Antimicrobial activity of intracanal dressings composed by natural products associated to chlorhexidine and its influence on dentinal colour change

Avaliação da ação antimicrobiana de medicações intracanais à base de produtos fitoterápicos associados à clorexidina e sua influência na alteração de cor da estrutura dentinária

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Abstract

Purpose: evaluate the antimicrobial activity of intracanal dressings and their influence on dentinal colour changes. Material and methods: eighty single-rooted human extracted teeth were decoronated and divided into eight groups (n=10) according to intracanal dressing protocols inserted into the root canals: G1–distilled water (DW); G2–2% chlorhexidine gel (CHX); G3–calcium hydroxide ($Ca[OH]_2$)+DW; G4–grape seed extract (GSE)+DW; G5–ginger extract (GE)+DW; G6–$Ca(OH)_2$+CHX; G7–GSE+CHX; and G8–GE+CHX. The antimicrobial activity was evaluated by colony-forming units (CFUs) counting and dentinal colour changes was evaluated by digital spectrophotometry. Data were statistically analysed by One-way ANOVA followed by Tukey´s post hoc test (antimicrobial evaluation) and non-parametric Wilcoxon followed by the Mann-Whitney-U test (colour change evaluation) (α=0.05). Results: the highest bacterial reduction was observed in groups 4, 6, 7 and 8, with no significant difference between them ($p<0.05$). Groups 4 and 7 showed the highest medians of dentinal colour change ($p<0.05$). Conclusion: the addition of CHX improved the antimicrobial activity of GE-based intracanal dressing, with no effect in GSE-based intracanal dressing; moreover, these protocols induced significant dentinal colour changes.

Keywords: Calcium hydroxide. Chlorhexidine. Ginger extract. Grape seed extract.

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Introduction

The pulp and periapical pathologies presents microbial aetiology[1,2], needing decontamination protocols, such as intracanal dressings, to reduce microorganisms from root canals. Moreover, the maintenance of dental aesthetics is important, especially regarding the colouration of dental structures[3]. Calcium hydroxide ($Ca[OH]_2$) is the most usual intracanal dressing, due to its ability to neutralise endotoxins and induce mineralisation on periapical tissues[4,5]. However, microorganisms such as *Enterococcus faecalis* (*E.faecalis*) demonstrated resistance to $Ca(OH)_2$[6].

The addition of antimicrobial substances represents a strategy to improve the decontamination of root canals[7]. Chlorhexidine (CHX) presents antimicrobial activity[8] and substantivity[9]. Furthermore, some studies suggest natural products with antimicrobial activity, such as grape seed extract (GSE)[10] and ginger extract (GE)[11]. However, there are no studies relating the antimicrobial activity of the association of CHX to natural products and its effects on colour change of dentinal structure.

The purpose of present study was to evaluate, *in vitro*, the antimicrobial activity of intracanal dressings composed by natural products and CHX, and its influence on dentinal colour changes. The tested hypotheses were that the addition of CHX to intracanal dressings results in better decontamination and it not result in colour changes of the dentinal structure.

Materials And Methods

This study was approved by the Ethics Commission of University of Passo Fundo (Passo Fundo, RS, Brazil) (protocol 735.208). Figure 1 (antimicrobial evaluation) and 2 (colour change evaluation) provides an illustration of methodologies of present study.

Evaluation of antimicrobial activity

Sample and inoculum preparation

Eighty single-rooted human extracted teeth were selected and dental crowns were sectioned, so that all roots retained a length of 15 mm. The root canals were enlarged in all extension using ProTaper system (Dentsply-Maillefer, Ballaigues, Switzerland) up to F3 file and NaOCl (Natupharma, Passo Fundo, RS, Brazil) as irrigant solution. Final irrigation with 3 mL of 17% EDTA (Biodinâmica, Ibiporã, PR, Brazil) and 5 mL of DW were performed. Finally, the root canals were dried with absorbent paper points.

The roots were fixed in plastic microtubes and sterilised in autoclave (Dabi Atlante, Ribeirão Preto, SP, Brazil) at 120°C for 30 minutes. A 100-µL aliquot of the culture of *E.faecalis* (ATCC 19433) was inoculated into the root canals. The remaining volume was completely filled with sterile BHI. The culture was maintained for 14 days, replacing the BHI every 48h. All procedures were performed in a laminar flow hood.

