EUROPEAN ENDODONTIC JOURNAL

ORIGINAL ARTICLE

Effect of Various Disinfection Protocols on Endodontic Biofilm and Growth Factors Release from Radicular Dentine: An *In Vitro* Study

Abhilasha GUGLIANI,[1] Sonali TANEJA,[2] Devi Charan SHETTY,[3] Vidhi Kiran BHALLA[4]

[1]Consultant Endodontist, ITS Centre for Dental Studies and Research, Uttar Pradesh, India
[2]Department of Conservative Dentistry and Endodontics, ITS Centre for Dental Studies and Research, Uttar Pradesh, India
[3]Department of Oral Pathology and Maxillofacial Surgery, ITS Centre for Dental Studies and Research, Uttar Pradesh, India
[4]Project Scientist, Dental Technology Innovation Hub, Maulana Azad Institute of Dental Sciences, New Delhi, India

ABSTRACT

Objective: The aim of this study was to evaluate and compare the effect of various disinfection protocols on bacterial biofilm and subsequent release of growth factors from radicular dentine.

Methods: One hundred and ninety two extracted single rooted premolars were obtained and contaminated with *E. faecalis* biofilm for 21 days. The samples were then divided into three main groups – Group I: Irrigation (I) only, Group II: Calcium hydroxide (CH) placement followed by final irrigation and Group III: Triple Antibiotic paste (TAP) placement followed by final irrigation. Each group was further then divided into four sub-groups according to the final irrigating solution used – Sub group A: Saline, Sub group B: 17% EDTA, Sub group C: 1% phytic acid and Sub group D: 0.2%. chitosan nanoparticles. After treatment, the samples were subjected to colony-forming unit (CFU) analysis to determine bacterial reduction and the release of TGF-β1 and VEGF from the root canals, which was quantified using Enzyme-Linked Immunosorbent Assay (ELISA). The data were analyzed using statistical tests.

Results: The maximum reduction in *E. faecalis* biofilm was observed in Group III (TAP), followed by Group II (CH), and finally Group I (irrigation only). Among the subgroups, the maximum reduction in bacterial biofilm was seen with chitosan nanoparticles, followed by phytic acid, EDTA, and saline. After 24 hours, the highest release of both TGF-β1 and VEGF was observed in the chitosan nanoparticles subgroup, followed by phytic acid, EDTA, and saline. Similar results were seen in the CH and TAP groups.

Conclusion: The study concluded that newer irrigating solutions, particularly 0.2% chitosan nanoparticles, showed superior antibacterial activity and better smear layer removal, leading to greater growth factor release from the radicular dentine. The study also highlighted that TAP placement resulted in maximum bacterial reduction, regardless of the final irrigant used. Furthermore, the release of TGF-β1 was significantly higher than VEGF in all groups.

Keywords: Dental biofilm, disinfection, growth factors, radicular dentine, regenerative endodontics

HIGHLIGHTS

- 0.2% chitosan nanoparticles demonstrated superior antibacterial activity and better smear layer removal, promoting greater growth factor release from radicular dentine.
- TAP placement led to the maximum bacterial reduction, regardless of the final irrigant used.
- TGF-β1 release was significantly higher than VEGF in all groups, highlighting its important role in the regenerative process.

Please cite this article as:
Gugliani A, Taneja S, Shetty DC, Bhalla VK. Effect of Various Disinfection Protocols on Endodontic Biofilm and Growth Factors Release from Radicular Dentine: An *In Vitro* Study. Eur Endod J 2025; 10: 1-10

Address for correspondence:
Sonali Taneja
Department of Conservative Dentistry and Endodontics, ITS Centre for Dental Studies and Research, Uttar Pradesh, India
E-mail:
drsonali_ taneja@yahoo.com

Received : March 14, 2024,
Revised : May 16, 2024,
Accepted : June 11, 2024

Published online: February 14, 2025
DOI 10.14744/eej.2024.84856

INTRODUCTION

Regenerative Endodontic Procedures (REP) emerged from the pioneering experiments of Nygaard Ostby (1) and Nygaard Ostby and Hjortdal (2), who established them as an ideal treatment modality for replacing diseased or necrotic pulp tissue with healthy tissue to revitalize the tooth. REP aims to preserve and regenerate pulp-like tissue within the root canal system, particularly in immature teeth with open apices, where the goal is to stimulate the natural healing process. A critical factor for successful regenerative treatment is the release of growth factors such as Transforming Growth Factor Beta 1 (TGF-β1), Vascular Endothelial Growth Factor (VEGF), Bone Morphogenetic Protein 2 (BMP-2), Epidermal Growth Factor (EGF), and basic Fibroblast Growth Factor (bFGF) from the dentinal matrix. Among these, TGF-β1 plays a crucial role in promoting odontoblastic differentiation, while VEGF is essential for angiogenesis and blood vessel formation, which are both vital to the success of regenerative therapies (3, 4).

