

Quantification of Calcium Ions in Chitosan, Smear Clear and Citric Acid after Root Canal Irrigation – An *In Vitro* Atomic Absorption Spectrophotometry Study

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Abstract

Background: The use of rotary or hand instruments during endodontic cleaning and shaping procedures forms smear layer inevitably. As the smear layer harbors microorganism and acts as a nidus of infection, the removal of SL provides a sterile environment in the root canal system and enhances the adaptation of sealer to the root canal. **Objectives:** This study was designed to analyze the calcium ion expelled, resulting from final irrigation with Smear clear, 0.2% Chitosan, 10% citric acid, and de-ionized water (control). **Materials and Methods:** Sixty human premolars were used in this study and biomechanical preparation was done. During instrumentation, 3% sodium hypochlorite was used and according to the irrigant used finally, grouping of the samples was done with 15 teeth in each group. Group 1 - Smear Clear (SybronEndo, Orange, CA, USA) - 17% ethylenediaminetetraacetic acid solution with cetrimide, polyoxyethylene and iso-octylcyclohexyl ether, Group 2 - 0.2% Chitosan, Group 3 - 10% citric acid, Group 4 - de-ionized water (Control). After final irrigation, these solutions were collected in a falcon tube and subjected to flame atomic absorption spectrometric study. Statistical analysis was carried out using one-way ANOVA and Tukey test. **Results:** The mean concentration of calcium ions of 1.9211 ± 0.4094 was noted with Smear clear and a value of 1.7891 ± 0.2837 observed with 0.2% chitosan. Statistically, these two groups did not differ. 10% citric acid eluted the least calcium ion. No calcium ion was detected in the control group. **Conclusion:** 0.2% chitosan is as potential as smear clear in removing the calcium ions and smear layer from root dentin.

Keywords: Calcium ions, Chitosan, Citric acid, Demineralization, Final irrigation, Smear clear

INTRODUCTION

A 3D obturation and gleaming endodontic treatment provides a pathway to the victory for a clinician.^[1] Favorable outcome of root canal treatment depends on consolidated efforts of proper instrumentation, good irrigation protocol, and fluid impervious hermetic seal. Cleaning and shaping of the canals along with effective irrigation plays a key role in the elimination of the microorganism and healing of the periapical tissue.^[2] Irrigants, which are an essential part of endodontic treatment, aid in the elimination of microbes and their byproducts beyond what might have been achieved by instrumentation alone.^[3] Therefore, numerous irrigants have been suggested to eradicate microbes, debris, tissue remnants, and smear layers from the root canal.^[4] Irrigants prevent the clogging of root apex. It also aids in the displacement of the suspended debris from canal space and dissolves the contents of smear layer. Single irrigant does not fulfill all the criteria of ideal irrigant. To likely attain the objectives of cautious and potent irrigation, the combined

use of multiple irrigants in a correct series is essential for more appropriate irrigation.^[5] Irrespective of the technique used during biomechanical preparation, all the endodontic instruments form debris and smear layer due to its action on root dentin.^[6] Smear layer prevents the entry of the intra canal medicaments in to the irregularities of complicated canal anatomy, and there is a risk of microleakage due to poor sealer adaptability of obturating material.^[7] Pashley put forward that smear layer harboring bacteria and their by-products may act as a nidus of infection and leads to the failure of the root canal.^[8]

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Hence adequate smear layer removal is a prerequisite for a successful endodontic therapy.

For the smear layer removal ultrasonics, laser and chelating agents are proposed during cleaning and shaping.^[9] Combined use of sodium hypochlorite and chelating agents are most commonly employed in the elimination of both organic and inorganic content of SL.^[10] Due to the limitations of NaOCl, like the displeasing taste, excessive toxicity, and inefficiency to remove smear layer when used alone, has opened on to the use of other chelating agents such as smear clear – an ethylenediaminetetraacetic acid (EDTA) formulation, citric acid, and tetracycline. Chelating agents improve the penetration of irrigants into the dentinal tubules which enhances disinfection, removes the smear layer decreases the microhardness of dentin which assists in easy preparation of irrigants into calcified and narrow canals.^[5]