Classification of treatment groups

After contamination, the 80 samples were irrigated with 5 mL of DW and randomly distributed into eight groups (n=10).

In groups 1 (DW) and 2 (CHX), the intracanal dressing was inserted with a 3 mL disposable syringe (Descarpack, São Paulo, SP, Brazil) and a 30 gauge needle (Navi-Tip - Ultradent, South Jordan, UT, Estados Unidos), until the complete filling.

In groups 3 ($Ca[OH]_2$+DW), 4 (GSE+DW) and 5 (GE+DW), the intracanal dressing was obtained from mixing 0.1g of the tested substance and 100µL of DW. Then, the root canals were completely filled with a Lentulo drill (Dentsply-Maillefer), placed 3 mm short to working length.

In groups 6 ($Ca[OH]_2$+CHX), 7 (GSE+CHX) and 8 (GE+CHX), the intracanal dressing was obtained from mixing 0.1g of tested substance and 100µL of 2% CHX. Then, the root canals were filled as described in groups 3, 4 and 5.

The roots were sealed with provisional restorative material (Cavitec – Caitthec, Rio do Sul, SC, Brazil) and stored for 14 days at 37°C and humidity.

Microbiological analysis

This analysis was performed in two stages: initial sample (S1) – after contamination and before intracanal dressing protocols; final sample (S2) – after intracanal dressing protocols. In both stages, the root canals were filled with sterile saline solution. Then, a sterile K-file #30 (Dentsply-Maillefer) was inserted, promoting contact with the walls for 30 seconds. Then, a sterile absorbent paper point worked into the root canal for 30 seconds and it was transferred to a tube containing 450µL of sterile saline solution. The material was homogenised and diluted to $1x10^{-3}$. Aliquots of 100µL of solution and each of the dilutions were cultivated on the surface of blood agar in duplicate; these samples were incubated for 18-24 h at 37°C. Then, the number of colony-forming units (CFUs) was counted on the plates.

The effectiveness of intracanal dressings was analysed by the percentage reduction of *E.faecalis* from the initial (S1) and final (S2) sample.

