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Ex vivo microbial leakage after using different final irrigation regimens with chlorhexidine

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ABSTRACT

Objective: To assess the influence of final irrigation protocols with chlorhexidine in the coronal leakage of *Enterococcus faecalis* in filled root canals. Material and Methods: Seventy single-root canals from extracted teeth were prepared using ProTaper instruments. The irrigation protocol accomplished an alternating irrigation with 5 mL of 2.5% sodium hypochlorite (NaOCl) and 17% EDTA between each file. The teeth were randomly divided into four experimental groups (n=15) according to the final irrigation regimen: group 1, without final irrigation; group 2, irrigation with 10 mL 2.0% chlorhexidine (CHX); group 3, with a final application of EC40™; and group 4, irrigation with the combination (1:1) of 0.2% CHX + 0.1% cetrimide (CTR). All the teeth were mounted in a two-chamber apparatus and the coronal access was exposed to *E. faecalis*. The presence of turbidity in the BHI broth over a period of 180 days was observed. The Friedman test was used for statistical analysis. Results: EC40™ varnish showed the least leakage at 180 days, and was statistically similar to 2% CHX. No significant differences were observed between the group without final irrigation and the 2% CHX group or 0.2% CHX + 0.1% CTR. Conclusions: In this *ex vivo* study, EC40™ showed the longest delayed coronal leakage of *E. faecalis*, although without significant differences from 2% CHX.

Key words: Bacterial leakage. Cetrimide. Chlorhexidine. *Enterococcus faecalis*. Irrigation.

INTRODUCTION

The use of irrigating solutions in root canal treatments is essential to dissolve organic tissue, to eliminate bacteria and to remove the smear layer formed on root canal surfaces by the action of endodontic instruments[40]. The alternating use of sodium hypochlorite (NaOCl) with chelating agents, like ethylenediaminetetraacetic acid (EDTA) or citric acid, has been recommended during root canal preparation to this end[25]. Chlorhexidine (CHX) has also been recommended as a root canal irrigant because of its antimicrobial activity, its capacity to adhere to dental hard tissues, its gradual release (substantivity), and its low grade of toxicity[22]. Kishen, et al.[21] (2008) showed that the bacterial adherence to dentin was significantly influenced by the last irrigant used on the root canal dentin; thus, 2% CHX used as the final solution, alone or after 17% EDTA-5.25% NaOCl, reduced the adherence of *Enterococcus faecalis.*

Vivacqua-Gomes, et al.[35] (2002) reported a decrease in coronal leakage in root canals irrigated with a combination of 1% NaOCl and 17% EDTA, or

with 2% CHX gel, than for those irrigated with only 1% NaOCl or with 1% NaOCl followed by 2% CHX gel. When applied in the gel form, a concentration of 2% CHX may exert antimicrobial activity[15,29] and delay recontamination of the root canals[6,18]. CHX varnishes have mainly been used for the control of dental caries. The EC40™ varnish has 35% CHX, 27% sandarac resin and 38% ethanol and is able to effectively reduce plaque acidogenicity[17]. EC40™ can penetrate and seal tubules in dentin[2], reducing the level of mutans streptococci in exposed root surfaces[38]. Nonetheless, we found no studies assessing the efficacy of EC40™ in root canals against *E. faecalis*.

CHX can be used alone or combined with surfactant agents such as cetrimide (CTR)[9]; in turn, CTR is a cationic surfactant largely used in endodontics in conjunction with other irrigants. When combined with CHX, it is capable of eliminating *E. faecalis* planktonic cultures[24] as well as biofilms[3]. Considering the antimicrobial efficacy of CHX in root canals, the objective of the present study was to assess the influence of different final irrigation protocols, using diverse concentrations and CHX vehicles, in the coronal leakage of *E. faecalis* in filled root canals.

MATERIAL AND METHODS

Seventy freshly extracted mandibular incisor teeth with single, straight root canals and fully developed apices were collected. Any remaining tissue was mechanically removed using a curette, without producing damage to the root surface. The teeth were cleaned with 0.5% NaOCl and then stored in 0.9% sterile normal saline solution at room temperature until use.

The crowns were removed and the coronal surfaces of the root were sectioned perpendicular to the long axis of the root using a turbine handpiece and a diamond bur. To ensure uniformity in the samples, the root length was set at approximately 15 mm from the coronal surface to the apex of the root. The protocol was approved by the Ethics Committee of the University of Granada.

