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Quantitative Assessment of Dentinal Tubule Disinfection in Absence of Biofilm on Root Canal Walls: An *in vitro* Study

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ABSTRACT

Introduction: This study aimed at assessing the quantitative effect of calcium hydroxide, 2% chlorhexidine gel, and 1.5% chlorhexidine linked to xanthan gel specifically against intratubular bacteria. **Methods and Materials:** Fifty-two semi-cylindrical bovine dentin specimens were infected with *Enterococcus* (*E.*) *faecalis* by centrifugation with subsequent 7-days incubation. The surface of specimens was disinfected with 3% H_2O_2. Scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and the count of bacterial colony-forming units (CFU/mg) were used to assess dentin infection. A total of 40 specimens were incubated for 2 weeks with one of the intracanal medication applied (10 samples for each group): 1) calcium hydroxide, 2) 2% chlorhexidine gel, 3) 1.5% chlorhexidine linked to xanthan gel and 4) sterile saline. Final passive ultrasonic irrigation with 3% sodium hypochlorite was performed in half of the total specimens. The effect of intracanal medications and irrigation against intratubular bacteria was assessed by bacterial culturing of dentin shavings. Two-Way ANOVA model was applied followed by post-hoc Tukey's test for multiple pair-wise comparisons of mean CFU/mg values. **Results:** SEM, CLSM, and bacterial culturing confirmed the absence of the surface biofilm on the root canal wall and showed vital intratubular bacteria at the depth up to 700 μm. Two-week application of 1.5% chlorhexidine with xanthan gel and 2% chlorhexidine gel significantly decreased intratubular bacterial counts compared with saline (P=0.0003 and P=0.0005, respectively). Subsequent passive ultrasonic irrigation with 3% sodium hypochlorite significantly reduced the number of intratubular bacteria in all groups except for the group with 1.5% chlorhexidine-xanthan gel (P=0.0054). **Conclusion:** This modified *ex vivo* model study showed ultrasonically activated irrigation with sodium hypochlorite had greater effect on intratubular bacteria counts compared with 2-week application of intracanal medications.

Keywords: Confocal Laser Scanning Microscopy; Electron Scanning Microscopy; Enterococcus faecalis; Root Canal Irrigants

Introduction

Endodontic treatment aims at reducing bacterial counts in the root canal system and preventing its recontamination [1]. Procedures allow to reduce bacterial counts in the root canal include mechanical instrumentation and application of antibacterial substances (irrigation solutions and inter-appointment dressings). With the increasing trend towards single visit endodontics, the role of inter-appointment dressings as disinfectants becomes questionable [2, 3]. At the same time, the authors of the recent Cochrane review concluded that there was no evidence to support neither single-visit nor multiple-visit root canal treatment [4]. The convenience and reduced time of a single-visit treatment may have the cost of a higher frequency of late postoperative pain and painkiller use [4]. The number of roots, time available, the clinician's skills, and the severity of the patient's symptoms are factors that can affect the number of required visits [1]. Sometimes it is impossible to perform one-

visit treatment, for example, in the case of acute periodontitis with swelling when root canals cannot be dried [1]. Therefore, clinicians face the problem of choice of the most effective intracanal medication for inter-appointment dressing.

The most common intracanal medications contain calcium hydroxide or chlorhexidine [5]. A number of experimental studies have indicated greater efficacy of chlorhexidine compared with calcium hydroxide [6-9] for disinfection of the root canals of teeth artificially infected with *Enterococcus (E.) faecalis*. A new formulation, xanthan gel chemically linked to the chlorhexidine molecule (CHX-Xanthan), has demonstrated *in vitro* its capacity to maintain adequate chlorhexidine concentrations and a highly stable pharmacokinetic profile [10]. Xanthan, a naturally derived biopolymer, is non-toxic, biocompatible, and biodegradable [11]. Chlorhexidine linked to xanthan has been effective both *in vitro* and *in vivo* for the treatment of plaque-induced periodontal diseases [12-14]. It was hypothesized that the ability of this formulation to maintain the bacteriostatic and bactericidal chlorhexidine concentrations for at least 2 weeks [13] would be beneficial in case of using it as an endodontic intracanal medication.

Many laboratory studies assessing intracanal medications focused on the antibacterial action against the surface biofilm [15, 16], or against the intratubular bacteria in the presence of surface biofilm [5, 7, 8, 17]. However, in clinical practice, the inter-appointment dressings are used after chemo-mechanical preparation and irrigation, when the main volume of biofilm is removed from the root canal surface. Therefore, it is important to assess the effect of intracanal medications on the intratubular bacteria in the absence of surface biofilm. Bacteria invading the dentinal tubules are less accessible to chemo-mechanical preparation and irrigation. These bacteria can survive for longer times causing root canal treatment failure [18].

The evaluation of the intratubular bacteria penetration and viability is a challenging task that cannot be performed by a single method. Scanning electron microscopy (SEM) allows to assess the morphology of bacteria, the presence of surface biofilm, and the depth of dentin infection [19]. Confocal laser scanning microscopy (CLSM) allows to assess bacterial viability [20]. However, both aforementioned methods fail to evaluate the whole volume of dentin as they inspect particular tooth split [18]. Bacterial culturing of dentin shavings with CFU (colony forming units) counting allows for quantitative assessment of the numbers of cultivable bacteria in the whole volume of acquired dentin shavings. This method was also widely used in the previous studies of endodontic antiseptics and intracanal medications [5, 21-23]. However, the method described in these studies is not suitable for the evaluation of the antibacterial effect specifically against intratubular bacteria. Even if the root canal was irrigated with sterile saline before acquiring of the dentin shavings, the superficial portion of shavings could still contain both intratubular bacteria and bacteria from the surface biofilm. Therefore, it was impossible to distinguish if bacterial growth was due to the presence of intratubular bacteria, or due to the presence of surface biofilm. The treatment with 3% H_2O_2 solution was previously shown to eliminate bacteria (*Escherichia coli*) from the surface of the specimen, but do not decrease bacterial counts inside the dentinal tubules [24]. Such pretreatment allows using the method of bacterial culturing to quantitatively assess the antibacterial action of various disinfectants specifically on the intratubular bacteria. Besides, the present study sought to replicate the clinical situation when surface biofilm was removed during chemo-mechanical preparation and irrigation, and the root canals were filled with intracanal medication.

