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Comparison of smear layer removal with the use of chitosan oligosaccharide, citric acid, and ethylenediaminetetraacetic acid in root canals of human lower pre-molars: Scanning electron microscope study

Jairo Jhoel Molina-Quispe, César Antonio Gallardo-Gutiérrez, Carmen Rosa Garcia-Rupaya, Miguel Angel Cabrera-Iberico

Department of Endodontics, Universidad Científica Del Sur, Lima, Perú

Purpose: The purpose of this study was to compare the efficacy of chitosan (CS)-derivative and its binding to citric-acid with chelators traditionally used in clinical practice.

Methods: 50 lower pre-molars were decoronated, standardizing a length of 15 mm, subsequently were instrumented with telescopic technique up to diameter 40.02, irrigated with 5% sodium hypochlorite. The samples were divided according to the treatment of smear layer (SL) (n = 10). Group I: 5% CS-oligosaccharide, Group II: 5% CS-oligosaccharide citrate, Group III: 10% citric acid, Group IV: 17% ethylenediaminetetraacetic-acid (EDTA), and Group V: distilled water. The samples were sectioned longitudinally, sputtered with gold, and observed with scanning electron microscope (SEM) under ×2000 and ×5000 magnifications. Data were analyzed using Kruskall-Wallis test followed by Mann-Whitney U test.

Results: In the apical third, CS-oligosaccharide citrate demonstrated better SL removal compared to the other groups ($p < 0.05$) but not with EDTA ($p > 0.05$). In the cervical and middle thirds, no differences were found ($p > 0.05$).

Conclusion: CS-oligosaccharide and CS-oligosaccharide citrate demonstrated similar chelating effect to citric acid and EDTA but were not superior.

Keywords: Citric acid; chitosan; ethylenediaminetetraacetic acid; scanning electron microscopy; smear layer.

Introduction

Biomaterials are substances designed to act within a biological environment, and these can be natural, synthetic, or semi-synthetic. Chitosan (CS) is considered a copolymer derived from chitin, which is a natural polymer found in nature (exoskeleton of crustaceans, plants, some fungi), has a high molecular weight (>1000KDa), its use is limited because it is slightly soluble in water, however, chitosan oligosaccharide (COS) has low molecular weight, which makes it more hydrophilic, in addition to having other ex-

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Correspondence: Jairo Jhoel Molina-Quispe. Department of Endodontics, Universidad Científica Del Sur, Lima, Perú
Tel: +51 965 66 28 72 e-mail: mjairo347@gmail.com

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cellent properties (1-3).

CS is considered a strong chelator (4). COS has a great capacity to act as a metal ion chelating agent (5) as well as ethylenediaminetetraacetic-acid (EDTA), which is usually used together with sodium hypochlorite (NaOCl) in irrigation protocols for the elimination of smear layer (SL) product of root instrumentation (6. Its chelating activity would be explained by the fixation of metal ions in dentin (7). Current literature reports up to two mechanisms for this effect (8). Another important property that CS possesses is the ability to remineralize dentin and act as a stabilizer of the collagen matrix (9). SL consists of a dust resulting from the interaction of the instruments inside the canal; therefore, it is composed of an organic part (biofilm, pulp remains, and bacteria) and an inorganic (hydroxyapatite of the dentin). The prolonged use of NaOCl alters the mechanical characteristics of the dentin and especially when alternate use is made with EDTA; since its harmful effect has been reported, manifested as considerable erosions in the dentin walls of the root canal system, therefore, a final rinse with NaOCl is not recommended (10). To assess the effects of NaOCl and EDTA on the dentinal walls, measurement scales were established (11). Due to the potential harmful effects, the use of chelators is not recommended beyond 5 min to achieve the SL removal (12). Regarding the comparison of chelating activity, Silva et al. (13). demonstrated that 15% EDTA presents a response similar to that of 0.2% CS, both irrigants achieving effective removal of SL.

