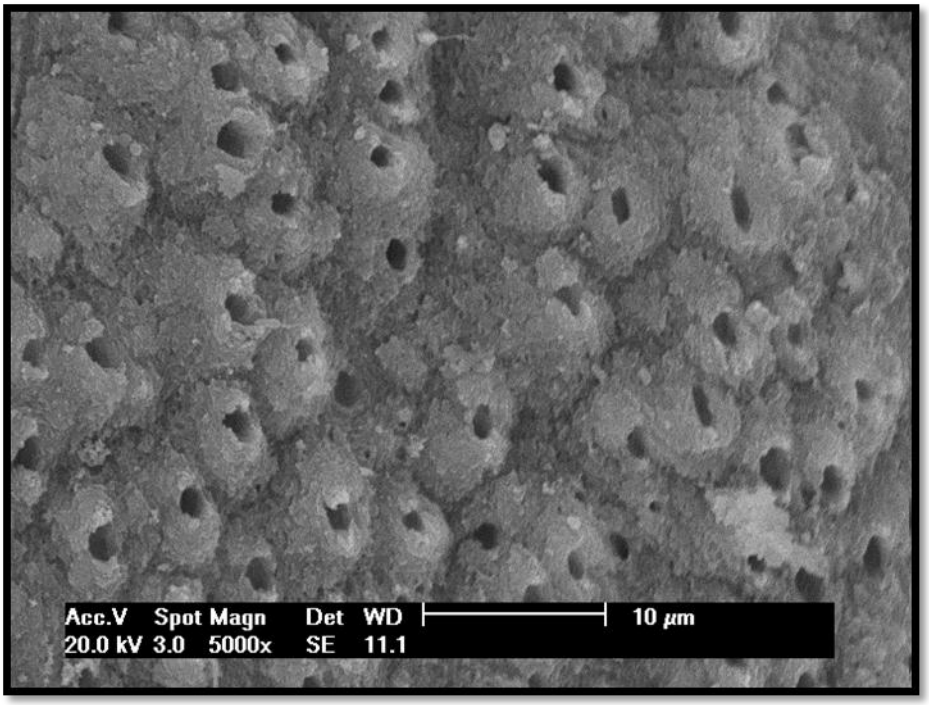


Figura 2 – magnificação de 10.000x – interface dentinária.

7.3 Imagens MEV – grupo 5 (DP+CHX liq)



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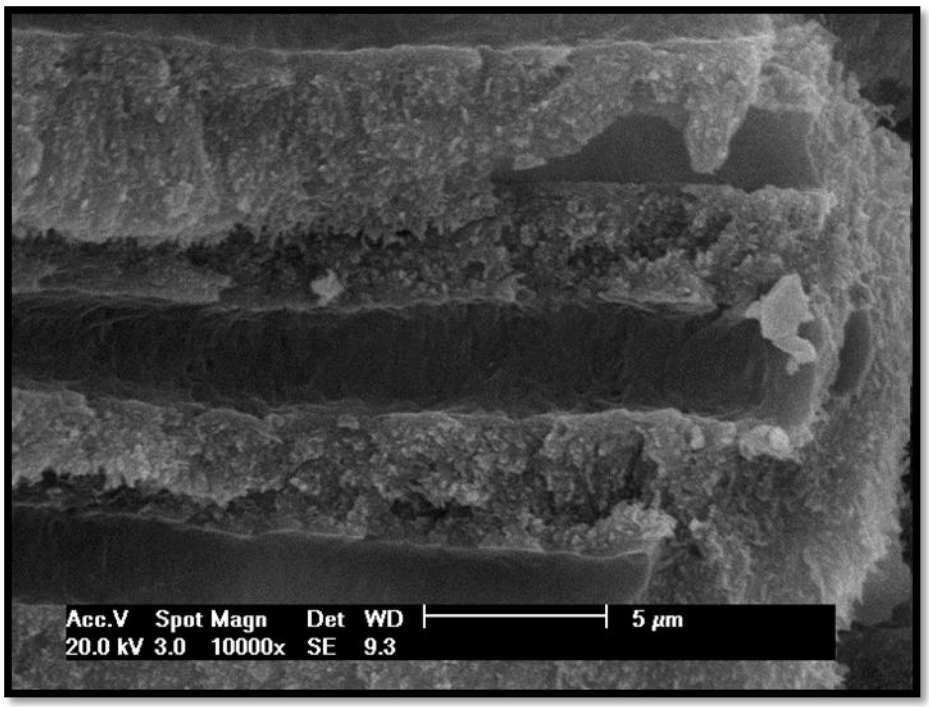

Figura 2 – magnificação de 10.000x – interface dentinária.