The aim of the present study was to evaluate the capability of a modified experimental model without surface biofilm to quantitatively assess the effect of intracanal medications on dentinal tubules disinfection. The second aim of the study was to compare the antiseptic effect of 2-week treatment with 3 intracanal medications (calcium hydroxide, 2% chlorhexidine and 1.5% chlorhexidine linked to xanthan gel) with and without 3% sodium hypochlorite passive ultrasonic irrigation on the intratubular bacteria counts.

Materials and Methods

The local ethical committee of I.M. Sechenov First Moscow State Medical University (Sechenov University) (protocol number 01-17) approved this *in vitro* study. Figure 1 presents the study design.

The preparation of the specimens

Twenty-six freshly extracted bovine teeth (incisors) were used in the study. Each tooth was decoronated with a diamond bur in a high-speed handpiece and the pulp was removed using pulp extractors. The teeth were prepared to the form of cylinders (height 6 mm, outer diameter 6 mm, inner diameter 2 mm) with a screw-cutting machine (TOS SV18RA, TOS, Czech Republic) and then vertically split into halves using a chisel and a hummer. This resulted in 52 specimens (semicylinders). The cementum was removed during the preparation to allow better bacterial penetration. In order to remove the smear layer, the specimens were consequently immersed in the ultrasonic bath (UltraEst, GeoSoft Dent, Moscow, Russia) containing the following solutions: 1) 3% NaOCl (TechnoDent, Industries, Lahore, Pakistan), 10 min; 2)

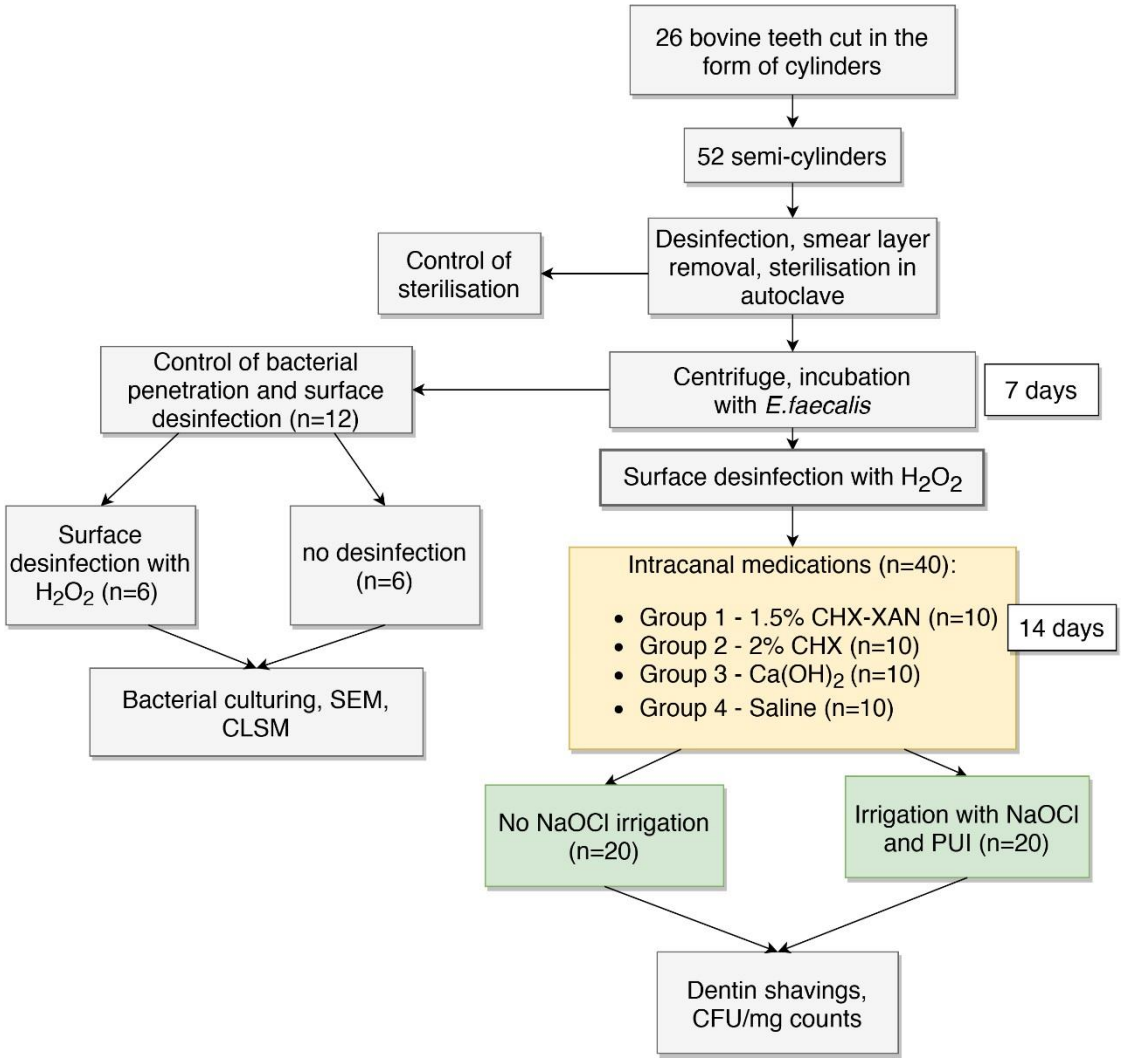

Figure 1. The design of the study has been shown (CFU-colony forming units, SEM-scanning electron microscopy, CLSM-confocal laser scanning microscopy, CHX-XAN-xanthan gel chemically linked to the chlorhexidine molecule, CHX-chlorhexidine)

