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Research Article

Assessment of the Effect of Chitosan Nanoparticles on the Ultra-Structure of Dentinal Wall: A Comparative *In vitro* Study

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Abstract

Objective: This study was conducted to evaluate the efficacy of 0.2% Chitosan Nanoparticles (CNPs), CNPs/EDTA (1:1) and 17% EDTA on smear layer removal ability.

Methods: Fifteen extracted human permanent mandibular premolars with single canals were decoronated and biomechanically prepared using protaper next rotary system and 2.5% Sodium hypochlorite, then randomly distributed according to the final irrigation protocol into 3 group; Group I (0.2% CNPs), Group II (CNPs/EDTA) (1:1), and Group III (17% EDTA). A standardized volume of 5 ml of each tested irrigant (s) was used for 3 minutes. Teeth were longitudinally split to evaluate the smear layer eradication using scanning electron microscope at magnifications of 500x and 1000x. The micrographs for each third were given scores by two blinded observers using Takeda., et al. (1999) scoring system.

Results: There was no statistically significant difference in the scores of the three groups at the coronal, middle, and apical thirds (P-value = 0.368, 0.054, and 0.0900) respectively. As for the combined total scores of each group, Group I (CNPs) showing a significantly higher smear layer elimination than both Group II (CNPs/EDTA) and Group III (EDTA) (P-value = 0.044).

Conclusions: None of the used chelating agents resulted in complete removal of the smear layer. The combined use of CNPs/EDTA (1:1) did not show superior performance over the other two groups.

Keywords: Chitosan; EDTA; Nanoparticles; Final Flush; Smear Layer; Scanning Electron Microscope

Introduction

Successful endodontic treatment relies on chemo-mechanical debridement of the root canal system through simultaneous use of instrumentation protocols and disinfectant solutions. However, mechanical preparation results in the production of a smear layer adhering to the root canal walls [1]. The smear layer may hinder intracanal medications and sealers penetration into the dentinal tubules, additionally it facilitates the adhesion and colonization of microorganisms. This is why attempts are made for removal of the smear layer to enhance the fluid tight seal of the root canal system [2]. Many Chelating agents have been proposed for smear layer removal including EDTA, MTAD, Citric acid and Qmix [3], Other methods include adjunctive devices such as Lasers and ultrasonics [4]. However, the most commonly accepted protocol for the removal of organic and inorganic components of smear layer is to utilize various concentrations of sodium hypochlorite (NaOCl) in combination with EDTA [5]. NaOCl and EDTA are not natural products, which increase the concerns about biocompatibility in order to minimize irritation and damage to the periapical tissues [6].

Chitosan is a natural polysaccharide, originating from the deacetylation of chitin, which is derived from the shells of crabs and shrimps. This polysaccharide has excellent biocompatibility, biodegradability, bio-adhesion and no reported toxicity to the human body. Moreover, it possess an acidic PH, with remarkable chelating capacity for different metal ions. At the nano-scale, nanoparticles possesses enhanced and unique physiochemical properties due to their ultra-small size, large surface area/mass ratio and increased chemical reactivity which make them an ideal alternative to their bulk counterparts [7].

In endodontics, the use chitosan nanoparticles as an antimicrobial agent has recently drawn considerable attention owing to their superior antimicrobial properties and low potential to produce microbial resistance. However, studies describing their effect on the smear layer removal are still few in literature. The null hypothesis tested was that there is no difference among the final irrigation solutions in terms of smear layer removal.

Material and Methods

Sample size

Based on the previous study by Kandil., *et al*. 2014 [1] and using power 90% and 5% significance level, Sample size was calculated as 15 samples for the study. Thus, the samples were distributed among 3 groups; each containing 5 samples.