A primary obstacle to the success of REP is pre-existing infection in the root canal system. Infections in the form of bacterial biofilms can hinder the migration and differentiation of stem cells, impeding the regenerative process (5). Biofilm formation in the root canal system prevents the release of growth factors from dentine, which in turn affects the viability of stem cells required for pulp regeneration. *Enterococcus faecalis* (*E. faecalis*) is one of the most persistent bacteria in the root canal, capable of forming biofilms that are resistant to conventional disinfection protocols, making it a major challenge in regenerative endodontics. The biofilm can cling to the canal walls and invade dentinal tubules, thus obstructing the necessary release of growth factors needed for successful treatment (6).

In cases of immature teeth with open apices, mechanical debridement is not advised, as it can compromise the integrity of the fragile root structure. In such cases, chemical irrigation becomes the primary method for disinfecting the canal system. The selection of appropriate irrigating solutions is, therefore, crucial for ensuring effective disinfection, removing biofilm, and facilitating the release of growth factors (7). While conventional irrigants like sodium hypochlorite (NaOCl) and Ethylene Diamine Tetracetic acid (EDTA) have been widely used in endodontics, they are often limited by factors such as incomplete smear layer removal, inadequate penetration into the dentinal tubules, and their inability to fully address bacterial biofilms (8, 9).

Emerging irrigating solutions such as phytic acid and chitosan nanoparticles are being explored for their superior ability to remove the smear layer, enhance antibacterial action, and facilitate the release of growth factors. Phytic acid, a highly acidic molecule, can effectively chelate calcium ions from dentine, leading to better dentine conditioning and growth factor release (10). Chitosan nanoparticles, due to their small size and polycationic nature, have been shown to interact with bacterial membranes, offering improved antibacterial properties and the potential for better penetration into dentinal tubules to remove biofilms and residual medicaments (11). These newer agents may offer advantages over traditional irrigants, particularly in the context of regenerative endodontic procedures.

In addition to the choice of irrigants, intracanal medicaments also play a pivotal role in controlling infection and promoting successful regeneration. Calcium hydroxide has long been recommended for its antimicrobial properties and ability to stimulate healing in the root canal system (12). Recently, triple antibiotic paste (TAP), consisting of a combination of metronidazole, ciprofloxacin, and minocycline, has gained popularity for its broad-spectrum antimicrobial activity and its ability to promote disinfection in regenerative procedures (13).

While individual studies have evaluated the effect of various irrigating solutions and medicaments on bacterial biofilm (14, 15), few studies have comprehensively assessed their effects on both biofilm removal and growth factor release from radicular dentine (16–18). The combination of these factors and their subsequent influence on regenerative outcomes has not been thoroughly explored in the literature. Furthermore, while studies have documented the release of TGF-β1 from dentine after chemical conditioning (19–21), literature on VEGF release in response to different disinfection protocols remains limited.

This study aimed to fill this gap by evaluating and comparing the effects of various disinfection protocols on bacterial biofilm removal and the subsequent release of TGF-β1 and VEGF from radicular dentine. The goal was to determine the most effective disinfection protocol that promotes both bacterial eradication and optimal release of growth factors, thereby enhancing the success of regenerative endodontic treatments.

The null hypothesis tested in this study was that there would be no significant difference in the effects of various disinfection protocols on bacterial reduction and the release of TGF-β1 and VEGF from radicular dentine.

MATERIALS AND METHODS

The manuscript of this laboratory study has been written according to Preferred Reporting Items for Laboratory studies in Endodontology (PRILE) 2021 guidelines (Fig. 1).

Ethical clearance was obtained from ITS Institutional Ethics Committee (IIEC) (Reference No.: ITS-CDSR/IIEC/2019-22/CONS/01). The study was conducted in the Department of Conservative Dentistry and Endodontics in collaboration with the Advanced Research Centre at ITS Dental College, Ghaziabad. The authors did not use any artificial intelligence (AI) assisted technologies (such as Large Language Models [LLMs], chatbots or image creators for this study. The study was conducted in accordance with the Declaration of Helsinki.

This study considered the treatment of radicular root dentine with REP based irrigation and medicament protocol. For each disinfection protocol antibacterial efficacy was evaluated quantitatively by determining the reduction in colony forming units followed by quantitative evaluation of TGF β1 and VEGF release using enzyme linked immunosorbent assay (ELISA).

Sample Size Calculation

The sample size was calculated by the following formula.