sterile distilled water, 1 min; 3) 17% ethylenediaminetetraacetic acid (EDTA) (MD-Cleanser, METABIOMED, PRC), 10 min [25]; 4) sterile distilled water, 1 min; 5) 3% NaOCl 10 min; 6) sterile distilled water, 5 min [26]. Then each specimen was placed in a glass tube with TS broth (Biocompas-C, Uglich, Russia) and sterilized with a steam autoclave (Series 2100 Prestige Medical; Prestige Medical Limited, Blackburn, UK) at 121°C for 30 min. To confirm the sterility, the tubes were incubated for 24 h at 37ºC and the turbidity of the culturing media was assessed.

Inoculation

The specimens were infected with 24 h culture of *E. faecalis* (ATCC 29212) suspended in TS broth (Biocompas-C, Uglich, Russia) with 5% of defibrinated sheep blood (ECOlab, Electrogorsk, Moscow oblast, Russia). The optical density of the bacterial suspension was adjusted to obtain a concentration of 1.5×10^8 colony-forming units/mL (corresponding to 0.5 McFarland standard test) by adding TS broth. Each sample was placed into individual Eppendorf tube containing bacterial suspension and 3 cycles of centrifugation were performed: 1400 gr 5 min, 2000 gr 5 min, 3600 gr 5 min (Eppendorf MiniSpin plus; Eppendorf, Hamburg, Germany) [18]. All specimens were incubated for 7 days at 37ºC. Every 2 days a fresh TS broth with 5% of defibrinated sheep blood was added in each Eppendorf tube [19].

The confirmation of surface biofilm removal and bacterial penetration into the dentin

Twelve specimens were used to assess the presence of surface biofilm and intratubular bacteria. From these 12 specimens 6 were treated with H_2O_2 for 20 min and 6 were left untreated.

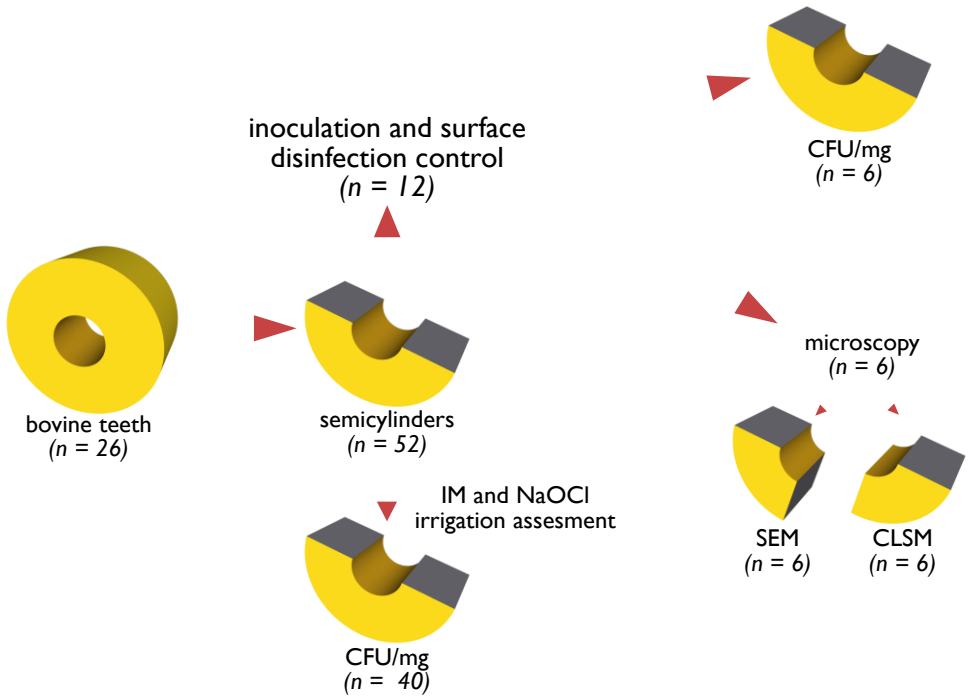

Figure 2. The shape and number of the specimens at the different stages on the study has been shown (IM-intracanal medications; NaOCl-sodium hypochlorite, CFU/mg-colony forming units per mg of dentin shavings)

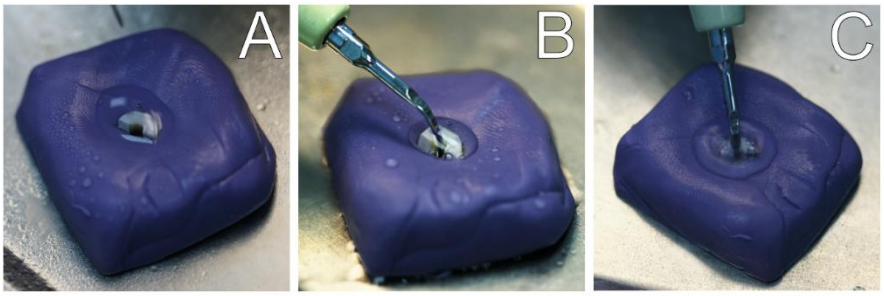

Figure 3. The irrigation of the "root canals" in silicon wells: *A*) the silicon well with specimen fitted inside and irrigating solution placed; *B*) ultrasonic tip inserted into the "root canal"; *C*) ultrasonic activation performed

Bacterial culturing method

Six specimens (3 with H_2O_2 surface treatment and 3 without) were used. The absence of surface bacteria was confirmed by culturing of: 1-sterile saline used to wash the samples; 2-sterile paper points used to dry the canals of the specimens; 3-shavings obtained by scrubbing of root canal walls with metal instrument (slowly rotating diamond bur) on Petri dishes containing brain-heart agar (Brain Heart Infusion Agar, HiMedia Laboratories, Mumbai, India). The Petri dishes were incubated for 24 h at 37°C and the presence of bacterial growth was assessed.

