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The influence of Nano Chitosan as Final Irrigation on Smear Layer Removal and Cleaning of Dentinal Tubules (In Vitro Study)

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Aim: This study aims to evaluate the effectiveness of newly chitosan nanoparticles (CNPs) irrigations and compare them with other golden standard irrigations in the removal of smear layer.
Materials and Methods: A total of Fifty-four single-straight rooted human mandibular premolar teeth were obtained after being freshly extracted and utilized. All the teeth were decoronated to leave a 12-mm root length. Each tooth was accessed and the root canal was prepared using Pro-Taper universal rotary files to size F3. During instrumentation and between each file, the canal was irrigated with 2 ml of 2.5% NaOCl followed by 5ml of distilled water to flush it. Samples were distributed into six groups. Each group contained 9 samples and received a final wash for 3 minutes. Subsequently, each sample was longitudinally sectioned and observed under SEM. Finally, collected data was analyzed statistically.
Results: Statistically significant difference was found among the groups in all three thirds ($p<0.01$) and no significant difference between 0.2% and 0.5% Nano Chitosan ($p>0.05$). Chitosan nanoparticles (CNPs) were highly efficient as chelating solutions apically, but equally effective in coronal and middle thirds to EDTA. The efficiency of NaOCl at cervical and middle levels was superior to CNPs. 2% CHX and distilled water had the least efficient on removing the smear layer at all levels.
Conclusion: Newly irrigation chitosan nanoparticles appear to be highly efficient in smear layer removal as final washing.

Keywords: Chitosan nanoparticles, EDTA, smear layer, SEM.

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Introduction

One may think of the canal system of the tooth as a three-dimensional (3D) device. It is made up of numerous minor collateral canals with branches, apical deltas, loop accessory canals, isthmuses which is a link between two root canals and microscopic channels called dentinal tubules in addition to main canal. To guarantee that irrigants reach all necrotic tissues and microorganisms in the intricate canal architecture, cleaning and shaping should be carried out in three dimensions.[1]

An important stage in endodontic treatment is irrigation. The irrigation eliminates the smear layers and necrotic tissues. It also removes bacteria and its products from canal.[2]

The smear layer is an uneven layer of denaturated debris that forms on dentinal walls as a result of endodontic instrument cutting action. It is an amorphous structure that contains both organic and inorganic phases. It consists of microcrystalline, organic particle debris, dentine particles, bacteria, residual necrotic and vital pulp tissues, and odontoblastic processes. This prevents sealants, intracanal medications, and root canal irrigants from entering the tubules.[3] To avoid this, a variety of irrigants are utilized, and the last irrigant is very crucial for smear layer removal and disinfection.[4] Although the surface smear layer is typically 1-2 µm thick, it can produce smear plugs up to 40 µm deep in the dentinal tubules. In addition to lowering dentin permeability, these plugs prevent antiseptics and dissolving agents from penetrating dentinal tubules. The removal of this layer strengthens the link between sealer and root canal walls, additionally increases antimicrobial efficacy of chemomechanical treatment by enabling antiseptic solutions to enter the dentine more deeply.[5]

Nowadays, numerous endodontic irrigants have been developed. These include oxidizing agents like Hydrogen peroxide (H2O2), chelating agents (like EDTA, bisphosphonate HEBP, natural agents (like triphala, chitosan, propolis), and antibacterial agents (like NaOCl, CHX and MTAD). The gold standard irrigatant is sodium hypochlorite (NaOCl) in range of 0.5% to 5.25% concentrations because of its antibacterial and tissue dissolving characteristics.[6] However, it has a limited effect on the inorganic portion of the smear layer. Therefore, removing the smear layer should be accomplished by irrigating with NaOCl and using decalcifying substances. Calcium ions in the dentin wall are reacting with chelating agents to form soluble calcium compounds that can be easily rinsed off.[7]