7.4 Imagens MEV – grupo 6 (DP+TC)

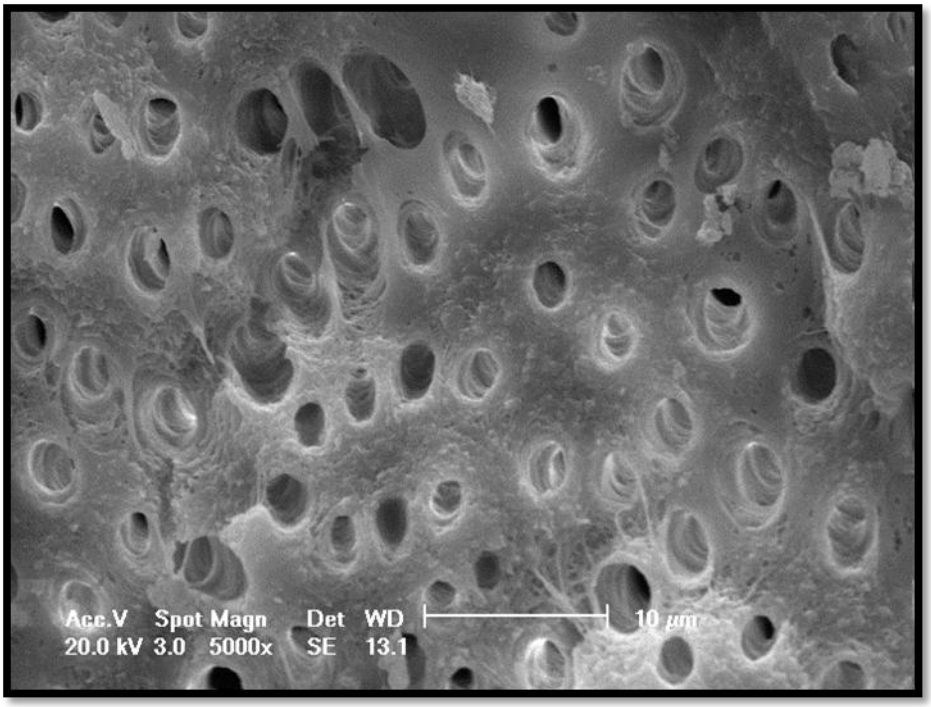

Figura 1 – magnificação de 5.000x – luz do canal.

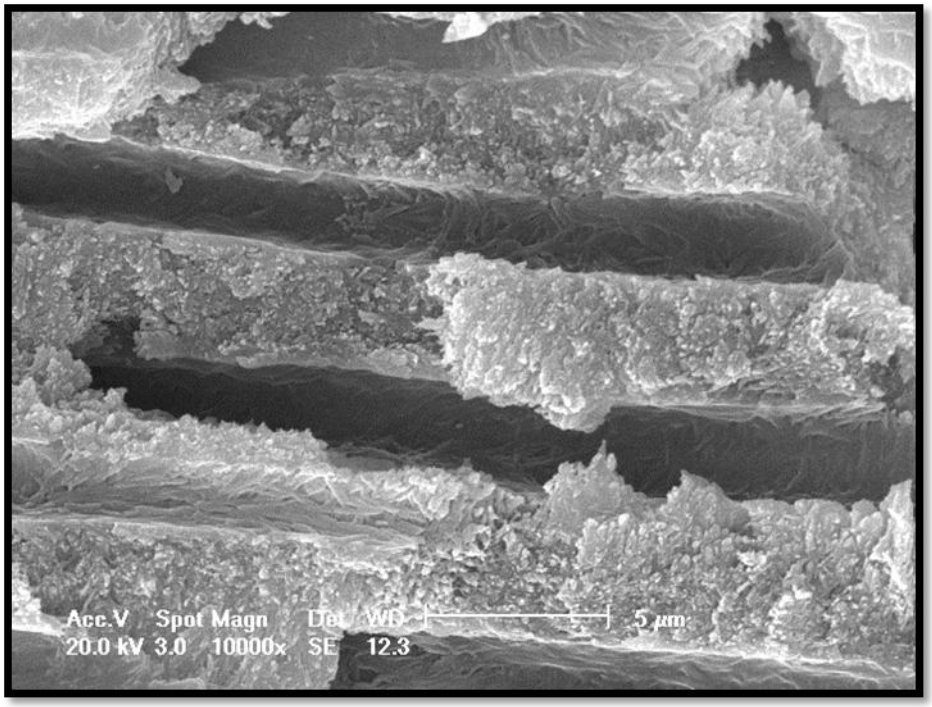

Figura 2 – magnificação de 10.000x – interface dentinária.