After an absence of bacteria on the specimens' surface was confirmed, dentin shavings were acquired from the depth of 500 µm by preparing the root canals of the specimens with diamond burs (Frank Dental) 029 in physiodispenser (70 rpm, torque 20) under a water cooling. The depth of drilling was continuously assessed by measuring the width of the specimen in several points with a sterilized micrometer. To collect the shavings an underwater cooling, the preparation was carried out to a sterile container (glass tube), in which both shavings and water were collected. Then the excess of water was removed using filter paper. Each specimen (tooth) was weighed before and after the preparation (Sartorius ED224S-RCE, Sartorius, Göttingen, Germany) and the weight of the obtained shavings was calculated. It should be noted that the specimen before the procedure was kept in TS broth, therefore it was wet. The excess of broth was removed by a filter paper. After the acquiring the

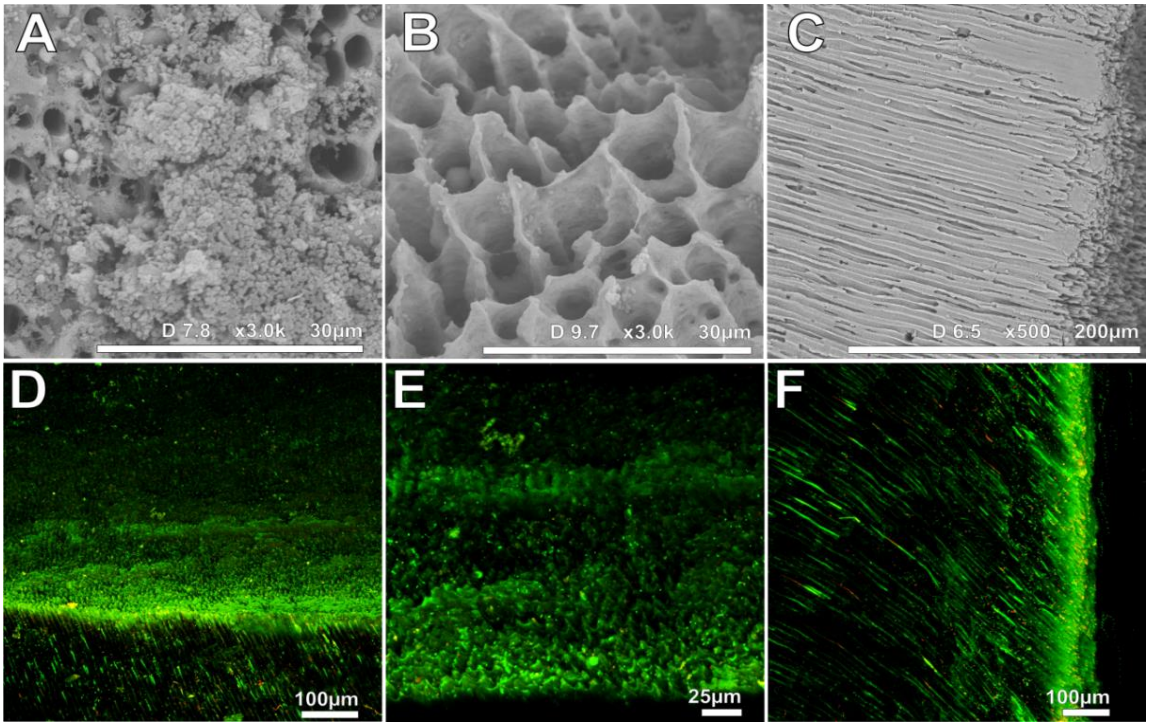

Figure 4. The assessments of the infected dentin blocks with confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM), 3000×) are shown. *A)* The root canal wall before the treatment with H_2O_2 assessed with SEM (3000×), showing visible biofilm; *B)* The absence of surface bacteria and the presence of bacteria remaining inside the dentinal tubules after H_2O_2 treatment; *C)* and *F)* The depth and density of bacterial penetration according to SEM (500×) and CLSM; *D)* and *E)* CLSM with 10× and 20× magnifications

shavings, the specimen was again placed on a filter paper in a laminar flow cabinet to remove the excess of water. The shavings were placed on agar plates and cultured (Brain Heart Infusion Agar, HiMedia Laboratories, Mumbai, India) with 5% defibrinated sheep blood for 24 h at 37°C, and CFU were counted [26].

SEM and CLSM

Six specimens (3 with and 3 without the treatment with H_2O_2) were again vertically split into halves; one half of each specimen was used for SEM and another half was used for CLSM evaluation (Figure 2).

Six halves for SEM evaluation were dehydrated through an ascending series of ethanol (30±100%), mounted on aluminum stubs with colloidal silver and stored in a silica-gel desiccator overnight. The specimens were sputter-coated with 15±20 nm gold-palladium (80:20) and inspected in a scanning electron microscope (TM3000 Hitachi; Hitachi High-Technologies Corporation, Tokyo, Japan) with various magnifications from 500× to 3000×.

The other six halves were kept for 15 min at room temperature in phosphate-buffer saline (pH 7.4) which contained 2.5 mg of SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen™ S34854; Thermo Fisher Scientific, NY, USA) and 2.5 mg of Propidium iodide (MP Biomedicals, LLC 195458, Ohio, USA). Then the specimens were inspected in a confocal laser microscope Eclipse Ti-E microscope with A1 (Nikon Corporation, Tokyo, Japan) confocal module and a Plan Fluor 10× DIC L N1 objective [20].

Both microscopic methods were used to assess the absence of surface bacteria (1), to confirm the penetration of the dentinal tubules with bacteria (2), and to assess the depth of bacterial penetration (3). The depth of bacterial penetration was measured using standard measuring tools integrated into the microscopes' default software under 1000× magnification [27].