The most popularly used chelating agents for the smear layer removal are based on EDTA as it chelates and expels the mineralized portion of the SL. It's seeking attention as a final irrigant due to its capability to isolate calcium from the tooth. EDTA combines with calcium ions of dentin with the formation of soluble calcium chelates. Various formulations of EDTA have been used as root canal irrigant.^[11]

Of late, a newer origination of EDTA was proposed with the trade name smear clear (SybronEndo, Orange, CA, USA) with the composition of 17% EDTA, Cetramide and two additional surfactants i.e., polyoxyethylene and iso-octylcyclohexyl ether for SL removal.^[12] In a study done by Nelson-Filho *et al.*, smear clear was able to remove the smear layer from the root canals of primary teeth as effectively as EDTA, suggesting that both solutions may be indicated for such purpose.^[13] Da Silva *et al.* assessed the efficiency of SmearClear and EDTA for smear layer elimination of permanent teeth after the instrumentation of the root canal and concluded that Smear Clear was as efficient as 14.3% EDTA.

Smear clear has also been examined for smear layer removal and cleansing of the root canal.^[14] The addition of cetrimide and cationic detergent improves the performance of the irrigant. The depth of penetration of irrigant is greatly enhanced due to the action of cationic surfactant. This surfactant decreases the surface tension and viscosity of the fluid permits the chelating agents to move to deeper parts of the dentinal tubules, and removes the debris much more effectively than EDTA alone.^[15] In addition, Smearclear possess added advantage of having antimicrobial properties. When compared to standard EDTA, Smear clear had better wettability and also greater cleaning potential. Jantarat *et al.* concluded in their study that the highest efficacy for smear layer removal and cleaning of the root canal walls was observed with smear clear.^[16] Its important action against *Enterococcus faecalis* and its ability to clear biofilm as compared to 2% CHX has drawn its attention as endodontic irrigant to remove the smear layer from root dentin.^[17]

The major drawback of EDTA is the intertubular and peritubular erosion of dentin. Root canal treatment failures are associated with erosion of dentin.^[5] EDTA is not found naturally in nature and is also considered a pollutant and has a detrimental effect on dentin.^[18]

Another routinely used chelating agent during root canal preparation is citric acid. Its advantage over EDTA is its biocompatibility, less cytotoxicity and it is effectiveness against anaerobic microbes. It is also effective in SL removal.^[1] The concentration of 10% with a pH of 1.1–1.8 is found to be effective in smear layer removal. Unique properties of citric acid like the formation of cementum and regeneration of periodontal tissues have gained its attention in dentistry. Although citric acid is effective in the extraction of calcium ions from dentin, it is not routinely advocated in clinical practice as the crystals get precipitated in the canal, which nurtures microorganisms and fails in the 3D seal of the obturation. It tastes sour and is not palatable.^[1]

Due to the disadvantages of EDTA-based irrigant – Smear clear and citric acid, researches are looking forward to seek alternative biocompatible irrigants such as chitosan.^[18]

Chitosan, a polysaccharide is found naturally and exuberantly in nature with the lower cost of production. It tissue compatible, ecological, mucoadhesive, harmless and has good chelating potential for numerous metal ions. Chitin is the precursor of chitosan, found in the crab and shrimp shells. Chitosan is procured by the deacetylation of chitin.^[19] Due to the known drawbacks of irrigants, newer and developing irrigants are gaining attention in endodontics. Most of the studies have compared EDTA and Chitosan and there is less literature on smear clear – a formulation of EDTA in comparison with chitosan. Therefore, this study was designed to determine the amount of calcium ion in 0.2% chitosan and comparing it with smear clear and 10% citric acid for smear layer removal.

MATERIALS AND METHODS

In this study, 60 non-carious freshly extracted human single-rooted premolars were collected and stored in distilled water in room temperature. Teeth with no caries or fractures extracted due to periodontal disease or orthodontic treatment were included in the study. Teeth with calcified canals, and accentuated curvatures were excluded from the study.