With the help of literature survey we have found the expected S.D of group 1, group 2 and group 3 are 5.4 ,4.9 and

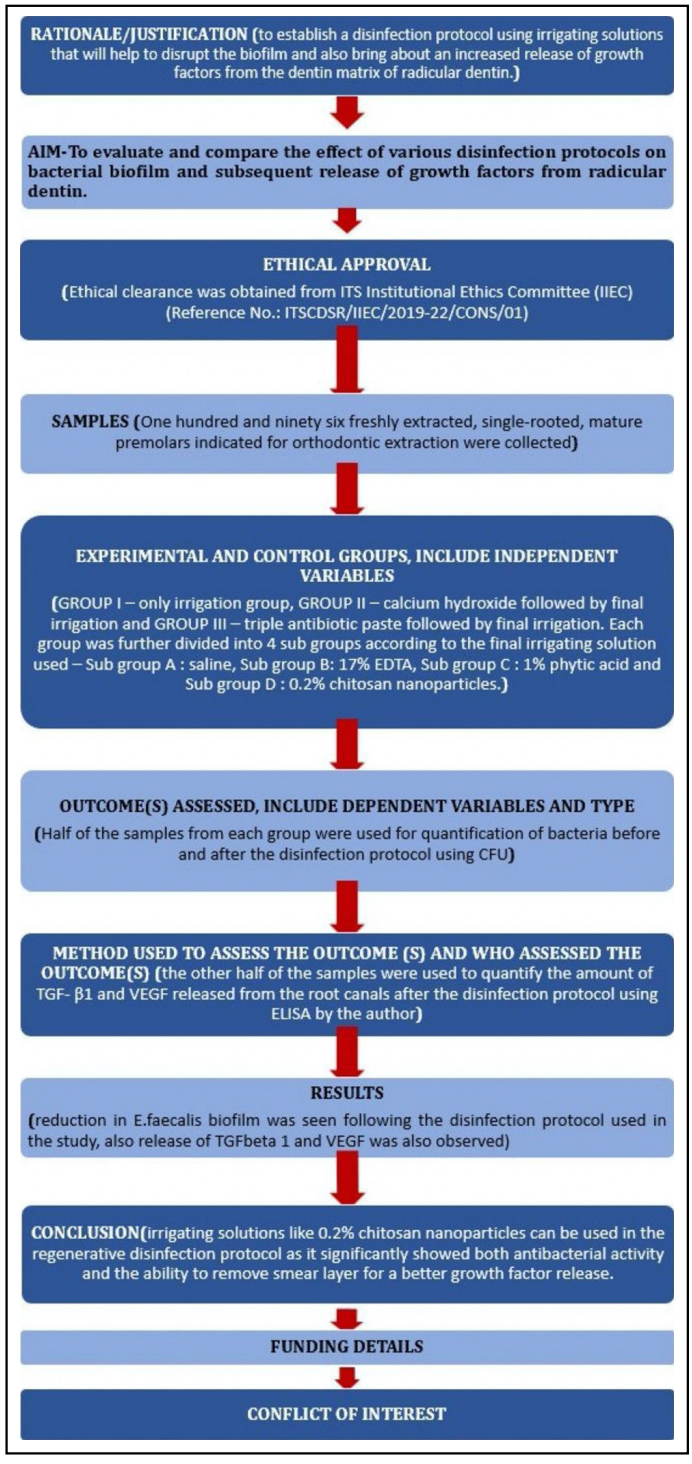

Figure 1. PRILE flow chart

PRILE: Preferred Reporting Items for Laboratory studies in Endodontology, EDTA: Ethylene diamine tetraacetic acid, CFU: Colony-forming unit, TGF-β1: Transforming growth factor beta 1, VEGF: Vascular endothelial growth factor, ELISA: Enzyme linked immunosorbent assay

4.8 respectively and mean difference is 4.6 of three groups for variables. Using the above formula with and software Open Epi, Version 3, we have found the sample size for each group is 64 and total is 192. Formula is:

$$n = \frac{(s1^2 + s2^2)\left(Z_{1-\frac{\alpha}{2}} + Z_{1-\beta}\right)^2}{D2}$$

The notation for the formulae is:

n= sample size of Groups

s1= standard deviation of Group 1

s2= standard deviation of Group 2

D= difference in group means

Z1-α/2 =two-sided Z value (eg. Z=1.96 for 95% confidence interval).

Z1-β= power=80%

Sample Collection

A total of 192 freshly extracted, single-rooted, mature premolars, each approximately 21 mm in length, were collected. Teeth with caries, internal or external resorption, highly curved roots, immature root apices, or previous endodontic treatments were excluded from the study.

Sample Preparation

The freshly extracted teeth were rinsed with phosphate-buffered saline before being stored in a 0.1% thymol solution at 4°C. Periodontal tissue was removed using ultrasonic scalers and curettes. The coronal portions of the teeth were removed, and the root segments were standardized by cutting them 7 mm from the apex. To simulate clinical conditions of regenerative endodontics, all root segments were instrumented with hand files (up to size 100) to create standardized canals with a 1-mm open apex. The samples were then autoclaved for 30 minutes at 121°C under 15 psi pressure to ensure complete sterilization (9). Nail varnish was applied to cover the external surface of the roots (17).