Collection and preparation of samples

Fifteen human permanent mandibular single-rooted premolar teeth extracted for periodontal disease or orthodontic treatment, were collected from the Department of Oral and Maxillofacial surgery at the Faculty of Dentistry-Cairo University. Conventional radiograph (Kodec, Rochester, New York, USA) was used to confirm that each tooth had a single canal with no internal calcifications, irregularities or any other anomalies. External root surfaces of teeth were debrided with a curette to remove calculus and periodontal tissues, and then placed in Sodium hypochlorite (NaOCl) (Clorox Co., Oakland, CA, USA) for 30 min. to remove soft tissue debris and teeth were then stored in saline till use. Crowns were flattened using a low speed diamond saw under copious irrigation to obtain approximately 15 mm uniform root lengths. Patency of the canals was established using k file #10 (MANI, Matsutain Seisakusho Co., Tochigi-Ken, Japan). Apices of all the roots were sealed using flowable composite (FiltekTM Z350 XT, 3M ESPE, St Paul MN, USA) then root canals were instrumented using Protaper Next rotary system (Dentsply Sirona, York, Pennsylvania, USA) starting with X1 (#17/.04), X2 (#25/.06), X3 (#30/.07) and finally X4 (#40/.06) using X-Smart Endo Motor (Dentsply Sirona, York, Pennsylvania, USA) with speed of 300 RPM and 2.5 N.Cm torque for all the files. The canal was irrigated with 3 ml of 2.5% NaOCl between each file size using a disposable plastic syringe with a 30-G needle (Sung Shim Medical Co., Bucheon, Gyeonggi, South Korea) to reach 1–2 mm from the apex without binding. Irrigation with 5 ml of saline (FIPCO, Borg Elarab, Alexandria, Egypt) followed and the canal was then dried using paper points #40 at the end of the instrumentation (Meta Biomed Co. Ltd, Korea).

Experimental groups distribution

The teeth were classified into three groups (n = 5) according to the final irrigation protocol as follows:

- **Group I:** 0.2% Chitosan Nano-Particles (CNPs).
- **Group II:** Chitosan Nano-Particles / Ethylenediaminetetraacetic Acid (CNPs/EDTA) (1:1).
- **Group III:** 17% Ethylenediaminetetraacetic Acid (EDTA) (Control).

Preparation of CNPs irrigation

CNPs were prepared at the Central Nanotechnology Characterization Lab, Agriculture Research Center, Ministry of Agriculture, Cairo, Egypt. The ionotropic gelation method was used as a described in a previous study [8] with modifications. First, a solution of 50 ml of 0.2% chitosan solution (2mg/ ml) was prepared by dissolving CS (Acros Organics, Belgium) in 2% (v/v) acetic acid solution (Sigma Aldrich, USA) in deionized water (Millipore-Sigma, Burlington, Massachusetts, USA) under magnetic stirring (Cimarec, Thermo Scientific, USA). The pH was adjusted to pH 4.8 with 0.5 M NaOH (Sigma Aldrich, USA) measured by pH meter (Orion 2-star, Thermo Scientific, USA). This was followed by constant stirring for 30 min. Afterwards, 6 ml of Tripolyphosphate (TPP) solution(Sigma Aldrich, USA) (11 mg/ ml) was added under stirring at 800 rpm to form the nanoparticles. Finally, the CNPs was stored in refrigerator and ultra-sonication of the suspension was done immediately before its usage.

Scanning electron microscopic assessment of smear layer removal

Following cleaning and shaping as previously described, the canals were randomly classified into three equal groups (n = 5) according to the final irrigation solution (s) used. A standardized

volume of (5 ml) of each solution was used for 3 minutes in each of the three groups. As for group (II); 2.5 ml of CNPs was used for 1.5 minutes, followed by 5 ml sterile saline, then 2.5 ml of EDTA (Prevest Dentpro Ltd., Jammu, India) for another 1.5 minutes. A final flush with 5 ml sterile saline then followed. Each root was then longitudinally split using double faced diamond disc at low speed under copious irrigation without penetrating into the canal lumen Splitting of the root into halves was then completed using a chisel and mallet (Dentsply Maillefer, Ballaigues, Switzerland) resulting in 30 halves from the 15 specimens. The two halves of each root were then examined under stereo microscope (Leica Microsystems, Switzerland) under magnification of (X16) to select the most representative half to be used for environmental scanning electron microscope (ESEM) (FEI Quanta FEG 250, Netherlands) analysis with an accelerating voltage of 30 K.V. All the samples were examined at magnifications of 500x and 1000x. The apical, middle and coronal thirds of each specimen were examined at 3, 7, and 12 mm, respectively from the apex. The micrographs of each third were coded and evaluated by two blinded well trained observers using a scoring method for evaluating smear layer. The 4 level score system described by Takeda., *et al.* (1999) [12] was advocated:

- **Score 1:** No smear layer and debris evidence on dentinal tubules.
- **Score 2:** Few dentinal tubules covered with a smear layer and debris.
- **Score 3:** Most dentinal tubules covered with smear layer and debris, a few tubules cleaned and opened.
- **Score 4:** Dentinal tubules completely covered with smear layer and debris. Each examiner scored all the micrographs independently in a blind manner. Four micrographs served as visual reference standards for the scoring system. When a conflict existed between the examiners, the micrograph of concern was discussed until an agreement on a definite score was reached. Finally, micrographs were decoded and the scores were tabulated.

The statistical analysis

Data was analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL) and explored for normality using tests of normality (Kolmogorov-Smirnov and Shapiro-Wilk tests). Smear layer scores showed non-parametric distribution. Numerical data was described as median and range. Comparisons of the 3 groups were done by Kruskal Wallis test. A p-value less than or equal to 0.05 was considered statistically significant. All tests were two tailed.

Results

The results of Smear layer assessment showed no statistically significant difference among the three groups (P-value= 0.368, 0.054 and 0.090) at the coronal, middle and apical thirds respectively. On the other hand, for the total score (mean of the three root levels), Group I (CNPs) showed significantly the highest amount of smear layer removal (P-value = 0.044), followed by both group II (CNPs/EDTA) and group III (EDTA) with no statistically significant difference between them (Table 1 and Figures 1 to 4).

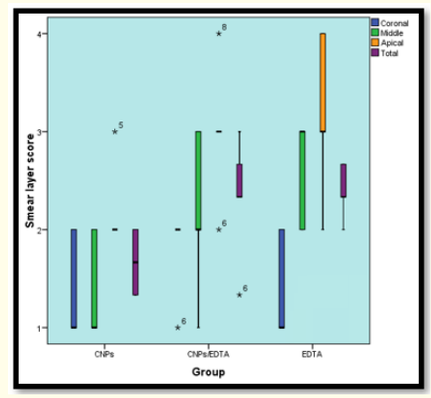

Figure 1: Box plot representing median and range values for smear layer scores of the three groups.

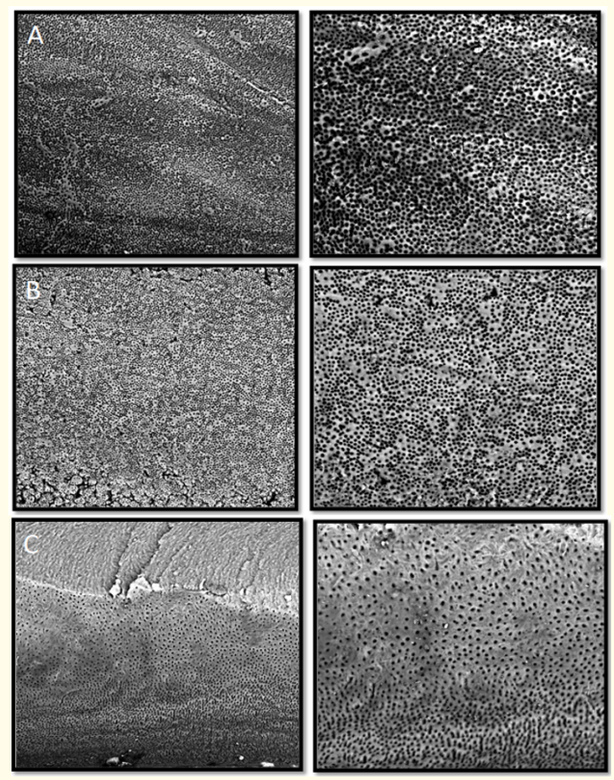

Figure 2: SEM micrographs showing smear layer removal in samples of Group I (CNPs) at 500X (Left side) and 1000X (Right side) (A) Coronal third, (B) Middle third, and (C) Apical third.