Access opening for all the teeth was done using no. 2 endo access bur (Dentsply), using high speed hand piece with continuous cooling. Working length was calculated using #15 k file (Mani) by keeping it 1mm short of apex. Cleaning and shaping were done with NITI protaper rotary system (Dentsply Maillefer) up to size F2 using a crown down technique using Endomate Endomotor and Handpiece (NSK). 1 ml of 3% sodium hypochlorite (Vensons India) was used during recapitulation. Free dentinal chips were cleared using 10 ml of deionized water (Millipore {India} Pvt. Ltd, Bangalore) with a conventional syringe (Dispovan - Hindustan syringes

and medical devices). All the canals were dried with paper point (Dia Dent Group International - Korea) & were ready to receive the final irrigation.

Sixty Extracted Human Premolar Teeth were divided into 4 groups with 15 teeth in each group according to the irrigating solution used as the final irrigant for a duration of 3 min as follows, Group 1: Smear clear, Group 2: 0.2% Chitosan, Group 3: 10% Citric acid, Group 4: Control- de-ionized water.

As control, de-ionized water was used to assure that this solvent would not be a source of Calcium contamination.

0.2% of chitosan solution was prepared according to the protocol followed in the previous study.^[18] 100 ml of 1% acetic acid (Nice Chemicals Pvt. Ltd, Kerala) was used to dilute 0.2g of chitosan (Everest Bio-Tech, 89.5% DA) which was mixed using a magnetic agitator (Spectralabwhirlmatic – 1) for about 2 h. The final irrigated samples were collected in 15 ml falcon tube (Tarsons) with each root penetrating through the perforated lid of falcon tube and crown outside the tube lid. Conventional syringe with #27 gauge needle was used to irrigate 5 ml of final irrigants. After passing through the root canal the irrigants passed through the patent foramen and was collected in falcon tube. After this, the tooth was removed from falcon tube and the tube received a new intact lid. The final irrigants collected were subjected to spectrometric determination of calcium ion concentration within the liquid. Independent scores were established and a mean value was calculated for individual group.

Flame Atomic Absorption Spectrophotometer, GBC 932 plus (Australia) was used to assess the calcium ion in the sample. It consists of an acetylene gas which measures the absorbance & calcium cathode lamp which is hollow. For calcium ion curve calibration adjustment, 100 mg/L concentration of standard calcium solution was used. Slit of the flame emitted had a width of 0.5 nm and a nitrous oxide acetylene flame with a current of 10.0 mA was used. The wavelength of 422.7 nm was used to determine the calcium ion concentrations in the irrigating solution. The concentration interval between 1 and 4 µg/ml was applied for a set of standard solutions.

Before the analysis, de-ionized water was used to dilute Smear Clear and citric acid (Sd fine Chemical Ltd. Mumbai), while 0.1% lanthanum solution- by mass/volume (Nice chemicals Pvt. Ltd, Kerala) was used to dilute Chitosan solution.

Statistical Analysis

The results obtained from the AASF analysis with reference to the values of calcium ions released were statistically analyzed using SPSS (Statistical Package for Social Sciences) for Windows version 20.0[®]. The nature and distribution of variables indicated that analysis by non-parametric methods was appropriate.

A comparison of the means in each group for the concentration of calcium was done by one-way ANOVA. Corresponding *P*-value were calculated. Pair-wise comparisons of the four

groups were performed using Tukey's multiple *post hoc* procedure. *P* < 0.05 was considered statistically significant.

Calcium content in all three groups has normal distribution since *P* > 0.05. Hence, one-way ANOVA followed by the Tukey test was used for analysis with a level of significance of 5% (*P* < 0.05).

RESULTS

Flame atomic absorption spectrophotometer analysis for the concentration of calcium showed that all solutions except the control group were able to remove calcium ions from the root canal walls.

The highest mean concentration of calcium ions was noted with Smear clear followed by 0.2% Chitosan. 10% citric acid eluted the least calcium ions whereas no calcium ions were released from the control group.

Table 1 shows the mean ± SD of the quantification of calcium ions extracted by the studied solutions, measured in µg/ml, which were as follows: Group 1 - (Smear Clear) 1.9211 ± 0.4094 µg/ml; Group 2 - (0.2% Chitosan) 1.7891 ± 0.2837 µg/ml; Group 3 - (10% Citric acid) 1.2522 ± 0.1949 µg/ml; and Group 4 - (Control group) 0.00 ± 0.00.