Ethylenediaminetetraacetic acid (EDTA) is the most widely used chelating agent and is considered gold standard final irrigation in endodontic because it has an excellent chelating action and provides sufficient opening dentinal tubule by acting on an inorganic component of smear layer.[8] Despite its best ability to remove smear layers. EDTA can cause negative effects such denaturation of collagen fibrils and peritubular and intertubular dentin erosion when used for longer than three minutes. These Mineral alterations in root canal dentin may impact the adhesive qualities of the dentin surface and hinder root canal sealing. Additionally, due to its cytotoxicity, extrusion of EDTA solution past the apical foramen should be avoided.[9]

There are two forms of chlorhexidine (CHX): liquid and gel. CHX possesses good lubricating properties, low toxicity, high substantivity, and antibacterial activity similar to that of NaOCl. Some researchers have recommended CHX as a good irrigation solution, particularly as a last resort and in situations where Enterococcus faecalis is persistent. In general, this popular chemical

agent is recommended for the treatment of recurring periapical infections due to its substantivity. However, CHX is unable to dissolve organic material and eliminate smear layer and cannot replace sodium hypochlorite.[10] CHX can also enhance root canal sealing by decreasing the radicular dentin's collagen-disruptive matrix metalloproteinases' activity.[11]

Nanotechnology is a technology that deals with objects of nanometer size, and the particles are called nanoparticles (NPs). Due to the limitations of traditional irrigation techniques, nanoparticle-based irrigants have become more common. Nano dentistry refers to the use of nanoparticles in the diagnosis and treatment of oral illnesses with the aim of enhancing general oral health. Recently, nanoparticles have been found to be beneficial in pharmaceuticals, irrigation solutions, sealants, and restorative materials.[12]

The word "nano" originates from the Greek word "dwarf.". There are numerous sizes and forms of nanoparticles. Nanoparticles vary in size from 1 to 100 nm. Among their many characteristics are their elevated chemical activity and extremely small dimensions.[13]

Chitosan has recently been popular in all life fields to reduce the harmful effect of toxic chemicals for its natural, more biocompatible alternative properties. Chitosan is produced either by enzymatically hydrolyzing chitin deacetylase or by deacetylating solid form of chitin from seashells of crabs and shrimps under alkaline circumstances. Because of its strong acidic chelating activity, broad-spectrum antimicrobial properties, and its ability to promote dentin remineralization, chitosan can be utilized in endodontic operations.[14] Chitosan can dissolve in aqueous acids like acetic acid and lactic acid, but it is not soluble in neutral and basic media. The degree of deacetylation, molecular weight, pH, temperature, and polymer crystallinity all play a role in chitosan's solubility.[15]

Chitosan has properties of non-toxic, antibacterial characteristics biocompatible, biodegradable, bioadhesive and chelating activity. This made it highly used in dentistry. Chitosan can eradicate the smear layer and open dentinal tubules without causing viable degradation. Depending on the end use or the physical properties needed in the nanoparticles, many techniques could be used to manufacture or assemble chitosan nanoparticles.[16]

To guarantee that the smear layer is completely removed, it must be properly identified. SEM is used to identify the smear layer and the opening of dentinal tubules.[17] The present study aimed to compare the smear layer removal and cleaning dentinal tubules in the cervical, middle, and apical thirds by Chitosan Nanoparticles and different irrigation solutions (chelating and non-chelating agents) in root canal.

The null hypothesis states the lack of difference between smear layer removal and cleaning dentinal tubules by adding nanoparticles to another irrigation solution.

Materials and methods

Ethics approval and consent to participate

Approval of the ethics and research committee at Collage of Dentistry/University of Mosul with code UoM.Dent.23/73.

Sample size estimation

The sample size was estimated using the G*power program. The sample size was calculated to be nine samples per group, with a 95% confidence level, 90% power, a standard deviation of 0.45, and a mean difference of 0.8.

Sample Selection

The University Ethics and Research Committee reviewed and approved this study

in compliance with code UoM.Dent.23/73. This research was carried out on fifty-four human tissue samples of lower mandibular premolars collected from the orthodontic center in Mosul from patients aged between 15 and 35 years. The external surface of the roots was cleaned carefully from any remaining soft tissues by dental scaler, and stored in pure distilled water in closed containers until used in order to avoid dehydration.