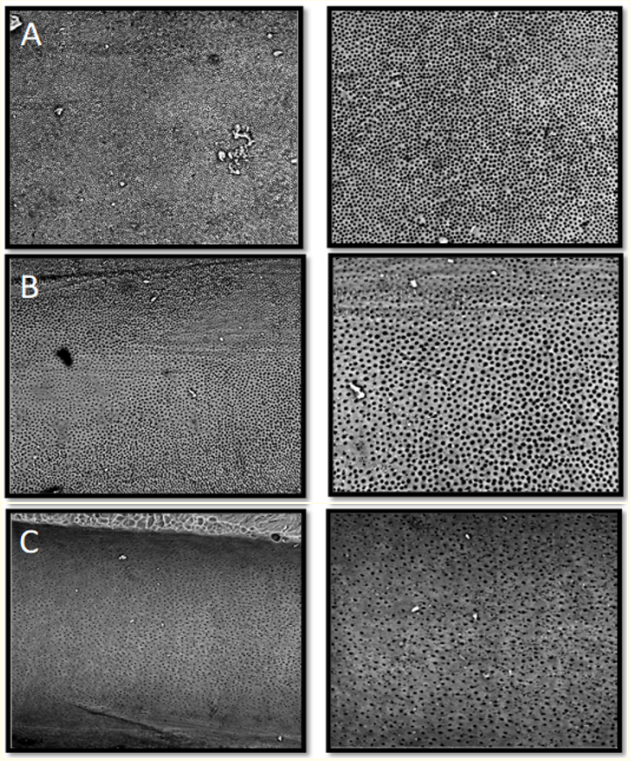

Figure 3: SEM micrographs showing smear layer removal in samples of Group II (CNPs/EDTA) at 500X (Left side) and 1000X (Right side) (A) Coronal third, (B) Middle third, and (C) Apical third.

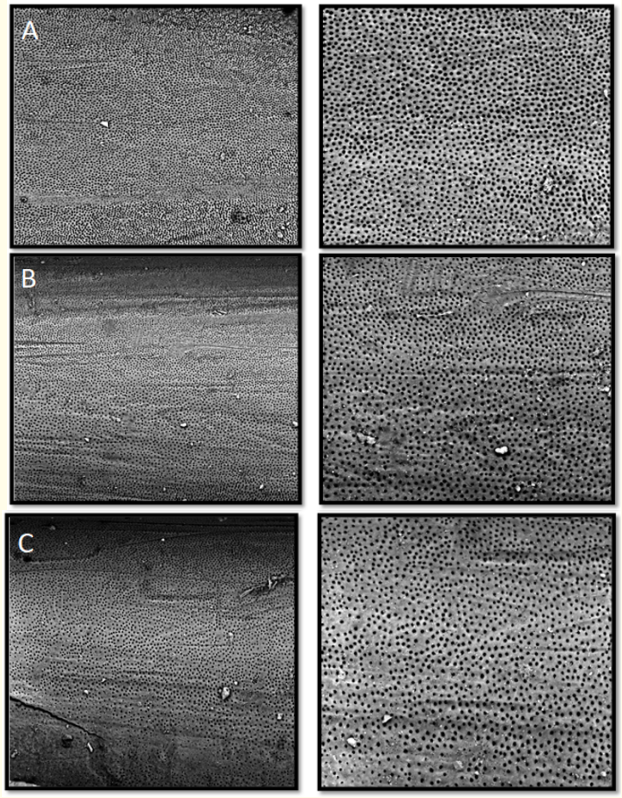

Figure 4: SEM micrographs showing smear layer removal in samples of Group III (EDTA) at 500X (Left side) and 1000X (Right side) (A) Coronal third, (B) Middle third, and (C) Apical third.

Root level	Group I (CNPs)		Group II (CNPs/EDTA)		Group I (EDTA)		P-value
	Median	Range	Median	Range	Median	Range	
Coronal	1	1-2	2	1-2	1	1-2	0.368
Middle	1	1-2	2	1-3	3	2-3	0.054
Apical	2	2-3	3	2-4	3	2-4	0.090
Total	1.7 ^{B}	1.3-2	2.3 ^{A}	1.3-3	2.3 ^{A}	2-2.7	0.044*

Table 1: The median, range values and results of Kruskal-Wallis test for comparison between smear layer scores among the three groups at each level

*: Significant at P ≤ 0.05, Different superscripts in the same row are statistically significantly different

Discussion

Although the question to remove or maintain the smear layer had remained debatable for many years, this bacteria-loaded layer may hinder the penetration of the disinfecting agents [9] and acts as a barrier between the filling material and the canal wall compromising the adequate seal formation [10]. Moreover, it is a loosely

adherent structure and a potential avenue for leakage between the root canal filling and the dentinal walls [11].