Surface treatment with H_2O_2

The root canal surfaces of 40 specimens (used for the assessment of intracanal medications and NaOCl irrigation) were treated with 3% H_2O_2 for 20 min. The absence of surface bacteria was confirmed in each specimen by plating on Petri dishes with brain-heart agar (Brain Heart Infusion Agar, HI Media Laboratories, Mumbai, India) of 1-sterile saline used to wash the samples; 2-sterile paper points used to dry root canals of the specimens; 3-shavings obtained by scrubbing of root canal walls with metal instrument (slowly rotating diamond bur). The Petri dishes were incubated for 24 h at 37°C and the presence of bacterial growth was assessed.

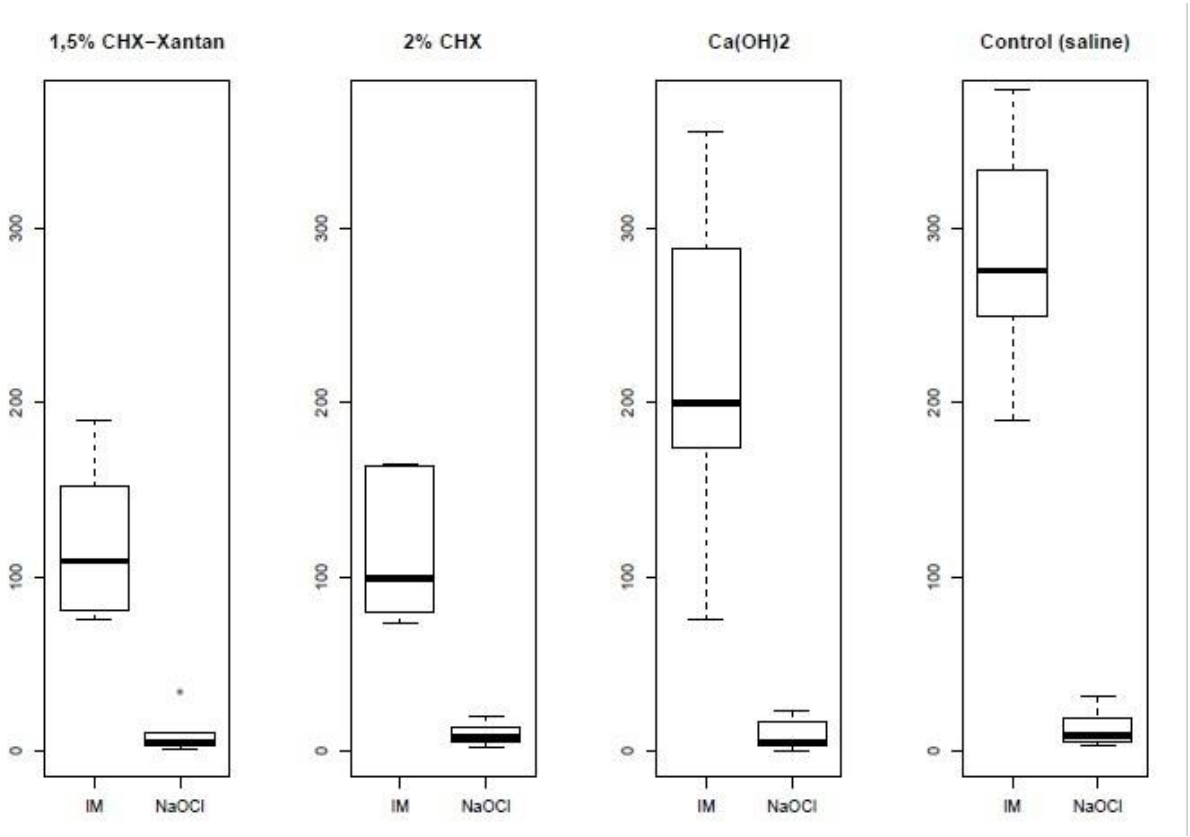

Figure 5. The CFU-counts (CFU/mg) in the dentin of specimens after the treatment with intracanal medications (IM) and after the irrigation with sodium hypochlorite (NaOCl) (CHX-XAN: - xanthan gel chemically linked to the chlorhexidine molecule; CHX: chlorhexidine; Ca(OH)2: calcium hydroxide)

Assessment of intracanal medications and irrigation

40 specimens were randomly assigned to one of the four groups (n=10) according to the intracanal medication: Group 1-1.5% chlorhexidine gel ("CHLO-SITE", Ghimas, Casalecchio di Reno, BO, Italy), Group 2-2% chlorhexidine gel (Chlorhexidine digluconate gel 2%; TechnoDent Industries, Lahore, Pakistan), Group 3- calcium hydroxide (Calcetin; TechnoDent Industries, Lahore, Pakistan), Group 4 (control)-sterile saline. The specimens were fully immersed into the Eppendorf tubes containing corresponding medications and incubated for 14 days at 37°C. After 2 weeks, chlorhexidine and calcium hydroxide were inactivated using 0.5% Tween-80 solution (Tween-80® [Polysorbate-80]; HiMedia Laboratories Pvt. Limited, Mumbai, India) and 0.5% citric acid (Citric acid monohydrate [Hi-LR]; HiMedia Laboratories Pvt. Limited, Mumbai, India), respectively. Specimens were thoroughly flushed with sterile saline. The surface treatment of the specimens was performed with 3% H_2O_2 solution. After that, the specimens in each group were randomly divided into 2 subgroups: first for the assessment of CFU/mg before the irrigation with sodium hypochlorite and second for the assessment of CFU/mg after the sodium hypochlorite irrigation. The specimens from the latter subgroups were irrigated with 3% sodium hypochlorite (3% Parcan; Septodont, Saint-Maur-des-Fossés, France). For this purpose, the root halves were placed into the cylindric wells formed out of silicon impression material to create a space for the depo of irrigating solution similar to a root canal (Figure 3). Five mL of sodium hypochlorite were used for each specimen and passive ultrasonic activation for 30 sec was performed (Piezon Master 600; EMS, Nyon, Switzerland) after the irrigation with 1 mL of sodium hypochlorite. Finally, the "root canals" were irrigated with sterile saline to remove the residuals of the irrigating solution.