The indiscriminate use of NaOCl together with EDTA could lead to endodontic failure due to the reduction of the mechanical strength of dentin and later to vertical fracture. CS associated with hydroxyapatite nanoparticles seems to improve the mechanical strength of dentin, restoring surface moisture and reinforcing the collagen structures (14). On the other hand, it is also said that EDTA and citric acid (CA) have an antagonistic effect on NaOCl, negatively modifying some of its properties (15) which seems not to occur when CS and NaOCl interact (16). Therefore, CS was recommended as a final irrigant and alternative to EDTA after the use of NaOCl, since it is capable of producing chelation and inhibiting bacterial adhesion to the root surface (17). In addition to having excellent antimicrobial properties (18), on the contrary, EDTA has limited antimicrobial activity (19). In vivo studies, CS has been associated with a decrease in colony-forming units (8,18) and with improvement in the post-operative symptomatology of pieces diagnosed with pulpal necrosis treated in single session (20), being soluble in acidic environment, it has been shown to have synergy with CA, taking the name of CS citrate (COSC), it has an excellent ability to remove SL (7).

Unlike chitin and CS, COS is less viscous and more soluble, which are desirable for a root canal irrigant. There are currently different methods for obtaining COS: Physical methods, such as ultraviolet radiation and chemical methods such as acid hydrolysis and enzymatic degradation (21). However, these different forms of preparation could influence the properties of COS, which is why discrepancies have been observed regarding the bioactivity of COS, for which Zhou et al. (22) recommend standardizing its preparation.

Considering previous literature, the purpose of this study is to compare the effect of 5% COS, 5% COS citrate (COSC), which is a mixture of CA and COS, 10% CA, and 17% EDTA on the SL removal inside root canals of human lower pre-molars, having proposed the superiority of the experimental chelating substances over CA and EDTA as alternative hypothesis.

Materials and Methods

This study was approved by Institutional Committee for Research Ethics of Universidad Científica del Sur, Lima, Peru (323-CIEI-CIENTIFICA-2022). The present study was carried out based on the PRILE guidelines for laboratory studies in Endodontology (23) (Fig. 1). Teeth have been obtained by a private clinic for research purposes; the authors do not know the patients who the teeth. The sample size was calculated by performing a pilot study, which consisted of 15 specimens with a 95% confidence level (1-α) and 90% statistical power, resulting in an adjusted sample size of 10 pre-molars per group. For the present study, single-rooted mandibular pre-molars with a single canal and minimal or no curvature (0°–10°) evaluated with ImageJ (NIH, USA) were selected. The exclusion criteria were teeth with more than one canal, calcified canals, fractured, fissured, and resorbed roots.

Preparation of 5% COS and 5% COSC

The COS solution was prepared as follows: 5 g of COS powder (Advanced Nutrients, California, USA) previously weighed on a precision balance (PIONEER OHRUS) was diluted in 100 mL of distilled water, on the other hand for COSC preparation was employed 5 g of COS powder (Advanced Nutrients, California, USA) previously weighed on a precision balance (PIONEER OHRUS) diluted in 100 mL of 10% CA (KMG Chemilab, Lima, Peru) with the help of a beaker. The solutions were taken to a magnetic stirrer Cimarec (Thermo scientific, Massachusetts, USA) programmed at 500 revolutions/min for 2 h.