Inclusion criteria: involve lower mandibular premolar with single rooted, single canal, closed apices, caries-free, no calcification, no internal or external resorption and previous root canal treatment.

Exclusion criteria, teeth with dilacerations, anatomical or morphological deformities, cracks or fractures and immature open apices were excluded. Teeth with single canal can be identified using angulated radiograph at mesiodistal and buccolingual directions. Moreover, a stereomicroscope (40X) was used to exclude any tooth with cracks or defects in the root.

Sample preparation

All teeth were decoronated using a diamond disc 0.2mm (NEXUS MEDODENT, Korea) perpendicular to long axis of tooth under water cooling to obtain root length 12mm measured by electronic digital caliper (Vizbrite, India).

The canals were accessed and pulp tissue extirpation were done using barbed broach. Initial canal patency to full working length was determined with ISO (10) K file (MANI, INC, JAPAN). Next, the correct working length was calculated by deducting1 mm from the length at which No. 10 K-file was visible at the apical foramen by necked eye.

Apex of each tooth was covered with pink wax and placed in polysiloxane elastomer impression material (Zetaplus, Zhermack, Badia Polesine, Italy) filled polyvinyl chloride tubing with a diameter of one inch in order to replicate the anatomical scenario in which the periapical tissues protected it. After that, the roots were fixed into the tube's middle.

ProTaper universal system rotary files (Shenzhen Denco Medical CO., Ltd, China) were used to prepare canal up to F3 with step-down technique using Eighteeth E-connect Cordless Endo-motor (Changzhou Sifary Medical Technology Co., Ltd, China) at (speed 250 rpm and torque 3 N-m).

About 2ml of 2.5% NaOCl (AQUA, TurKiye) solution for (1) min was used to irrigate the canal during instrumentation and between each file, then flushing with 5 ml of distilled water. A guage 30 double-sided Vented needle was used and inserted not more than 1mm from the apex.

Preparation of Irrigating Solution

0.2% Chitosan nanoparticles solution

The preparation of 0.2% of nano chitosan solution were aggregated by dissolving 2 gm of nano chitosan powder (78 nm) (Shaanxi Sangherb Bio-Tech Inc CO., Ltd, China) was weighed in high-precision portable balance (Mettler Toledo PL-S Classic Light, UK) in V:100 ml of 1% acetic acid. A magnetic stirring machine (DAIHAN LABTECH CO., LTD) was employed for stirring the mixture within 2 hours at room temperature (±23°C) till a crystalline homogenous solution (PH 3.8).[18]

0.5% Chitosan nanoparticles solution

To prepare of 0.5% chitosan nanoparticle. About 1 ml of glacial acetic acid was added to 100 ml of distilled water to obtain 1 % of acetic acid. Then 5 gm of nano Chitosan powder (78 nm) (Shaanxi Sangherb Bio-Tech Inc CO., Ltd, China) was weighed in high precision balance and added to the solution above of % acetic acid. The mixture was agitated using magnetic stirrer to produce viscous solution (PH 4.1) for 2 hours at room temperature (±23°C).[19]

Sample grouping

Based on final irrigation was used, six groups (n =9) were developed and each final irrigant was rinsed the canal as the following:

G I: Samples were lastly washed by 3 ml of 0.2% CNP for 3 minutes.

G II: Samples were lastly washed by 3 ml of 0.5% CNP for 3 minutes.

G III: Samples were lastly washed by 3 ml of 17% EDTA (Imicryl, TurKiye) for 3 minutes.

G VI: Samples were lastly washed by 3 ml of 3% NaOCl (AQUA, TurKiye) for 3 minutes.

G V: Samples were lastly washed by 3 ml of 2% CHX (AQUA, TurKiye) for 3 minutes.

G VI: Distilled-water.

Then the canals washed with (10ml) of distilled-water for 2 minutes and dried with F3 absorbent paper points. To avoid contamination of canals during sectioning from debris, the entrance of canals was covered with cotton balls.