Dentin shavings were acquired from the depth of 500 µm by preparing root canals with diamond burs (Frank Dental, Gmund, Germany) 029 in Physio-Dispenser (Surgic Pro Non Optic, NSK, Tochigi, Japan) 70 rpm, torque 20 under water cooling. The specimens were weighed before and after the preparation and the weights of acquired dentin shavings were calculated. The shavings were collected in the individual sterile glass tubes with TS broth and then placed on agar plates (Brain Heart Infusion Agar, HiMedia Laboratories, Mumbai, India) with 5% defibrinated sheep blood and cultured for 24 h at 37°C. CFU per mg of dentin shavings were counted [24].

Statistical analysis

The data were imported into Statistical Software (IBM SPSS Statistics version 22; SPSS Inc, Chicago, IL, USA). The normality of the distribution of CFU counts in the study groups was confirmed by the Shapiro-Wilk test. Leven's test has shown the equality of error variances. To assess the effect of intracanal medications on bacterial counts, two-ANOVA was performed (Tables 1 and 2), followed by the Tukey post hoc test for pair-wise comparisons (Table 3). Cohen's F effect size was calculated for all independent variables in the model. The level of significance was set at 5%. Sample size calculations were based on the preliminary study. The preliminary mean CFU/mg in groups after the application of calcium hydroxide and 2% chlorhexidine gel were 220±45 and 110±38, respectively. To get 80% power with α-level set at 0.05, minimum sample size of 4 group specimens has been defined (on the basis of mean and standard deviation values from the results of preliminary study).

Results

Bacterial penetration and surface treatment with H_2O_2

All bacteriological samples taken from the group treated with H_2O_2 (1- sterile saline used to wash the samples; 2- sterile paper points used to dry the canals of the specimens; 3- shavings obtained by scrubbing of root canal walls with a metal instrument) were negative. The corresponding samples from the specimens without H_2O_2 treatment were positive.

Both SEM (was used for the measurement of penetration depth) and CLSM (was used for the confirmation of the viability of intra-tubular bacteria) demonstrated comparatively dense and uniform bacterial penetration into the dentin with an average depth of 700 µm from the root canal lumen (Figure 4C, F). The depth of bacterial penetration was measured using default measurement tool in the microscopes' software. According to the results of SEM, the root canal wall surface was free from bacterial biofilm after H_2O_2 treatment (Figure 4B) in contrast to the teeth without such treatment (Figure 4A). CLSM images of teeth after surface H_2O_2 treatment confirmed the viability of bacteria inside the dentinal tubules even close to the root canal lumen (Figure 4D-F). No differences in the green and red signal intensity were detected on CLSM images of teeth with and without surface treatment with H_2O_2 (even at the maximal possible magnification). Therefore, the absence of surface bacteria without the elimination of intra-tubular bacteria was confirmed by both bacteriological method and SEM allowing to further assess the influence of intracanal medications specifically on the intratubular bacteria.

Assessment of intracanal medications and irrigation with sodium hypochlorite

Microbiological culturing showed that none of the medications were capable of eliminating *E. faecalis* from dentinal tubules either after 2 weeks of treatment with intracanal medications or after the passive ultrasonic irrigation with sodium hypochlorite solution (Figure 5, Table 1). According to two-way ANOVA, the type of medication had a significant effect on dentin infection of the specimens (P=0.002) with the effect size 0.77. Post-hoc Tukey test revealed significantly lower CFU/mg numbers in the groups with 1.5% and 2% chlorhexidine compared with the control group (P=0.0003 and P=0.0005, respectively). The CFU/mg counts in the calcium hydroxide group didn't differ significantly compared with the control group (P=0.4821). Irrigation with sodium hypochlorite had the most prominent effect on CFU/mg (P<0.001): the effect size was 1.86. We observed a substantial decrease in CFU/mg in groups with 2% chlorhexidine gel, calcium hydroxide and saline after the irrigation with sodium hypochlorite (P=0.037, P<0.001, and P<0.001, respectively). The interaction of the two factors influenced CFU/mg significantly (0.003) with the effect size 0.74. No significant differences were found among groups after sodium hypochlorite irrigation (P>0.05 in all groups).

Table 1. The number of *E. faecalis* (CFU/mg) in the dentin of specimens treated with different intracanal medications.

Medication	CFU/mg after IM		CFU/mg after NaOCl irrigation	
	Mean (SD)	95% CI	Mean (SD)	95% CI
1.5 % Chlorhexidine gel-xanthan	116.2 (45.1)^{A}	60.2-172.2	9.8 (7.2)^{C*}	0.83-18.8
2% Chlorhexidine gel	121.4 (48.9)^{A}	60.6-182.2	10.6 (13.5)^{C*}	-6.2-27.4
Calcium hydroxide	218.8 (108.2)^{B}	84.5-353.1	9.6 (9.9)^{C*}	-2.7-21.9
Saline (control)	286.0 (73.7)^{B}	194.4-377.6	13.4 (11.6)^{C*}	-1.0-27.8

* *statistically significant differences compared with CFU without the irrigation with sodium hypochlorite;* ^{A,B,C} *Heterogeneous subsets indicating the statistically significant differences between different intracanal medications; CFU-colony-forming units; IM-intracanal medications; SD-standart deviation; CI-confidence interval*

Table 2. The results of two-way ANOVA

	F value	*P*-value
Group (type of IM)	6.333	0.00170
NaOCl irrigation	110.880	$6.31e^{-12}$
Interaction(Group*NaOCl irrigation)	5.848	0.00265

IM-intracanal medications; NaOCl-sodium hypochlorite

Discussion

The present study assessed the effect of intracanal medications and NaOCl irrigation specifically on the intra-tubular bacteria in the absence of root canal wall biofilm.