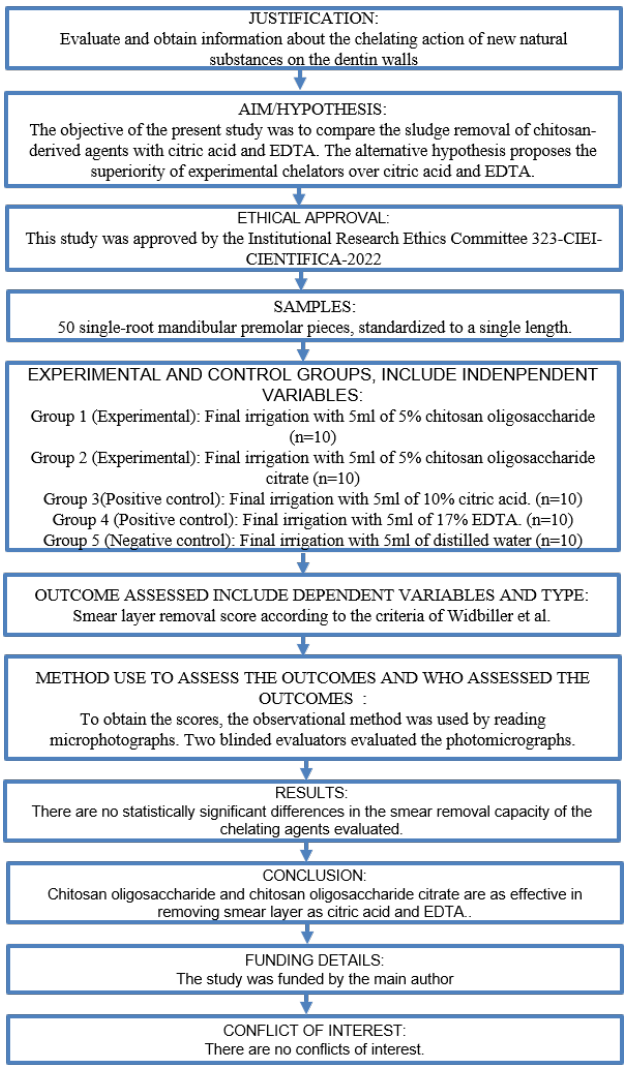

Fig. 1. PRILE flowchart.

Sample Preparation

The samples were left submerged in 10% formalin (KMG Chemilab, Peru) for 1 week for disinfection (24). Subsequently, radiographs of the samples were taken with the help of RVG Eagle equipment (Dabi Atlante, Brasil) in proximal projection to rule out the presence of an extra canal. Later, it was decoronated transversely with a carbide disc (Syndent, China) in such a way that a uniform length of 15 mm was established in all the pieces with the aim of making the apical thirds uniform. Afterwards, the later the conductometry was carried out with a C-Pilot #10 file (VDW, Germany). Moreover, the foramen was debrided, exceeding the anatomical apex by 1 mm. Immediately afterward, the apex was covered with yellow wax and the sample was stored in an individual container with heavy-body silicone (Speedex, Coltene, Switzerland) simulating a closed system. Manual instrumentation was performed with k files with a telescopic technique up to a K file # 40.02 (D – Perfect, Guangdong, China) so that there is a dynamic exchange of the irrigant within the root canal (25), accompanied with a volume of 2.5 mL of irrigating solution 5% NaOCl (Delta Química, Peru) between each instrument used (26). Once the instrumentation is finished, it is irrigated with an additional 5 mL of NaOCl.

A 5 mL syringe and a 30G needle (NaviTip, Ultradent, USA) with an open-end were used since closed-end needles are less effective in carrying the irrigant toward the most apical portions of the preparation (27). Subsequently, the prepared samples units were divided according to the treatment of the SL:

- Group I: Final irrigation with 5 mL of 5% COS
- Group II: Final irrigation with 5 mL of 5% COSC
- Group III: Final irrigation with 5 mL of 10% CA
- Group IV: Final irrigation with 5 mL of 17% EDTA (Maquira, Brasil)
- Group V: Final irrigation with 5 mL of distilled water.

The first 3 mL of the chelator were irrigated with back-and-forth movements, at the 4 mL; irrigation was supplemented with manual dynamic activation for 1 min with a standard 40.02 gutta-percha cone (D–Perfect, China) that was cut 1 mm before working length with 2 mm amplitude inward and outward movements (28). The last milliliter was irrigated again with to-and-fro movements. Finally, the canal was dried with standard number 40 paper cones (D–Perfect, China), until evidence of the absence of moisture in the cones.

Evaluation of SL Removal

The samples were removed from their silicone container and sectioned into two longitudinal halves. The entrance was previously sealed with Teflon, then the cut was started in the buccal-lingual direction with the help of a carbide disc at low speed without invading the root canal lumen. Later with the help of fine dentin curette (Dentsply Maillefer, Switzerland) complete division was achieved, preserving the half that best exposed the apical third. The split samples were dehydrated by immersing them in 30%, 50%, and 75% ethanol for 10 min and 100% for 30 min. Once all the samples were dehydrated, they were assembled into coded pieces and sputtered with gold-palladium (80–20%) in the Q150R plus coater (QUORUM, UK) for further evaluation under the scanning electron microscope (SEM).