Preparation of samples for viewing under scanning electron microscope

Each sample was fixed using a bench vice and high-speed diamond disc was used to create two parallel vertical grooves without perforating the canal on the buccal and lingual faces of each root. A tiny cotton plug was used to close canal opening after a blue gutta-percha cone (F3) was inserted inside of it. The gutta-percha cone served as an indicator of the depth grooves to prevent he disc from penetrating the canals and causing contamination from sectioning-related debris. Next, a chisel was used to split the root into two sections. All the measurements were performed at three levels: coronal (8– 11 mm from apex), middle (6–8 mm from apex), and apical (1–3 mm from apex) thirds by creating three horizontal grooves using a tapered fissure carbide bur perpendicular to the canal on each half of roots and each half was scanned under Scanning Electron Microscope (SEM) (ZEISS EVO 10, Germany) (Collage of pharmacy\ Ninevah University).[20]

SEM Examination

Ethyl alcohol 30% for 10 minutes, 50% for 20 minutes, 70% for 20 minutes, 90% for 30 minutes, 100% for 30 minutes, and 100% for 30 minutes. was used to dehydrate each half. Specimens were fixed on coded stubs, dried with air, calibrated under vacuum, coated by gold sputter, and investigated by SEM at 15 kV accelerating voltage, 10 mA, and a magnification of 2000 X. the images were taken from central part of each level to detect whether the smear layer presents and visualizes opening of tubules. Elimination of the smear layers were observed by one observer who is unknown to the irrigation schedules employed for each sample.[21]

Subsequently, the image assessment was based on Hülsmann's categorization about smear layer and degree of tubules opening.

- Score 1: No smear layer; dentinal tubules are open.
- Score 2: Small amount of smear layer; some dentinal tubules are open.
- Score 3: Homogeneous smear layer covering the root canal wall; only a few dentinal tubules are open.
- Score 4: Homogeneous smear layer covering the root canal wall; no dentinal tubules were open.
- Score 5: Heavy, inhomogeneous smear layer covering the entire root canal wall; no open dentinal tubules.

Statistical Analysis

Descriptive and analytical statistics were done by IBM SPSS 24 software window. According to the test of normality (Shapiro Wilk test) which states that the measurements do not follow a normal

distribution, which prompted us to use nonparametric tests to find differences between the groups at different thirds. Kruskal-Wallis Test and Mann-Whitney U Test were used. The level of statistical significance for Kruskal-Wallis Test (p value<0.01) and for Mann-Whitney Test (p value>0.05).

Results

Results of kruskal Wallis test showed that chitosan nanoparticles exhibited highly significant difference values than other test groups for removal of smear layer in all section of canal (P<0.01)(Table 1).

The score of smear layer removal of six groups at three different levels are shown in (Table 1). A significant difference was observed in the apical third between 02%,0.5% CNPs and other groups and no clear significant difference with 17% EDTA in coronal and middle thirds, but 3% NaOCl was a significant difference with 0.2%,0.5% CNPs in coronal and middle thirds. 2% CHX and distilled water showed the least ability to remove smear layer and were statistically different from other test groups.

Mann Whitney test was done between GI and GII and showed no statistically difference (Table 2).

GV and GVI show higher smear layer and debris at all levels of tooth. GI and GII showed better eliminating smear layer at apical level than cervical and middle levels. GIII showed less eliminating smear at apical third than GI and GII. GI, GII and GIII showed comparable effect in removing smear layer and less debris was present. GIV exhibited better removal smear at cervical and middle levels this can be shown in figure 1.