The experimental model used in the present study was a modification of the models proposed by Haapasalo and Ørstavik and Ma *et al.* [26]. The main difference is the introduction of surface treatment with H_2O_2 to remove surface biofilm while preserving viable intra-tubular bacteria. The aim of the surface biofilm removal was to prevent the contamination of the dentin shavings with bacteria from the surface. We also sought to replicate the real clinical situation when the intracanal medications are applied after chemo-mechanical preparation of the root canal, *i.e.* after the maximal possible removal of the biofilm from the root canal walls.

In the pilot study (data not published) we compared different antiseptics and treatment times for the removal of *E. faecalis* surface biofilm while preserving intra-tubular bacteria. Twenty-min treatment with 3% H_2O_2 solution proved to be effective for this purpose. In the present study, the absence of surface biofilm was confirmed by the three methods: CFU-counts, SEM and CLSM. Firstly, all bacteriological samples from the root canal surface were negative. Secondly, SEM demonstrated the absence of bacterial biofilm on the root canal wall and the presence of bacteria in the dentinal tubules. Thirdly, CLSM confirmed viability of the intra-tubular bacteria. Both microscopic methods allowed to assess the depth of bacterial penetration (which was up to 700 μm). Besides, further bacterial culturing of the dentin shavings obtained from the depth 500 μm resulted in the growth of bacterial colonies, confirming their viability. These data prove that in the present experimental model there was no surface biofilm and there were viable intra-tubular bacteria at the depth. This allowed assessing the antiseptic effect of intracanal medications and NaOCl specifically on the intra-tubular bacteria.

The majority of the recent studies of root canal biofilms and dentin infection used confocal laser scanning microscopy. However, CLSM doesn't allow to assess bacteria in the whole volume of dentin as it inspects only particular split surfaces [19]. In the present study, we used CFU to compare the antibacterial effect of intracanal medications and NaOCl irrigation. Although this method counts only reproducing cells and can over-quantify killed cells that entered viable but nonculturable state [28], it represents cultivable bacteria in the whole volume of the acquired dentin shavings. The previous studies using culturing of the dentin shavings did not include the step of surface disinfection; therefore, the samples were possibly contaminated with surface bacteria. It was reported that the root canals were irrigated with sterile saline before bacterial sampling. Although this irrigation could remove unattached bacteria, it was insufficient to remove the attached bacterial biofilm. Therefore, the resultant CFU-counts might not reflect the presence of bacteria solely in the dentinal tubules.

In the present study, we quantitatively assessed the influence of the 2-week application of three intracanal medications (1.5% chlorhexidine-xanthan gel, 2% chlorhexidine gel, and calcium hydroxide paste) and subsequent 3% sodium hypochlorite irrigation on the intra-tubular bacteria. Both medications containing chlorhexidine significantly reduced bacterial counts compared with control group (saline). After the subsequent irrigation with sodium hypochlorite, there was a significant decrease in bacterial counts in all study groups. However, neither the intracanal medications nor sodium hypochlorite irrigation could eliminate *E. faecalis* from the dentinal tubules.

Table 3. Pair-wise comparisons between different groups (Tukey's test)

Compared groups		Diff	Lower	Upper	*P*-Value Adjusted
CHX-XAN	2%CHX	-5.2	-112.7	102.3	0.9999
CHX-XAN	Ca(OH)2	-102.6	-210.1	4.9	0.0700
CHX-XAN	Saline	169.8	62.3	277.3	0.0003*
2%CHX	Ca(OH)2	97.4	-10.1	204.9	0.0980
2%CHX	Saline	164.6	57.1	272.1	0.0005*
Ca(OH)2	Saline	67	-40.3	174.7	0.4821
2%CHX+SH	Ca(OH)2+SH	1.0	-106.5	108.5	1.0000
CHX-XAN+SH	2%CHX+SH	0.2	-107.3	107.7	1.0000
2%CHX+SH	Saline+SH	3.8	-103.7	111.3	1.0000
CHX-XAN+SH	Ca(OH)2+SH	-0.8	-108.3	106.7	1.0000
Ca(OH)2+SH	Saline+SH	2.8	-104.7	110.3	1.0000
CHX-XAN+SH	Saline+SH	3.6	-103.9	111.1	1.0000
CHX-XAN	CHX-XAN+SH	-106.4	-213.9	1.1	0.0540
2%CHX	2%CHX+SH	-111.8	-219.3	-4.3	0.0369*
Ca(OH)2	Ca(OH)2+SH	-208.2	-315.7	-100.7	0.0000126*
Saline	Saline+SH	-272.6	-380.1	-165.1	0.0000001*

** Statistically significant*

Our results are in agreement with some of the previous studies showing higher efficacy of chlorhexidine gel compared with calcium hydroxide for the reduction of CFU both on the root canal surface [15, 16] and in the dentin shavings [5-8, 17, 29, 30].

In the study by Menezes *et al.* [16], first microbiological sampling from the root canals with paper points revealed no CFU after the application of calcium hydroxide and 2% chlorhexidine; while second sampling (after 7 days) reveled 5.8 and 0 CFU in these groups, respectively [16]. These results are similar to those reported by Atila-Pektaş *et al.* [15]. In their study 1-week application of 2% chlorhexidine gel resulted in the absence of bacterial growth in the samples acquired with sterile paper points. Minor bacterial growth (less than 100 CFU) was reported after 1-week calcium hydroxide application. In should be noted that samples acquired with paper points reflect the presence of biofilm on the root canal wall, but not the presence of intra-tubular bacteria.