Root canal preparation

All the teeth were prepared by the same operator. The patency of each canal was confirmed by inserting an ISO size 10 K file (Dentsply Maillefer, Ballaigues, Switzerland) until the tip was just visible at the apical foramen. The working length was determined by subtracting 1 mm from the length. The root canals were prepared using ProTaper instruments (Dentsply Maillefer) with the crown-down technique recommended by the manufacturer; all were enlarged with a F3 file (finishing file number 3; taper 0.09-0.05; size 30). A size 10 K file was used between each ProTaper instrument to verify the patency of the canal.

Irrigation protocols

Next, the irrigation was carried out using a 3 mL Luer-Loc syringe coupled to a 30-gauge needle tip placed passively into the canal up to 2 mm from the apical foramen without binding. Alternating irrigation of 5 mL of 2.5% NaOCl and 5 mL of 17% EDTA solutions was performed between each file. After the root canal preparation, the teeth were flushed with 10 mL of 2.5% NaOCl and then randomly divided into four experimental groups (n=15) according to the final irrigation protocol. Group I was without final irrigation. The other three groups, before the final irrigation regimen, were flushed with 5 mL distilled water and dried with F3 paper points (Dentsply Maillefer). Group II received a final flush with 10 mL of 2.0% CHX solution; Group III a final application of EC40™ varnish (Biodent, Nijmegen, The Netherlands) using a microbrush at an established length of 15 mm from the canal orifice; and Group IV, a final flush with 10 mL of 0.2% CHX + 0.1% CTR. To prepare this combination, both solutions were prepared at double concentration and mixed 1:1 to obtain the desired concentrations in the mixture. All the root canals were dried after the final irrigation protocols with F3 absorbent paper points.

Ten teeth served as controls: 5 positive (roots that were not obturated) and 5 negative (teeth with intact crowns).

The root canals were filled using a F3 master gutta-percha point (Dentsply Maillefer) and AH Plus® sealer (Dentsply Maillefer). Lateral condensation was performed using a #25 spreader (Dentsply Maillefer). The gap left by the spreader was filled with #25 gutta-percha points. The excess gutta-percha was cut off using a hot instrument, and the coronal portion of the warm gutta-percha was firmly condensed vertically. All these samples were stored at 37ºC for 72 hours to allow the sealer to set.

Microbial leakage test

The external surfaces of all teeth, except 2 mm around the apical foramen, were covered with two layers of nail varnish in order to prevent bacterial leakage through the lateral canals. The negative controls were fully covered by two layers of varnish.

The microbial test consisted of a 2-chamber method[10]. The upper chamber contained 1.5 mL Eppendorf plastic tubes (Elkay, Shrewbury, MA, USA) tapered and cut to receive the root end. The interface between the tooth and the Eppendorf tube was put into the rubber cork of a penicillin bottle previously cut to fit inside the lower chamber. The junctions between the root, the Eppendorf tube and the rubber cork were sealed with cyanoacrylate

adhesive (Super Glue-3, Henkel Ibérica, S.A., Barcelona). The mounts were sterilized for 45 minutes in hydrogen peroxide gas plasma (Sterrad-50, Advanced Sterilization Products, Johnson & Johnson, Irvine, CA, USA).

After sterilization, the apparatus was placed in a glass flask (the lower chamber) containing sterile Brain Heart Infusion broth (BHI, Scharlau Chemie S.A., Barcelona, Spain). The root tip had 2-3 mm immersed in the broth. The junctions between the Eppendorf tubes and the glass flasks were tightly sealed with Parafilm M™ (Pechiney Plastic Packaging, Chicago, IL, USA) and cyanoacrylate adhesive.

An initial bacterial suspension containing 6 x 10^8 CFU/mL of *E. faecalis* (ATCC 29212) was obtained in a turbidimeter (bioMérieux S.A., Densichek Plus, NC, USA). The Eppendorf reservoirs were filled with 1 mL of the initial suspension keeping the bacterial suspension in contact with the coronal portion of the filled roots. The mounts were always handled in sterile conditions under a laminar flow hood (Nuaire, Plymouth, MN, USA) to avoid bacterial contamination. They were placed in an oven at 37ºC during a period of 180 days. The culture medium in the upper chamber was replaced with freshly grown broth twice a week. The viability and purity of the bacteria were checked every week by seeding some in blood agar plates and observing the colony morphology.