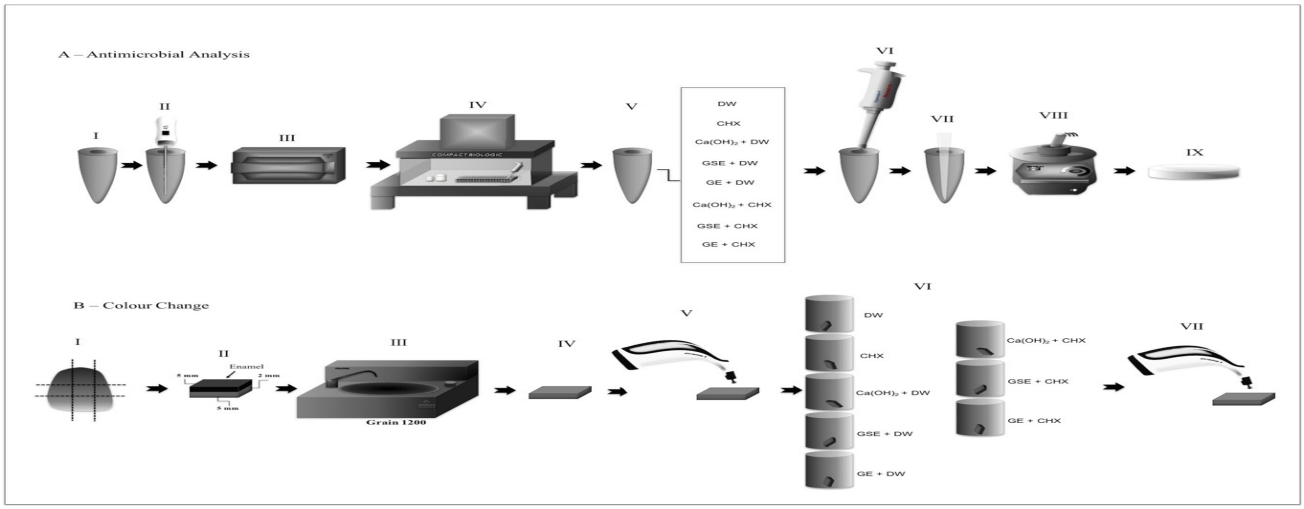

Figure 1 – Antimicrobial Analysis: (i) 80 root canals with 15 mm of length; (ii) roots instrumentation; (iii) sterilization procedure; (iv) bacteria culture and inoculation; (v) root canals filled with tested intracanal dressings; (vi) root canal filled with sterile saline solution; (vii) sterile absorbent paper point inserted into the root canal space filled with sterile saline solution; (viii) material homogenized and diluted; (ix) CFU performed in the plates.

Evaluation of colour change

Sample obtaining and preparation

The buccal portions of remaining eighty dental crowns were used to make blocks measuring approximately 5 mm long, 5 mm wide and 2 mm thick, containing enamel on the front and dentine on the back, respectively. The cutting was performed in a metallographic machine (IsoMet 1000, Buehler, Lake Bluff, Illinois, USA). The blocks were ground with abrasive paper until the dentin was exposed, washed with DW and dried with absorbent paper.

Classification of treatment groups

The eighty dentin blocks were randomly distributed in the eight previously described groups (n=10). The dentin blocks were immersed in plastic tubes containing 2 ml of the intracanal dressings and stored for 14 days at 37°C and humidity. Then, the dentin blocks were washed with 5 mL of DW and dried with absorbent paper.

Reading of dentinal colouration

The reading was performed in two stages: before and after 14 days of the immersion in the intracanal dressings, by using digital spectrophotometry. Each of the samples was initially submitted to colour evaluation using a digital spectrophotometer (Vita Easyshade – Vita Zahnfabrik, Bad Säckingen, Germany), consisting of a fiber optic cable and an integrated sphere. Barium sulfate (Vetec Química Fina Ltda., Rio de Janeiro, RJ, Brazil) was placed in a plastic container and used as a white reference material for total reflection. The dark pattern was obtained

using the integrated sphere of the measuring device. The initial measurements were made for each sample, and the beam of light from the sphere of the measuring device was directed at the central portion of the dentin surface of the made sample. The values from 1 (lighter) to 16 (darker) provided by the digital spectrophotometer itself were recorded. Ten measurements were made for each sample and the initial colour score of each sample was the average of these 10 scores.

After 14 days and the removal of the tested intracanal dressing, the samples from each group were analyzed again, for colour change evaluation, using the digital spectrophotometer, according to the methodology described above. The mean of 10 scores of each sample were recorded again, as previously described. Finally, the final mean values of the scores from 1 to 16 were reduced from the respective initial mean values of each sample, obtaining the final score of each sample. Then, the colour change was measured by the average of the final scores of each sample of each group, and these data were statistically analyzed.