Discussion

In order to facilitate the removal of pulpal tissue, bacteria, and endotoxins, proper shape of the restrictive dentin which is a necessary part of the root canal preparation process. It also creates space for large volume of irrigants to flow through the canal in order to accomplish the goal of a successful cleaning and get the canal ready to receive filling material for an efficient apical seal therapy.[22]

Nanoparticle-sized materials are produced via nanotechnology and possess unique physicochemical properties such as high surface area and amplified chemical reactivity. This leads to a higher concentration of atoms near the surface, in comparison to their microscale or macroscale sized counterparts.[23] Lately, natural biomaterials introduced in endodontics to restore structural strength that destroyed by synthetic chemical solutions. Biomaterials like CNP that limits bacterial penetration, prevents dentinal micro-fractures, and improves the mechanical properties of the root dentin. Because of its greater capability of chelating for various metal ions and low price.[24]

The anatomical crowns were decoronated to root length 12mm in order to remove any variation between samples and to standardize and facilitate instrumentation.[25] Apex of each tooth was covered with pink wax and placed in polysiloxane elastomer impression material filled polyvinyl chloride (PVC) tube in order to replicate the anatomical scenario and provide closed canal system that prevent extrusion of irrigation from apical apex and permit recapitulation canal patency.[26] Chitosan nanoparticles are soluble in weak acid solutions and could also be soluble in water. They are employed to eradicate bacteria and remove the smear layer because of their deeper absorption and penetration into dentinal tubules and intertubular dentine.[27]

Table 1: Kruskal Wallis analysis for smear layer removal scores in each tooth section.

SEM	GI (0.2% CNP)	GII (0.5% CNP)	GII (17%EDTA)	GIV (3%NaOCl)	GV (2%CHX)	GVI(Distilled water	P-value	Sig
Apical							0.01	High sig
(Median+SD)	4.00+1.000	4.00+1.000	3.00+1.616	3.00+1.225	5.00+0.394	5.00+0.000		
MR	20.17	20.17	27.67	22.72	30.78	44		
Middle							0.001	High sig
(Median+SD)	3.00+0.527	2.00+0.527	3.00+1.118	2.00+0.500	5.00+0.781	4.00+0.707		
MR	19.26	19.16	19.98	17.33	31.78	44.78		
Coronal							0.00	High sig
(Median+SD)	2.00+0.707	3.00+0.72	3.00+1.093	4.00+1.130	4.00+0.781	4.00+0.707		
MR	19.06	19.94	20.39	17.72	40.50	39.39		

***P<0.01, SD: Standard devation, Sig: Significance MR: Mean Rank, CNP: Chitosan nanoparticles, EDTA: Ethylenediamineteraacetic acid, NaOCl: Sodium hypochloride, CHX: Chlorohexidine**

Table 2: Mann-Whitney analysis for intergroup compares between 0.2% and 0.5% CNPs.

Site	Groups	Mann-Whitney U	P- value	Sig.
Apical	G I	40.50	1.00	Not Sig
	G II			
Middle	G I	36.00	0.65	Not Sig
	G II			
Coronal	G I	26.50	0.2	Not Sig
	G II			