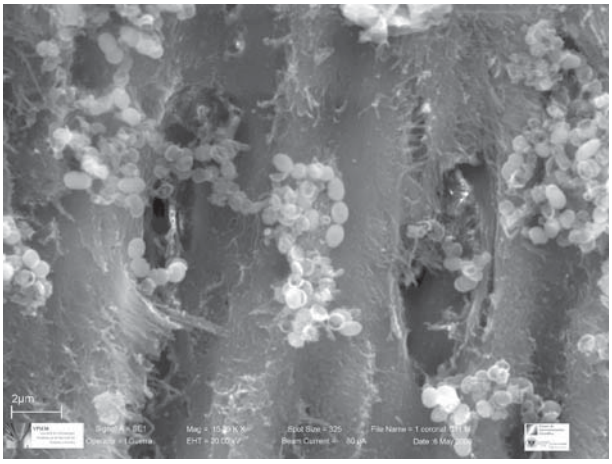

Figure 1- Scanning electron microscopy (SEM) micrograph. *E. faecalis* biofilm attached to the root canal wall and entering the dentinal tubule

The lower chambers of all mounts were observed daily, the turbidity time was recorded for each specimen as an indicator of entire root canal contamination. Once turbidity was present, a sample of the turbid broth was streaked onto blood agar plates and the bacteria were identified to ensure that there was no contamination other than *E. faecalis*. The presence of *E. faecalis* in the root canal walls was confirmed under scanning electron microscopy (Figure 1).

Statistical analysis

The number and percentage of specimens not leaking were determined for each group in the five evaluation periods (45, 60, 90, 120 and 180 days). Since the assessment of the days of bacterial leakage did not show a normal distribution, the Friedman test was used to evaluate the significance of the differences over time for the four irrigating protocols, as well as for the paired comparisons. Moreover, the significance and sign of the slope regression over time served to study the intra-group behaviour of the irrigating protocol. The level of statistical significance was set at 5%. All statistical analyses were performed with the SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 shows the percentage of specimens with no leakage at the different periods of evaluation, and the comparisons among the groups. No leakage was observed in the negative control group at any time period of study. The positive control group leaked on the second day.

The global comparison among the groups, simultaneously taking into account the five evaluation periods using the Friedman global model, gave significant differences with a value

Table 1- Specimens not leaking in the different time periods (initial sample n=15 in each group)

	Evaluated times (days)				
	45	60	90	120	180
FINAL PROTOCOL	n (%)	n (%)	n (%)	n (%)	n (%)
2.5% NaOCl*	13 (86.7)	12 (80.0)	11 (73.3)	9 (40.0)	3 (20.0)
2% CHX*,**	15 (100.0)	15 (100.0)	10 (66.7)	7 (46.7)	3 (20.0)
EC40™ varnish**	15 (100.0)	15 (100.0)	14 (93.3)	9 (60.0)	5 (33.3)
0.2% CHX + 0.1% CTR*	12 (80.0)	12 (80.0)	9 (60.0)	8 (53.3)	1 (6.7)

The same superscript small asterisks show statistically similar groups according to the Friedman test. In all cases $p<0.05$ is considered a statistically significant difference.

of $p=0.021$. The results of the pair-by-pair comparisons between protocols accounting for the five evaluation periods can be viewed in the Table. EC40™ exhibited a similar behaviour to 2% CHX. Yet, in the 90 day period, the EC40™ group had only one leaked sample, in contrast with the five samples showing leakage in the 2% CHX group. EC40™ had significant differences when compared to the 2.5% NaOCl group and the group 0.2% CHX + 0.1% CTR. No significant differences were observed between the 2.5% NaOCl group and the 2% CHX group or 0.2% CHX + 0.1% CTR.

The slopes of the regression models relating to the percentage of non-leaked specimens with the time period were statistically significant and negative for all the irrigating protocols, thereby showing an intra-group reduction of non-leaking over time.

DISCUSSION

Obtaining hermetic tri-dimensional sealing of a root canal is essential for isolating the microorganisms that may remain after biomechanical preparation and preventing their entry from the oral cavity[30,32]. In this sense, coronal restoration is an important requisite for long-term endodontic success, and restorative materials should provide a permanent, leak-proof seal. Defective temporary or permanent restoration, during or after root canal treatment, is known to be a main cause of coronal leakage[8].

Several studies have shown that coronal micro-leakage can occur with any of the different obturation techniques used[10,26]. However, the irrigation procedure seems to play a key role in the success of endodontic treatment, as it can delay or decrease bacterial contamination of the treated canal[21,35]. Therefore, in order to enhance efficient antimicrobial action over time, the use of irrigating solutions with a residual antiseptic capacity, such as CHX, is recommended[22], although it is not effective for smear layer or debris removal[33].