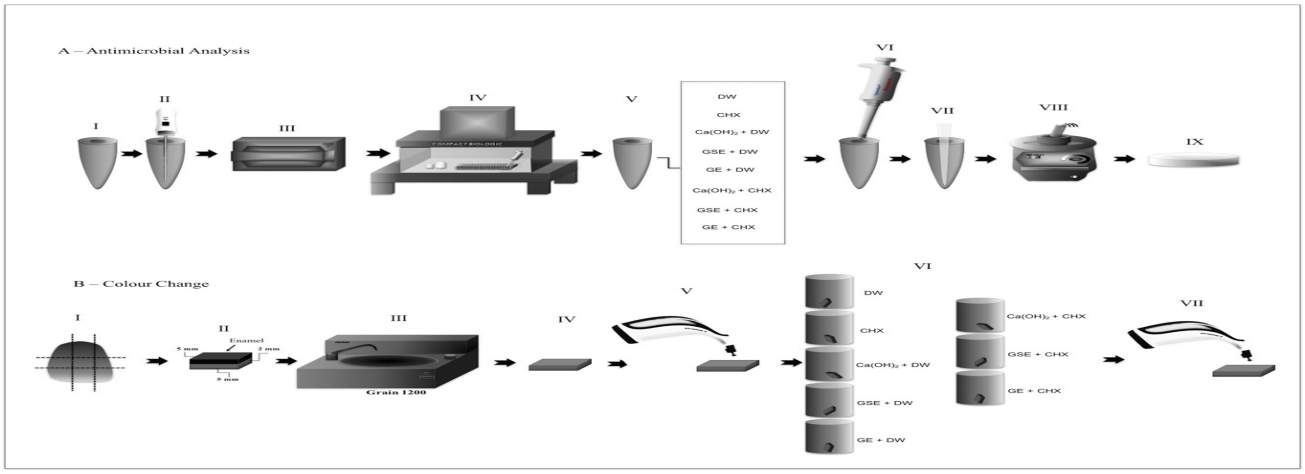

Figure 2 – Colour Change Analysis: (i) 80 remaining crowns (ii) dentin blocks from buccal portion measuring 5 mm long, 5 mm wide and 2 mm thick (iii) grounding of dentin blocks with abrasive paper (iv) grounded dentin blocks (v) initial reading of dentinal colouration (vi) immersion of dentin blocks it the tested intracanal dressings (vii) final reading of dentinal colouration.

Statistical Analysis

For the microbiological test, the statistical analysis was performed by calculating the percentage reduction of *E.faecalis* from initial and final counting of CFUs, using the formula: %=100-[(Final value/Initial value)x100]. One-way ANOVA test followed by Tukey´s post hoc procedure were performed (α=0.05). For the colour change test, non-parametric Wilcoxon test followed by the Mann-Whitney-U test were applied (α=0.05). Data were analysed using SPSS version 17.0 (SPSS, Chicago, IL, United States).

Results

The mean and standard deviation of *E.faecalis* percentage reduction are presented in Table 1. The highest percentage reduction of *E.faecalis* was observed in groups 4(GSE+DW), 6(Ca(OH)2+CHX), 7(GSE+CHX) and 8(GE+CHX), with no statistically significant difference between them ($p<0.05$).

The median and minimum-maximum score values of colour change are presented in Table 2. Groups 4(GSE+DW), 5(GE+DW), 7(GSE+CHX) and 8(GE+CHX) revealed colour change, with groups 4(GSE+DW) and 7(GSE+CHX) being statistically different to all other groups ($p<0.05$).

Table 1 – Mean (standard deviation) of percentage of Enterococcus faecalis reduction (%)

Group	N	Bacterial reduction (%)	P value
1. DW [a]	10	11.72 (0.68)	P < 0.05
2. CHX [b]	10	70.83 (3.90)	P < 0.05
3. $Ca(OH)_2$+DW [c]	10	50.62 (3.33)	P < 0.05
4. GSE + DW [d]	10	96.45 (3.54)	P < 0.05
5. GE + DW [e]	10	58.79 (3.15)	P < 0.05
6. $Ca(OH)_{2+}$CHX [d]	10	98.69 (3.22)	P < 0.05
7. GSE + CHX [d]	10	96.93 (3.48)	P < 0.05
8. GE + CHX [d]	10	99.27 (3.74)	P < 0.05

* Data are presented as mean (standard deviation). P values are significant using analysis of variance on ranks. Different index letters represent statistical significant different at the post hoc procedure (Tukey test).

Table 2 – Median (minimum-maximum) of score values of color change in the dentin structure for all evaluation periods and treatment groups

Group	N	Baseline	14 days
1. DW	10	1.8 (1.0-6.0) [a]	1.8 (1.0-6.0) [a,A]
2. CHX	10	2.0 (1.0.-7.0) [a]	2.0 (1.0-3.0) [a,A]
3. $Ca(OH)_2$+DW	10	2.1 (1.0-7.0) [a]	2.1 (1.0-8.0) [a,A]
4. GSE + DW	10	1.0 (1.0-6.0) [a]	16.0 (9.0-16.0) [b,B]
5. GE + DW	10	2.0 (1.0-6.0) [a]	8.5 (2.0–16.0) [b,C]
6. $Ca(OH)_2$+CHX	10	2.0 (1.0-6.0) [a]	2.0 (2.0-8.0) [a,A]
7. GSE + CHX	10	1.5 (1.0-6.0) [a]	16.0 (9.0-16.0) [b,B]
8. GE + CHX	10	1.5 (1.0-6.0) [a]	9.0 (2.0-13.0) [b,C]

* After intragroup analysis, different lowercase letters represent statistical significant difference of change values between Baseline and 14 days period of observation. After intergroup analysis, different uppercase letters represent statistical significant difference of change values after 14 days of proposed treatments.