SEM Evaluation

Coded samples were observed using FIB-SEM Scios 2

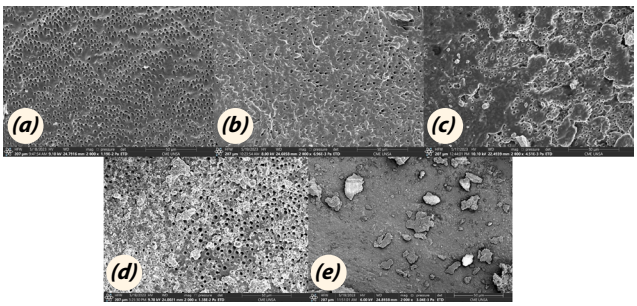

Fig. 2. Widbiller scoring system. **(a)** Score 0; **(b)** Score 1; **(c)** Score 2; **(d)** Score 3; **(e)** Score 4. Gold-Palladium stain.

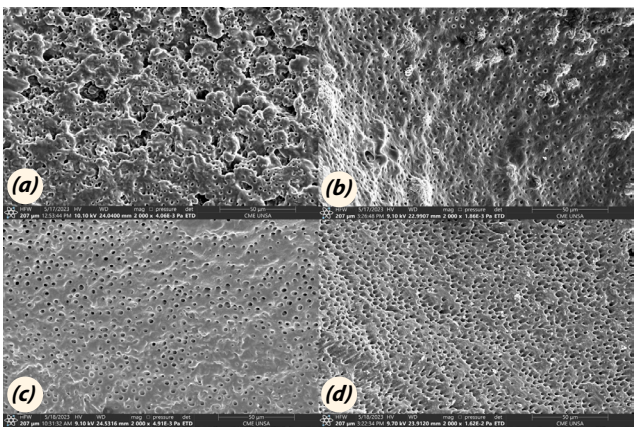

Fig. 3. Photomicrographs under ×2000 magnification. **(a)** Middle third from group I; **(b)** Apical third from group II; **(c)** Cervical third from group III; **(d)** Cervical third from group IV. Gold-Palladium stain.

LoVac (ThermoScientific, USA) where the samples were analyzed at 2.5 mm, 6 mm, and 12 mm of the apex (apical, middle, and cervical third) obtaining six photomicrographs (two for each third), under magnifications ×2000 and ×5000 (29), giving 300 images were examined and scored according to the criteria mentioned by Widbiller et al. (11): (Fig. 2).

- Score 0: No SL
- Score 1: Presence of SL ≤25% of the surface
- Score 2: Presence of SL >25% and ≤50% of the surface
- Score 3: Presence of SL >50% and ≤75% of the surface
- Score 4: Presence of SL >75% of the surface.

Statistical Analysis

The significance level for statistical analysis was set at 0.05 with the SPSS for windows version 22 (SPSS Inc., USA), the mean and standard error of the mean was obtained, and the non-parametric Kruskal–Wallis test was used to compare data on the effectiveness of SL removal. Subsequently, the non-parametric Mann–Whitney U-test was used to make the pair-wise comparison.

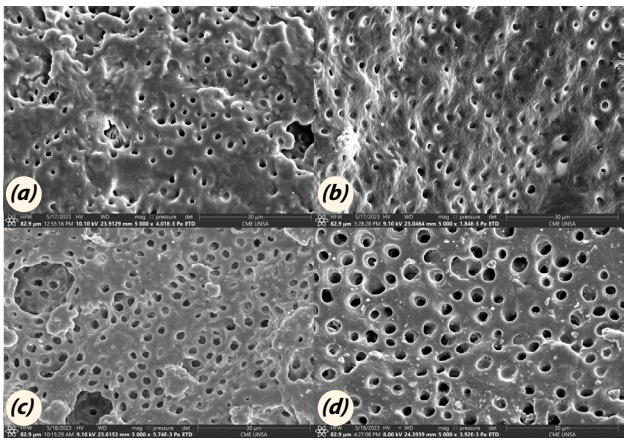

Fig. 4. Photomicrographs under ×5000 magnification. **(a)** Middle third from group I; **(b)** Apical third from group II; **(c)** Cervical third from group III; **(d)** Cervical third from group IV. Gold-Palladium stain.