Table 2 shows the comparison of Groups (1, 2, 3, and 4) for the concentration of Calcium (in µg/ml) by one-way ANOVA. A significant difference was observed between four groups (1, 2, 3 and 4) for the concentration of Calcium (in µg/ml) since *P* = 0.000 < 0.05 (*F* = 161.0154) at a 5% level of significance. It can be interpreted that, the mean concentration of Calcium (in µgm/ml) scores are different in different groups.

Further, if *f* is significant, to know the pair-wise comparisons, the Tukeys multiple *post hoc* procedure was applied and the results are presented in the following table.

Table 3 shows the Tukeys multiple *post hoc* procedures for the pair-wise comparison of four groups (1, 2, 3, and 4) for concentration of Calcium (in µg/ml): It can be interpreted as follows:

1. No significant difference was observed between Group 1 - Smear clear (1.9211 ± 0.4094) and Group 2 – 0.2% chitosan (1.7891 ± 0.2837) with concentration of calcium (in µg/ml) at 5% level of significance (*P* > 0.05). It can be interpreted that, group 2 (0.2% Chitosan) can substitute group 1 (15% EDTA).

Table 1: Mean, SD, standard error (SE) of concentration of calcium

Group	N	Mean	SD	SE
Group 1	15	1.92	0.41	0.11
Group 2	15	1.79	0.28	0.07
Group 3	15	1.25	0.19	0.05
Group 4	15	0.00	0.00	0.00

Table 2: One-way ANOVA test for Comparison of four groups for concentration of Calcium (in $\mu\text{g/ml}$)

Sources of variation	Sum of squares	Degrees of freedom	Mean sum of squares	F-value	P-value
Between groups	3	34.5473	11.5158	161.0154	0.00001*
Within groups	56	4.0051	0.0715		
Total	59	38.5524			

* $P < 0.05$ **Table 3: Tukeys multiple *post hoc* procedures for the pair wise comparison of four groups (1, 2, 3 and 4) for concentration of Calcium (in $\mu\text{g/ml}$): Dependent variable: Calcium**

Group	Group 1	Group 2	Group 3	Control
Mean	1.9211	1.7891	1.2522	0.0
SD	0.4094	0.2837	0.1949	0.00
Group 1	-			
Group 2	$P=0.5350$	-		
Group 3	$P=0.0002^*$	$P=0.0002^*$	-	
Control	$P=0.0002^*$	$P=0.0002^*$	$P=0.0002^*$	-

* $P < 0.05$

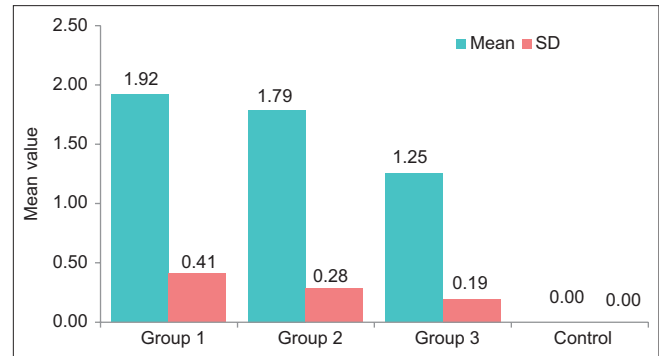
- A significant difference was observed between Group 1 - Smear clear (1.9211 ± 0.4094) and Group 3 – 10% citric acid (1.2522 ± 0.1949) with the concentration of Calcium (in $\mu\text{g/ml}$) at 5% level of significance ($P < 0.05$). It can be interpreted that, the mean concentration of calcium (in $\mu\text{g/ml}$) is significantly higher in group 1 Smear clear as compared to group 3 (10% citric acid).
- A significant difference was observed between Group 2 – 0.2% chitosan (1.7891 ± 0.2837) and Group 3 – 10% citric acid (1.2522 ± 0.1949) with the concentration of calcium (in $\mu\text{g/ml}$) at 5% level of significance ($P < 0.05$). It can be interpreted that, the mean concentration of Calcium (in $\mu\text{g/ml}$) is significantly higher in group 2 (0.2% chitosan) as compared to group 3 (10% citric acid).
- A significant difference was observed between the control group when compared with other experimental groups ($P < 0.05$).

Graph 1 shows that the mean calcium ion concentration of smear clear and 0.2% chitosan was similar to each other and significantly different from other solutions.