*** p>0.05, Sig: Significance.**

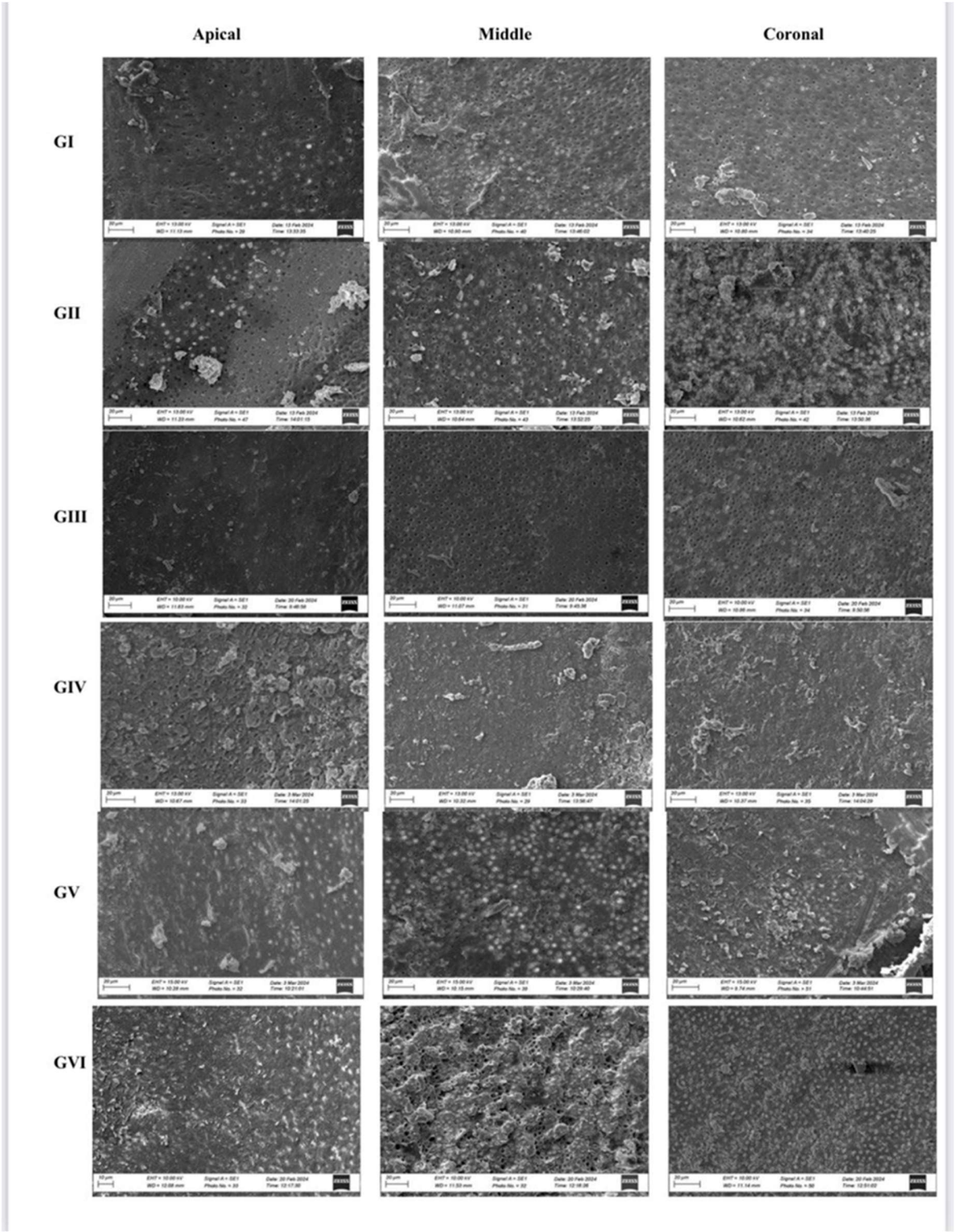

Figure 1: Scanning electron microscope photographs at 2000x magnification showing root samples treated with GI (0.2% CNP), GII (0.5% CNP), GIII (17% EDTA), GIV (3% NaOCl), GV (2% CHX) and GVI (Distilled water) at three different levels (apical, middle and coronal).

In previous study, Silva et al.[28] Darrag [29] concluded that irrigation of 0.2% chitosan for 3 minutes sufficiently cleared the smear layer, better fallouts and caused degraded dentine less than EDTA. This contrasted with EDTA, which causes erosions and excessive removal of peritubular and intratubular dentin when used longer than 1 min. For easy and standardized comparison, the final irrigations were administrated in same volumes for same application time to all groups. The application times were selected depending on prior study, that suggested the application time of 1ml of chitosan and its nonparticles is 3 min.[30]

In research of Ratih et al.[31] that concluded, 3 min application period resulted in stronger bond and greater apical sealing than 1 min, independent final irrigation utilized. Thus, the application time of 3 min is chosen when using chitosan nanoparticle for final washing. Applying irrigants for a longer period of time is necessary for increased sealing capability and binding strength, this works best in the clear apical third canal from smear.

According to descriptive analysis, there was the highest smear layer present for GVI (distilled water), that agreement with previous research. This may be due to its solely intended to be as lubricant and whatsoever has no impact on microorganism or dentin smear layer. It was utilized as control group for this reason.[32] The GIV (3% NaOCl) in our study have higher smear layer removal than CNPs at middle and cervical thirds this because it is nonspecific deproteinizing substance that can break down the smear layer's organic phase, this lead to thinning of smear layer which facilitate irrigation penetration.[33]

In study of Nanda et al. [34] noticed when comparison the effect of irrigation (3% NaOCl, 17% EDTA, and MTAD) with saline which using as control in clear from smear layer showed 3%NaOCl is more efficient than saline. Sodium hypochlorite solutions consist of Hypochlorous acid , a chemical components which behaves like solvent upon contact organic tissue. Also It release chlorine, which insert in a chemical process in contact with amino group of protein, and formation of chloramines that compromise metabolism of cells.