7.5 Imagens MEV – grupo 7 (DP+$Ca(OH)_2$)

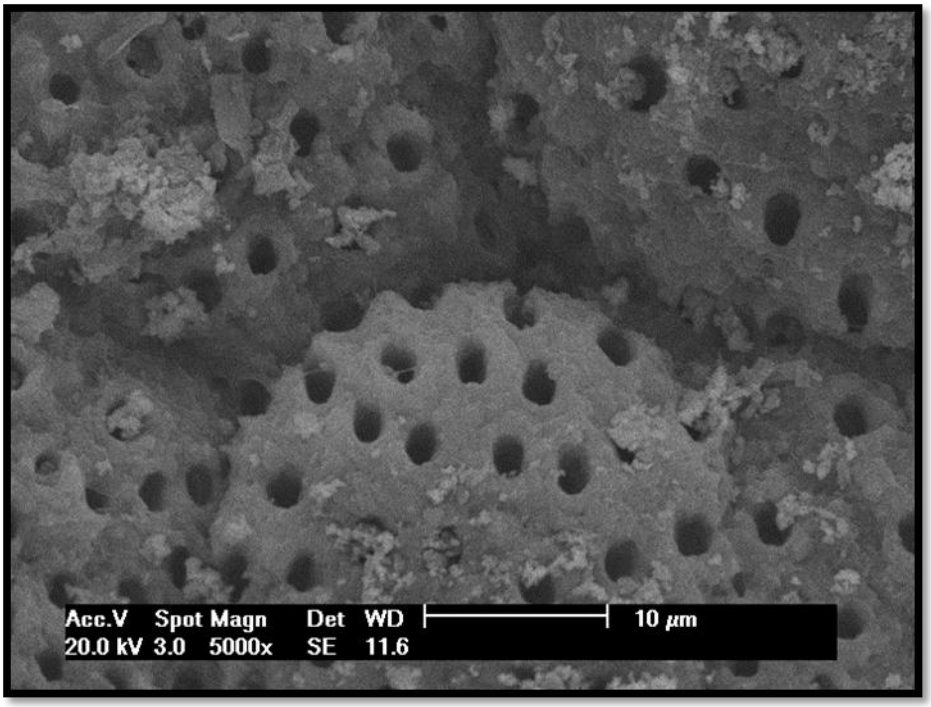

Figura 1 – magnificação de 5.000x – luz do canal.

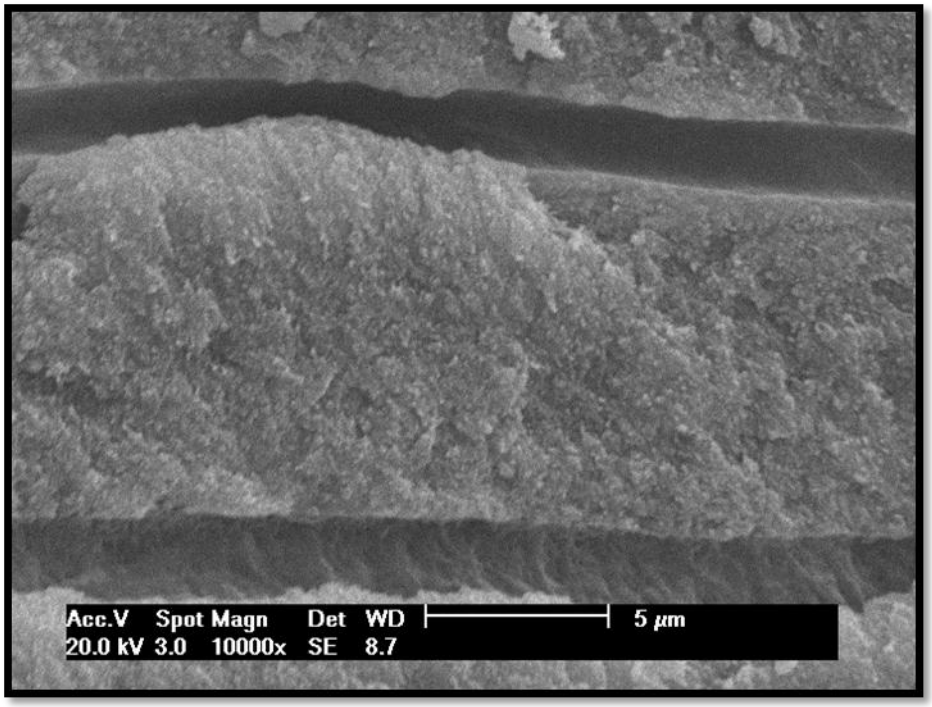

Figura 2 – magnificação de 10.000x – interface dentinária.