DISCUSSION

The application of chelating solutions in root canal therapy has been evaluated in previous studies and has proved its role in the improvement of the final cleaning potential and sealing ability of the canal.^[20] Therefore the purpose of this study was to evaluate, the capacity of Smear clear and 10% citric acid and compared to 0.2% chitosan to remove the inorganic smear layer which was quantified, by atomic absorption spectrophotometer with flame, for the chelated calcium ions released in these solutions after their use in root canal therapy.

One of the main inorganic elements of dentin is the calcium which is present in hydroxyapatite crystals. Therefore, the

**Graph 1: Comparison of the Groups 1, 2, 3 and 4 for the concentration of calcium (in $\mu\text{g/ml}$)**

irrigating solutions that have direct action over calcium ions were chosen in this study. In a previous study by Jantarat *et al.*, smear clear efficiently removed SL and showed a good outcome as an irrigant.^[17] Chitosan and citric acid were effective in the removal of calcium ions from the dentin according to previous research.^[18]

Numerous methods such as Fourier transform infrared, Flame photometry, atomic Absorption spectrometry and scanning electron microscope (SEM) are employed to estimate the decalcifying effect of various chemicals with precise calibration.^[21]

In the present study, chelated calcium ions were assessed using flame atomic absorption spectrometry as authors in the earlier study have stated the limitations of SEM analysis, where they could not discriminate between Smear layer and sclerotic dentin under SEM.^[22] Therefore; an alternative method was followed to evaluate the smear layer removal using AASF,^[18] as this technique assesses single element, which is economic as compared to recent multi-element techniques such as inductively coupled plasma atomic emission spectrometry.^[23]

According to the results obtained in a study by Marques *et al.*, it was interpreted that, there was a direct association between the chelating potential of irrigants to remove smear layer and its capability to remove calcium ions. The action of chelators and decalcifying agents on the calcium element of the hydroxyapatite matrix within the dentin causes the exposure of the collagen and also reduces its microhardness.^[24] Hence, it was interpreted that the irrigant with the greatest calcium extraction, represented efficient demineralization and effective cleaning action as supported by Silva *et al.*^[18] and Slutzky-Goldberg *et al.*^[25]

In the present study, the results obtained from the quantification of chelated calcium ions showed that except the control group (de-ionized water), all the other experimental groups (smear clear, 0.2% chitosan, and 10% citric acid) were able to extract Calcium from root dentin. The groups irrigated with smear clear and 0.2% chitosan did not differ statistically and showed similar outcome for calcium ion removal. Both these groups were different from the samples irrigated with 10% citric acid which eluted a lesser quantity of calcium ions. 10% citric acid removed more calcium compared to deionized water which did not remove any calcium from the root dentin.

Various aspects such as duration of application, pH, the solution concentration and the quantity of the solution affect the performance of the irrigant.^[20] Dua *et al.* concluded that the best outcome for the smear layer removal was obtained when smear clear was used for 3 min.^[26] Similarly, in the present study, smear clear irrigated for 3 min, showed favorable results in terms of calcium ions removal from the root canal. In the current study, chitosan was irrigated for 3 min which was supported by the claim of Pimenta *et al.* who stated that chitosan employed for 3–5 min as the final irrigant showed the best chelating effect on dentin.^[27]

Due to its acidic pH of 3.2, Chitosan shows an outstanding chelating potential for different metal ions.^[28] The working principle of chitosan for dentin calcium ion elimination is mainly by chelation, adsorption, and exchange of the ions.^[18] Chitosan is hydrophilic in nature, and it favors close contact with root dentin. The cationic nature of chitosan is due to the numerous free hydroxyl and amino groups which is accountable for the ion interaction amidst calcium ions of dentin and chelators. The protonated amino group that is present in this polymer attracts other molecules and gets adsorbed into root dentin and hence penetrates deep into the dentinal tubules for its action.^[29] As chitosan is insoluble in water, it is dissolved in solutions of various acids like acetic and Hydrochloric acid.^[30]