The effect of the tested medications on the intra-tubular bacteria is similar to their effect on the surface biofilm. Chlorhexidine gel had a significantly higher antimicrobial efficacy as measured by the number of CFU counted after the cultivation of dentin shavings obtained from the depths of 200 µm [6, 8, 29], and 400 µm [7]. In some studies, the application of chlorhexidine for 1-5 [29] or 14 days [17] resulted in the absence of growth in the samples of dentin shavings from the depths 200-400 µm, while there was growth after the application of calcium hydroxide. Chlorhexidine gel also showed better antibacterial efficacy than calcium hydroxide (7 days) according to the measurement of optical density of cultivated dentin shavings [30]. The antibacterial effect of calcium hydroxide is mainly obtained from its high alkalinity. Besides, it depends on the ability to defund into the dentinal tubules and to sustain the pH level sufficient to exert antibacterial activity. It has been shown that nano-calcium hydroxide had significantly greater depth of dentinal tubules penetration than conventional calcium hydroxide [31, 32]. The introduction of nano-size particles provides the material with unique physicochemical properties and allow to achieve greater therapeutic efficacy [33].

A xanthan gel chemically linked to the chlorhexidine molecule had previously demonstrated *in vitro* its capacity to maintain adequate chlorhexidine concentrations and a highly stable pharmacokinetic profile inside the periodontal pocket [10]. It has been shown that in xanthan-based syringable gel system (ChloSite, Casalecchio di Reno, Bologna, Italy) chlorhexidine digluconate maintained the bacteriostatic and bactericidal concentrations for at least 2 weeks (>0.10 µg/ml) [13]. According to Paolantonio *et al.* [12], xanthan-based chlorhexidine gel can be used during scaling and root planning with better outcomes. We hypothesized that a prolonged release of chlorhexidine from the formulation linked to xanthan gel for 1-2 weeks [13] would be beneficial for the elimination of bacteria in the dentinal tubules. However, we found no increase in antibacterial action of CHX-xanthan formulation compared with the conventional 2% chlorhexidine gel. These results are in agreement with the study by Perinetti *et al.* [14] in which the effect of chx-xanthan formulation did not differ from the effect of 1% chlorhexidine gel in microbiological tests. On the other hand, in the present study, the tested medications containing chlorhexidine were equally effective despite the lower concentrations of chlorhexidine in the CHX-xanthan formulation.

We found that irrigation with sodium hypochlorite was effective in decreasing bacterial counts irrespective of the intracanal medication used. This is in agreement with the previous studies showing that sodium hypochlorite is capable of eliminating of more than 99% of bacteria from the root canal system [34]. It has been shown to penetrate the dentinal tubules to the depth up to 376 µm depending on the concentration, temperature of the solution, and the time of exposure [35] . The depth of penetration could also depend on the viscosity of the substance: it was shown that NaOCl gel penetrated less into dentinal tubules of bovine teeth than NaOCl solution [36]. The depth of NaOCl penetration was assessed by bleaching of the stain and measured by light microscopy [35], or by CLSM [37]. It was also shown in CLSM study that 1% NaOCl solution was effective against bacteria mostly at the first 200-µm zone from the main canal, while after the irrigation with 6% NaOCl, dead bacteria could be seen throughout the 500-µm-deep zone [20].

We used passive ultrasonic irrigation, because it was reported to be the most widespread method of agitation of the irrigants in dentistry [38]. It promotes the cavitation effect by producing bubbles that rupture close to the dentin walls, in addition to the formation of a micro-acoustic current that promotes hydrodynamic agitation of the liquid potentiating cleaning [39]. The previous studies have indicated that sonic and ultrasonic activation increased the depth of penetration of irrigating solutions [40].

The present study has some limitations. This was an *ex vivo* study, using extracted teeth. On the one hand, the advantages of the *in vitro* model compared with the clinical study include the ease of modification if necessary, control of variables, low cost, and ease of replication [41, 42]. Contrarily, the study performed *ex vivo* may not accurately represent *in vivo* biofilm behavior and lacks the reaction or challenges imposed on a biofilm *in vivo* due to the host immune response [41]. We used bovine teeth instead of human, due to their availability and similar sizes and shapes allowing for the acquiring of similar amounts of dentin shavings. Bovine teeth have larger dentinal tubules and are more permeable for medications [43-45]. We used a mono-microbial model (*E.*

faecalis); however, *in vivo*, root canals are invaded by a variety of bacterial species that interact to form a polymicrobial biofilm. *E. faecalis* exhibits different starvation endurance in mono-species and multi-species biofilms, depending on which microbe it is in association with [46]. In our study the cementum was removed during the preparation to allow better bacterial penetration and we didn't cover the surface with a varnish, because toxic components of varnish could influence the viability of intra-tubular bacteria. In the present study, we used CFU to test the effectiveness of intracanal medications and irrigation. According to our results, none of the antiseptics could eliminate *E. faecalis* from the dentinal tubules. The presence of viable bacteria in all specimens suggested that in favorable conditions they could multiply and recontaminate the root canal space. Further research is required to develop new medications and/or irrigating techniques that will promote the effective disinfection of dentinal tubules. The assessment of the intracanal medications substances after different periods of application (1 week, 3 weeks) could also provide further valuable information.

Conclusion

None of the medications and antiseptics completely eradicated *E. faecalis* from the dentinal tubules. Within the discussed limitations, this *in vitro* study has shown that 1.5% chlorhexidine with xanthan gel and 2% chlorhexidine gel were significantly more effective compared with control (saline) for decreasing bacterial counts in dentinal tubules after 2 weeks of application. Calcium hydroxide didn't show a significant antibacterial effect compared with control. However, subsequent passive ultrasonic irrigation with 3% sodium hypochlorite significantly reduced bacterial counts in the dentinal tubules irrespective of the intracanal medication used. Therefore, it seems that sodium hypochlorite irrigation with ultrasonic activation is more important for the reduction of intra-tubular bacteria compared with the application of intracanal medications.

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