** DW= distilled water; CHX= chlorhexidine; $Ca(OH)_2$= calcium hydroxide; GSE= grape seed extract; GE= ginger extract.

Discussion

The bacterial growth model of *E.faecalis* was based on previous study[10] and this microorganism was chosen because of its ability to penetrate dentinal tubules and colonise the root canal by biofilm[12]. According to Guerreiro-Tanomaru *et al.*[12] (2013), 14 days is a sufficient period for the formation of a biofilm of *E. faecalis* on dentin. For this reason, a 14-day culture period was adopted. Moreover, counting of CFUs was used to calculate the percentage reduction of *E.faecalis* because it allows bacterial quantification from the root canals in an acceptable way[13].

The antimicrobial mechanism of $Ca(OH)_2$ is known to be dependent on the dissociation and diffusion of hydroxyl ions, elevating the pH and the inhibiting enzymatic activities[4]. Previous studies reported that 14 to 21 days are necessary to obtain an elevated pH and effective antimicrobial activity of $Ca(OH)_2$[4]. For this reason, 14 days of maintenance of the tested protocols in the root canals was adopted in the present study.

Neither of the intracanal dressings were able to promote complete elimination of *E.faecalis*, being in accordance with previous studies where *E.faecalis* was not completely eliminated using decontamination strategies[7,8,10]. *E.faecalis* is a facultative anaerobic microorganism that is highly resistant and usually found in cases of failure of endodontic therapy[14]. It shows virulence factors, ability to persist with nutrients limitation and survival starvation[15]. Moreover, this microorganism colonises the root canals by biofilm formation, being difficult its removal[12]. These characteristics explain the *E.faecalis* survival rates in the present study.

Previous studies showed that *E.faecalis* remains viable after exposure to $Ca(OH)_2$ [7]. It is in accordance with the present study, where the group 3($Ca[OH]_2$+DW) was less effective against *E.faecalis* after 14 days of exposure. The ions released by $Ca(OH)_2$-based intracanal dressings need to diffuse and reach concentrations to present antimicrobial action. Moreover, physical and biological barriers such as the buffer effect of dentin, remaining tissues and fluids can decrease the antimicrobial activity of $Ca(OH)_2$ [16]. For these reasons, the present study proposed new alternatives to increase the elimination of *E.faecalis*.

The groups that added 2% CHX showed higher reduction of *E.faecalis*. It confirms the first hypothesis of the study and are in accordance with previous studies, which obtained an effective elimination of *E.faecalis* by addition of 2% CHX to intracanal dressings[7]. Its antimicrobial mechanism occurs by the interaction between CHX molecules (positively charged) and bacterial cellular wall (negatively charged). Then, CHX induces changes in cellular osmotic equilibrium, resulting in the precipitation of cytoplasmic content and the death of microorganisms[8]. Therefore, the antimicrobial properties of tested intracanal dressings and CHX may explain the greater elimination of *E.faecalis* from these groups.

The GSE presents in its composition proanthocyanidins and phenolic components, which act in structural components of the bacterial cell wall[17]. It can help to explain the results of the present study, where no statistically significant difference was observed between groups 4(GSE+DW) and 7(GSE+CHX), revealing that GSE showed an elevated percentage reduction of *E.faecalis*, even with no addition of 2% CHX. Moreover, the proanthocyanidins preserves the mechanical properties (10) and stabilises the collagen degradation of the dentin[18]. It contributes to the maintenance of dentinal substrates for the root canal filling and coronal restoration using

resin materials. It provides satisfactory bond strength and prevents microbial leakage, creating a favourable environment for regeneration in the periapical region[18].

GE is used in dentistry due its anti-inflammatory and antimicrobial activity[19]. The group 5(GE+DW) showed a better ability to reduce *E.faecalis* when compared to group 3($Ca[OH]_2$+DW). Furthermore, the association of GE and CHX (group 8) resulted in the highest mean percentage reduction of *E.faecalis* (99.27%). These findings are according with previous study, where the use of GE resulted in great reduction of *E.faecalis* from root canals[11]. It is known that *gingerol* is the main active substance of GE and is responsible for the beneficial properties of this product[19], such as antibacterial activity against endodontic pathogens[11] and specific mediated immune response[20].