In the present study, when group 1 and group 2 were evaluated for calcium ion extraction, group 1- Smear clear eluted more calcium ions from root dentin as compared to group 2 – Chitosan but there was statistically no significant difference between these two groups. This is in accordance with the SEM study by Vallabhaneni *et al.*^[15] In a study by Khedmat *et al.*, smear clear and 17% EDTA had no statistically significant difference for the smear layer removal.^[31] Although there is the scarcity of literature that has compared Smear Clear and Chitosan for calcium ion removal, there are many studies which have evaluated 17% EDTA and Chitosan and have proven that these two groups had a similar capacity for smear layer removal.^[18,32] 17% EDTA applied for 10 min caused a massive erosive effect and dissolution of peritubular and intertubular dentin.^[5] Pimenta *et al.* laid out that 0.2% Chitosan exhibited little dentinal erosion as compared to 17% EDTA.^[27] By this it can be concluded that Chitosan was more biocompatible than smear clear – an EDTA based irrigant.

In the present study, when Group 1 - Smear Clear and Group 3 - Citric acid were compared, the mean concentration of Calcium was significantly higher in Group 1 as compared to Group 3. In a previous study, similar results were obtained for SL removal, especially in the apical 3rd of the root which indicates superior properties of smear clear over 10% citric acid for SL removal.^[31] In contrast, the findings of Vallabhaneni *et al.* showed that 6% citric acid showed better SL removal due to difference in volume of 10 mL which was used in their study in contrast to 5 mL of irrigant which was used in our study.^[15]

Studies by Vallabhaneni *et al.*, Dua *et al.*, Pimenta *et al.*, confirms that the concentration and the application time of the irrigants have an effect on decalcifying effect on dentin.^[15,26,27]

Comparison of Group 2 – Chitosan and Group 3 – Citric acid revealed that the mean concentration of Calcium is significantly higher in group 2 as compared to group 3 which is in accordance with a study by Suzuki *et al.*^[33] They concluded that a greater amount of smear layer removal with minimal erosion of the dentin was observed with Chitosan – citrate as compared to 10% citric acid. They highlighted that chitosan – citrate solution had a greater cleaning potential of the root canal walls due to its chelating property as compared to citric acid. In another research by Silva *et al.*, 10% citric acid removed lesser calcium as compared to 0.2% chitosan from root canal.^[18]

In the current study, it was observed that 0.2% Chitosan sufficiently removed calcium ions from the dentinal surface which indicates satisfactory removal of smear layer. Whereas the concentration of EDTA in Smear Clear was 17%. Lower concentration of chitosan was equally potent as compared to smear clear. Hence when both these chelating agents show the same effect, a solution with the least concentration should be prioritized. As Chitosan is enormously present in nature, its procurement is more cost effective, and it causes little erosion of dentin compared to smear clear – An EDTA formulation; therefore preference should be given to Chitosan over smear clear.^[14,34]

The chelating behavior of Chitosan demonstrated in the present study indicates that this solution acted on the inorganic portion of the smear layer, favoring its removal.

In the present study, the concentration of Chitosan used was 0.2% which was in accordance with the previous study.^[35] It has been noted that the benefits of Chitosan are two folded; due to its capacity of removing the smear layer and also it can prevent the reestablishment of the microorganisms on the root dentin.^[36]

The present study observations suggested that irrigation of the root canal with Smear Clear and 0.2% chitosan effectively removed the inorganic content of smear layer as demonstrated by loss of calcium ions. From this study, it is obvious that 3 min application of 0.2% Chitosan is as effective as Smear Clear for smear layer removal from root dentin.

CONCLUSION

Based on the results obtained it could be concluded that a comparison of the chelating solutions: smear clear, 0.2% chitosan, and 10% citric acid for the root dentin decalcification was possible by AASF. And among the solutions assessed, smear clear removed a larger quantity of calcium ions followed by 0.2% Chitosan. Therefore, Chitosan as a chelating agent can be used similar to EDTA. The greater effect on the root dentine demineralization was observed in 0.2% Chitosan, similar to smear clear and better than 10% Citric acid. The decalcifying effect of 10% Citric acid was better than the control group. The use of the chelating agents and other root canal irrigants in the study successfully removed the Calcium from the root dentin surface which might have caused significant removal of inorganic content of smear layer. Further studies must be conducted, like SEM study, and with varying concentrations and pH of the solutions, as well as promoting the association among them, which may lead to formulating a solution with active agents that favor effective smear layer removal.

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