Diverse *in vitro* methods for evaluating the sealing ability of root canal filling materials have involved the use of dyes, scanning electron microscopy, fluid filtration techniques, electrochemical methods, radioisotopes, and bacteria[34]. Evaluation of coronal leakage by bacteria provides data that are more biologically significant and clinically relevant than other methods[34]. In our study, the 2-chamber model described by Imura, et al.[19] (1997) and modified by De-Deus, et al.[10] (2005) was applied. It is a static model that does not exactly simulate clinical conditions, requiring a long period of observation, and not allowing for quantification of the number of penetrating bacteria[30]. In addition, the presence of leakage evaluated as positive turbidity is a binary variable that is not very accurate. The bacteria selected for our study was *E. faecalis*, often isolated from necrotic or improperly filled root canal systems, as well as from properly filled ones[16]. E. faecalis ATCC 29212 is a strain of reference widely used in antimicrobial susceptibility studies, and it exhibits a great capacity for forming biofilms[20].

Mandibular incisors with single oval canals were used in this study. It is known that the prevalence of long oval root canals in the apical third of mandibular incisors is ≥ 50%[39], representing a great challenge for proper cleaning and disinfection of the root canal[1,27]. In canals with these anatomical conditions, hand and rotary instruments work in reaming motion and would leave some un-instrumented recesses, which have the potential to harbour persistent bacteria[7,36]. In this sense, instruments with greater taper, like the ProTaper, are more efficient than NiTi- hand files, but in some cases, at the expense of remaining dentin-wall thickness[13]. Moreover, the quality of the root fillings in oval canal-mandibular incisors may be compromised[31]; however, different obturation techniques in oval-shaped canals have not shown statistical differences[10,11].

Preparation of root canals was similar in all the groups of our study and included sequential use of 2.5% NaOCl and 17% EDTA with a final flush of 2.5% NaOCl. However, all groups were not treated with the same amount of final irrigating solution, which may have influenced the results. The regimen of final irrigation has been shown to influence the capacity of adherence of *E. faecalis*[21]. Alternating use of NaOCl and EDTA can prove to be beneficial. NaOCl can dissolve organic matter and necrotic tissue as well as exert strong antimicrobial action[14]. In turn, EDTA is able to remove the smear layer[40] formed during preparation of the root canal, although its antimicrobial activity is still a matter of debate. Using NaOCl as the final irrigating solution is widely recommended[40], but it has not demonstrated any residual antibacterial activity[23].

A 2% CHX solution is recommended as the final irrigant in the root canal preparation[22]. Nevertheless, the use of a CHX varnish such as EC40™ has not been tested in endodontics. Our results indicate that the delay of leakage was greater in the EC40™ group, while the 2% CHX group showed an intermediate effectiveness. Vivacqua-Gomes, et al.[35] (2002) obtained better results with a 2% CHX gel than with a 2% CHX solution, suggesting the importance of the vehicle of application. Moreover, the antimicrobial substantivity of CHX depends on the number of CHX molecules available to interact with the dentin[22], and EC40™ has a very high concentration of this active agent. White, et al.[37] (1997) evaluated substantial antimicrobial activity in instrumented root canals using 2.0% and 0.12%

CHX as irrigants; their results indicated that the antimicrobial activity remaining in the 2% CHX-treated teeth was significantly greater than in the 0.12% CHX-treated teeth.

Another factor to bear in mind is the vehicle used. EC40™ can be considered a slow release device, and varnishes were formulated with the objective of prolonging the delivery of the active agent. Attin, et al.[5] (2008) observed a greater release of CHX on bovine dentin fissures treated with CHX varnishes as compared to dentin treated with CHX preparations, in gel or in solution. Their study found the varnish providing the best release of CHX to be EC40™. Attin, et al.[4] (2003) and Derks, et al.[12] (2008) compared the efficacy of different concentrations of CHX in varnish (EC40™, Cervitec) in reducing the levels of mutans streptococci, lactobacilli and in plaque formation in interproximal plaque and saliva. According to their findings, application of the highly concentrated varnish EC40™ results in a higher decrease of mutans streptococci in plaque sites and saliva. Furthermore, it should be emphasized that the 2% CHX was a solution, and since the root canals were dried with paper points, it would have been better removed than the varnish.

Although it was recently demonstrated that the association of CHX and CTR enhances the *in vitro* capacity to eradicate *E. faecalis* biofilm[3], our results for the group 0.2% CHX + 0.1% CTR showed no differences with respect to the control group. This may be due to the relatively low concentration of CHX, promoting a less intense substantive action.

Notwithstanding, high concentrations of CHX in the form of a varnish (EC40™) would appear to ensure some delay in coronal leakage. Further investigations are needed to evaluate the efficacy of different vehicles and concentrations of CHX and to assess how they might affect the complete sealing of the root canal.

CONCLUSION

In this *ex vivo* study, high concentrations of CHX in the form of a varnish (EC40™) would appear to ensure some delay in coronal leakage.

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