Single rooted mandibular premolars with single oval canals were selected for this study as this cross section can't be ultimately touched and cleaned by the rounded cross-sectioned endodontic files, leaving large areas of untouched canal walls and accumulated hard-tissue debris in irregularities within the root canal space. Research has shown that these untouched areas may reach up to 35% of the total canal walls [12,13] Thus, the efficacy and the role of irrigating solution can be clearly observed.

The use of chitosan irrigation at 0.2% concentration was justified in previous studies that demonstrated its efficiency in smear layer removal at this low concentration [14-17]. Nanoparticles have shown advanced physical and chemical properties in comparison to their parent materials in terms of ultra-small size, larger available surface area and increased chemical reactivity [18].The concept of using CNPs followed by EDTA in group II of this study was elaborated to achieve ultimate chelating effect as previously proposed in a study by Geethapria., *et al.* 2016 [19]. Hence, this combination was used to be compared with the use of each agent per se.

SEM studies may give subjective results that often depend on the selection of the observed areas and the operator's interpretation. In our study, the micrographs were captured at fixed lengths from the root apex (3, 7 and 12 mm from the root apex) to standardize the readings. Moreover, two calibrated and blinded experts scored the micrographs independently to increase the reliability and accuracy of the results obtained. Studies used magnifications ranging from 35x to 5000x [20-23] where low magnifications allowed for a large surface area to be observed, it did not allow for surface details to be noted. On the other hand, higher magnifications allowed for a closer examination, but with limited area of observation. To overcome these problems, intermediate magnifications of 500x and 1000x were used.

Regarding the results of smear layer removal, the significant difference in the combined scores between Group I (CNPs) and the other two groups can be attributed to its mechanism of action and the presence of chitosan in nano form might have been the reason for the fortified effect and significant superiority of group I. Currently, there are two theories to explain the chelation process of chitosan. The first, known as the model of the bridge, which states that two or more amino groups of one chitosan chain will bind to the same metallic ion [24]. The other theory supports that only one amino group of the structure of the substance is involved in the binding, that being the metallic ion "anchored" to the amino group [25]. Either of the two mechanisms could be responsible for the chelation of calcium ions in dentin resulting in the depletion of inorganic matter from the smear layer [26].

The results were in accordance with the results of previous studies by Pimenta., *et al.* (2012) [26], Silva., *et al.* (2013) [27], Del Carpio-Perochena., *et al.* (2015) [28], Neha., *et al.* (2017) [29], and Mathew., *et al.* (2017) [30] who demonstrated that there was no statistically significant difference between chitosan and EDTA at each root level in terms of smear layer removal. However, the results were in contrast to results by Darrag (2014) [31], Hassan and Negm (2017) [32], Kamble., *et al.* (2017) [15] and Mittal., *et al.* (2018) [33] who showed that chitosan was superior to EDTA in the removal of smear layer at different root thirds, while our results showed this significant superiority with group I (CNPs) only when the total scores were considered. This might be attributed to the difference in experimental design as well as the scoring systems used. Combining EDTA with CNPs in group II resulted in significantly inferior results in terms of the total smear layer scores compared to group I where CNPs irrigant was used per se. This might be attributed to the use of CNPs at half volume (2.5 mL) and for half duration of application (1.5 min.)

Conclusions

Within the limitations of this study, it could be concluded that:

- 0.2% CNPs is effective in removing smear layer from the root canal dentin which is a promising observation for future research.
- None of the used irrigants resulted in complete removal of the smear layer.
- The apical third always retained more smear layer than the middle or coronal thirds.
- The combined use of CNPs/EDTA (1:1) did not show superior performance over the other two groups.

Conflict of Interest

The authors deny any conflicts of interest in this study.

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