Kassaee et al.[35] concluded that CNP appears to be more effective permeable solution than 17% EDTA, NaOCl and ordinary Chitosan. Because of small nano size of CNP, has low contact-angle and higher penetration, under comparable conditions. Chitosan is a natural final irrigation solution, which used recently in endodontics because it has properties of bio-degradability, bio-compatibility to human cells, antibacterial, bio-adhesion, and lack of toxicity. CH and CNPs can be an alternative option instead of EDTA with these properties.[36]

CNP demonstrated better smear layer removal than CH, this implies that decreased in particle sizes, stronger surface area, and higher chemical reactivity may have contributed to improve its chemical and physical characteristics.[37]

Bajpe et al., [38] Concluded that 0.2% CNP solution was more efficient than 17% EDTA and 18% etidronic acid irrigants in eliminating thin layer of debris. According to this present study findings, 0.2% CNP and 0.5% CNP solutions had comparable effect on eliminating smear layer as 17% EDTA at coronal and middle thirds. While they were better smear removal than 17% EDTA at apical third which is in consent with prior studies that found that 0.5% CNP has higher capability to eliminate smear layer from apical third than 17% EDTA.[39,40]

The apical portion of the root canal has a narrower diameter, making it more difficult to eliminate the smear layer. The nano-size of chitosan nanoparticles can increase the removal of the smear layer by enhancing the

flow of irrigation fluid into dentinal tubules, and the hydrophilicity of Chitosan polymer allows it to be readily adsorbed by root canal walls and delivered deeper into dentinal tubules by maintaining close contact with the root canal dentin.[40.41]

Previous study was observed that CNP demonstrated superior smear removal than EDTA. This is in accordance with this investigation. The lesser capability of EDTA to eliminate smear layer in the apical third may be attributed that EDTA has property of chelating activity and reacts with calcium ions in dentine and forms soluble calcium chelates, and apical third has less amount of water soluble non-collagen proteins which EDTA eject calcium from them, therefore less EDTA decalcification. EDTA decalcified dentine to a depth 20–30 microns in 5 min, thus higher concentration and prolonged contact time of solution may increase cleaning but increasing demineralizing of dentine. The effect of EDTA may be restricted by tubular-sclerosis of dentin in apical third.[42,43]

Statistical analysis of our study showed 2% CHX had more smear layer surviving at all thirds than other experimental groups. This is consistent with study of Dewi et al. [44] Although CHX possess wide spectrum of antibacterial activity, substantivity and low toxicity than NaOCl but not able to resolve necrotic residues and remove smear layer, so not utilize as a regular major irrigation.[45]

This study has several limitations. First, no assessment was made of the impact of the chitosan on dentin degradation. Second, the initial micro-hardness of the root dentin was not assessed resulting in the inability to compare data for the same solution. To assess the impact of chitosan on micro-hardness of root dentin over time, further reseach is needed. As a substitute for chelating solutions, the chitason solution's capacity to remove smear layers ought to be examined. It should be mentioned that using irrigating solutions for more than three minutes may cause the dentin to erode.

Conclusion

1. Chitosan nanoparticles solution showed higher smear layer removal than 17% EDTA in apical level.
2. Chitosan nanoparticles solution showed comparable effective to EDTA in coronal and middle levels.
3. Chitosan nanoparticles solution has been proposed as a substitute for well-known root canal irrigants, such as sodium hypochlorite, which has some drawbacks, including increased toxicity and bad odor, CHX inability to remove the smear layer, and EDTA that cause dentine erosion and degradation.

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Data availability

Data related to this research will be available upon request to corresponding author.

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