Results

Two blind observers performed evaluation independently after examining 20 specimens (magnifications ×2000 and ×5000) for calibration purposes. Intra-and interexaminer reliability for SEM assessment was verified by the Cohen's kappa test showing values above 0.95 indicating a strong agreement.

The removal of SL was evaluated in the cervical, middle, and apical thirds under magnification ×2000 and ×5000 (Fig. 3 and 4, respectively). It was observed that the greatest amount of SL was found in the apical third, at this level group II proved to be slightly more effective in terms of SL removal compared to the other groups under magnifications ×2000 and ×5000. When applying the Kruskal–Wallis test (Tables 1 and 2, respectively) statistically significant differences were found ($p < 0.05$).

The experimental groups (I-II) showed significant differences in all thirds when compared the negative control ($p = 0.000$) (Tables 1 and 2). In the apical third, differences were found for magnification ×2000 and ×5000 between groups II-III ($p = 0.027$ and $p = 0.049$, respectively) and between groups II-I ($p = 0.040$ for both magnifications) (Tables 3 and 4). In cervical and middle third, no statistically significant differences were found between groups I-II-III-IV ($p > 0.05$ for both magnifications).

Discussion

Chitin is the second most abundant polymer on earth, as the main precursor of CS and COS, and it can be of animal (crustacean) or vegetable (algae and fungi) origin (1). Chemically, CS is similar to COS since they have more N-glucosamine units than N-acetylglucosamine, which is more abundant in chitin. This structure explains chela-

Table 1. Presence of SL by thirds at ×2000

Radicular third	Group	Mean	Standard error of the mean	P-valor (Kruskal–Wallis test)
Cervical third	I	0.80	0.249	0.000
	II	0.50	0.269	
	III	0.40	0.267	
	IV	0.50	0.224	
	V	4.00	0.000	
Middle third	I	0.90	0.407	0.000
	II	0.80	0.416	
	III	0.40	0.221	
	IV	0.40	0.221	
	V	4.00	0.000	
Apical third	I	1.70	0.559	0.000
	II	0.60	0.221	
	III	1.40	0.427	
	IV	0.90	0.433	
	V	4.00	0.000	

Table 2. Presence of SL by thirds at ×5000

Radicular third	Group	Mean	Standard error of the mean	P-valor (Kruskal–Wallis test)
Cervical third	I	0.70	0.300	0.000
	II	0.50	0.269	
	III	0.40	0.221	
	IV	0.50	0.224	
	V	4.00	0.000	
Middle third	I	0.80	0.416	0.000
	II	0.70	0.396	
	III	0.30	0.213	
	IV	0.30	0.153	
	V	4.00	0.000	
Apical third	I	1.80	0.573	0.000
	II	0.30	0.213	
	III	1.10	0.433	
	IV	1.00	0.422	
	V	4.00	0.000	

tion when amino groups interact with calcium. The low molecular weight of COS gives it an advantage over CS since this characteristic makes it highly soluble in any medium (5). Moreover, due to the fact that CS is capable of being solubilized in acidic environment (30), it has been proposed to solubilize the COS in distilled water in this study (31).

Regarding the preparation of the experimental irrigating substances of groups I and II, it was observed that magnetic stirring for a period of 2 h at 500 RPM and room temperature was sufficient to observe the complete dissolution of the COS powder in distilled water (neutral pH) and CA (acid pH). The rheological behavior of the experimental substances COS and COSC at 5% showed fluidity, corroborating the excellent solubility and low viscosity.

Some studies reported the superiority of CS derivatives over EDTA in the removal of SL, using agents such as CS derived from fungi (2), COS together with calcium hypochlorite (8), and CS nanoparticles at 0.2% (32), these results could not be confirmed in the present study, probably because CS was associated with acetic acid and it was accompanied with calcium hypochlorite, which could perform better SL removal.