Several methods are advocated to evaluate the colour change of dental structures[21,22]. The digital spectrophotometry was used in the present study because it is a precise method in the measurement of dentin coloration[22]. The method of attributing scores corresponds to a new proposal to assess the colour change, since the device itself measures and assigns a score, and by the difference before and after the tested protocols it is possible to detect whether these color changes have occurred. In this scenario, the colour change was more significant in the groups of GSE-based intracanal dressings, with no agreement with the second hypothesis of the present study. It can be explained by the darkened coloration of this substance due to the presence of tannins in its composition[23], and because of the pasty consistency, which facilitates its adherence to the dentin structure. Moreover, the final washing of samples was performed with DW. If a washing with substances with ability to promote smear layer removal had been performed, the results could be different and the colour change might not have been as significant as those shown by the present study. Anyway, higher care is necessary to promote an effective removal of this intracanal dressing, since the colour change present in the dentinal surface can be reflected in the dental enamel. In this case, despite an effective removal of microorganisms, aesthetics are compromised and this will interfere with the degree of satisfaction of the patient undergoing this therapeutic modality.

Besides the aesthetic damage, the dentine colour change promoted by GSE and GE-based intracanal dressings may have another implication, promoting the formation of a chemical smear layer and obliteration of the dentinal tubules. Then, a reduction of dentinal permeability [24] and bond strength of filling material to the root dentin[25] may occur, making it difficult to perform an adequate filling. Therefore, further studies evaluating the use of final irrigants with ability for smear layer removal[24,25] are required, providing an effective removal of these intracanal dressings at the same time that it is removing the microorganisms from root canal system.

Conclusion

Despite the limitations of present study, it was possible to conclude that the addition of 2% CHX improved the antimicrobial activity of GE-based intracanal dressing whereas its addition did not improve the antimicrobial activity of GSE-based intracanal dressings; moreover, these protocols induced significant colour changes in the dentinal structure.

- The authors deny any conflicts of interest.
- We have no financial affiliation (e.g., employment, direct payment, stock holdings, retainers, consultantships, patent licensing arrangements or honoraria), or involvement with any commercial organisation with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years. Any other potential conflict of interest is disclosed.

Resumo

Objetivo: avaliar a atividade antimicrobiana de medicações intracanais e sua influência na alteração da cor dentinária. Materiais e métodos: oitenta dentes humanos extraídos unirradiculares foram seccionados e divididos em oito grupos (n = 10), de acordo com os protocolos de medicação intracanal inseridos nos canais radiculares: água destilada G1 (DW); G2-2% de gel de clorexidina (CHX); hidróxido de cálcio G3 – (Ca [OH] 2) + DW; extrato de semente de uva G4 (GSE) + DW; extrato de gengibre G5 (GE) + DW; G6-Ca (OH) 2 + CHX; G7 – GSE + CHX; e G8-GE + CHX. A atividade antimicrobiana foi avaliada por contagem de unidades formadoras de colônias (UFCs) e as alterações de cor dentinária foram avaliadas por espectrofotometria digital. Os dados foram analisados estatisticamente por ANOVA one-way, seguida pelo teste *post hoc* de Tukey (avaliação antimicrobiana) e Wilcoxon não paramétrico, seguido pelo teste de Mann-Whitney-U (avaliação da mudança de cor) (α = 0,05). Resultados: a maior redução bacteriana foi observada nos grupos 4, 6, 7 e 8, sem diferença significativa entre eles ($p < 0,05$). Os grupos 4 e 7 apresentaram as maiores medianas da alteração da cor dentinária ($p < 0,05$). Conclusão: a adição de CHX melhorou a atividade antimicrobiana da medicação intracanal baseado em GE, sem efeito na medicação intracanal baseado em GSE; além disso, esses protocolos induziram alterações significativas na cor dentinária.

Palavras-chave: Hidróxido de cálcio. Clorexidina. Extrato de gengibre. Extrato de semente de uva.

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