The experimental union of chelators has been shown to have a synergistic effect in the removal of SL, as demonstrated by 4% COSC (CA plus CS) which was as effective as 2% CS and 10% CA (7). Other authors report that the application of COSC for 5 min was sufficient to remove

Table 3. Intra-group comparison at ×2000

Radicular third	Irrigant (Intra-group comparison)	P-valor (Mann–Whitney U test)
Cervical third	IV-III	0.512
	IV-II	0.823
	IV-I	0.362
	IV-V	0.000
	III-II	0.689
	III-I	0.168
	III-V	0.000
	II-I	0.314
	II-V	0.000
	I-V	0.000
Middle third	IV-III	1.000
	IV-II	0.155
	IV-I	0.343
	IV-V	0.000
	III-II	0.155
	III-I	0.343
	III-V	0.000
	II-I	0.629
	II-V	0.000
	I-V	0.000
Apical third	IV-III	0.299
	IV-II	0.282
	IV-I	0.289
	IV-V	0.000
	III-II	0.027
	III-I	0.815
	III-V	0.000
	II-I	0.040
	II-V	0.000
	I-V	0.000

the SL, with minimal erosion (33). It was also reported that the 0.2% CS achieved better SL removal at the apical level than the MTAD (CA-containing substance), however, this could not be replicated at the coronal and middle levels (34). The present study agrees with all these findings, demonstrating better COSC activity at the apical level, confirming the synergy of CA and COS. This finding cannot be compared directly since there are no studies that evaluate the binding of these agents.

On the other hand, other studies have shown that 0.2% CS and 17% EDTA achieved similar SL removal, without any significant differences (12,13,35,36) even using devices such as the EndoVac (37), concluding that CS is an alternative to EDTA, this study agrees with these results since both COS, COSC, and EDTA achieved equally effective SL removal.

Evaluating the apical third, which is the most crucial part of the root, some authors have shown that 0.2% CS was superior to 17% EDTA in SL removal (38), using ultrasonic devices for irrigant activation (39) or using the EndoVac (9). In contrast to the findings of the present study, it was not possible to demonstrate the superiority of COS and COSC over EDTA in the apical third. This may be explained by the use of the manual irrigation activation in this study.

NaOCl is the irrigant most used by endodontists (4), however, its interaction with traditional chelants such as EDTA and CA alters some of its properties (15). On the contrary, it has been shown by iodometric titration that CS does not affect the properties of NaOCl (16).

The limitation of this study covers the use of SEM for the evaluation of SL removal since only a portion of the entire root canal system is evaluated. The ideal evaluation would be with microcomputed tomography (micro-CT).

For future research on chelating activity, it is suggested to delve into the effects of sonic, ultrasonic, and laser devices

Table 4. Intra-group comparison at ×5000

Radicular third	Irrigant (Intra-group comparison)	P-valor (Mann–Whitney U test)
Cervical	IV-III	0.687
	IV-II	0.823
	IV-I	0.730
	IV-V	0.000
	III-II	0.888
	III-I	0.503
	III-V	0.000
	II-I	0.622
	II-V	0.000
	I-V	0.000
Middle	IV-III	0.726
	IV-II	0.473
	IV-I	0.473
	IV-V	0.000
	III-II	0.326
	III-I	0.326
	III-V	0.000
	II-I	1.000
	II-V	0.000
	I-V	0.000
Apical	IV-III	0.841
	IV-II	0.154
	IV-I	0.358
	IV-V	0.000
	III-II	0.049
	III-I	0.477
	III-V	0.000
	II-I	0.040
	II-V	0.000
	I-V	0.000

on COS and COSC, as well as on the erosive activity of these substances.

With a $p > 0.05$, the alternative hypothesis was rejected. The experimental substances were not superior to 17% EDTA and 10% CA in terms of SL removal.

Conclusion

This study demonstrated that under laboratory conditions, COS and COSC were as effective in removing SL as the traditional chelators EDTA and CA. At the apical level, it was demonstrated that the experimental union of COS and CA (COSC) achieved better results. Both experimental irrigants could be an alternative to traditional chelators.

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Ethical Approval: The study protocol was approved by the Científica University Ethics Commitee (date: 11.11.2022, protocol no: 323-CIEI-CIENTIFICA-2022).

Informed consent: Written informed consent was obtained from patients who participated in this study.

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