Biofilm Formation

The root canals were inoculated with a 20 µL suspension of *Enterococcus faecalis* (ATCC 29212) using sterile micropipettes. The canal opening was sealed with sterile cotton. To maintain humidity, cotton dampened with distilled water was placed in Eppendorf tubes along with the teeth, and the tubes were incubated for 3 weeks at 37°C. Every two days, 20 µL of Brain Heart Infusion (BHI) broth was added to provide nutrients for the bacteria (15).

Sample Categorization

After biofilm formation, the root segments were randomly divided into three main groups (n=64 per group) according to the type of disinfection protocol used:

- **Group I:** Only irrigation (Fig. 2).
- **Group II:** Calcium hydroxide followed by final irrigation (Fig. 3).
- **Group III:** Triple antibiotic paste (TAP) followed by final irrigation (Fig. 4).

Each group was further divided into four subgroups based on the final irrigating solution:

- Subgroup A: Saline
- Subgroup B: 17% EDTA
- Subgroup C: 1% Phytic acid
- Subgroup D: 0.2% Chitosan nanoparticles

Half of the samples in each group were used to quantify bacterial levels before and after disinfection using CFU analy-

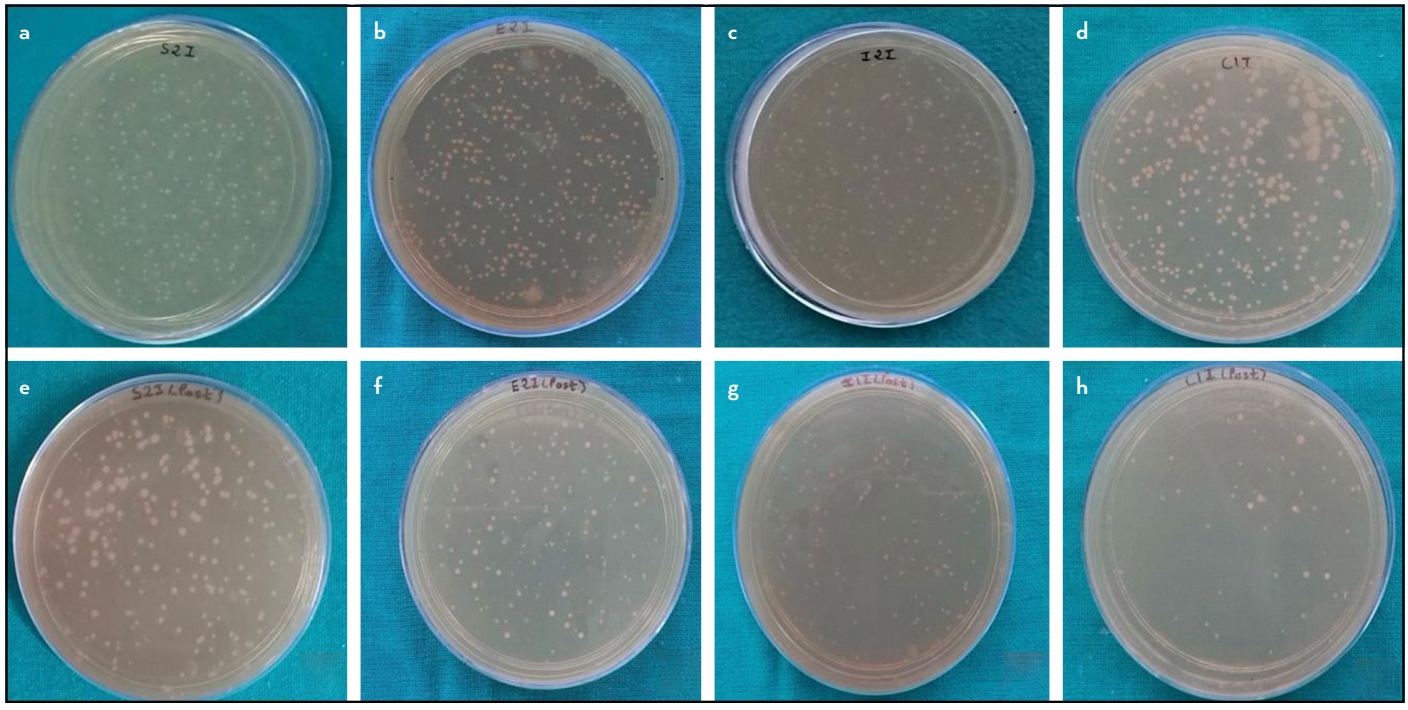

Figure 2. Irrigation only group. (a) Saline-Predisinfection (b) EDTA-Predisinfection (c) Phytic acid-Predisinfection (d) Chitosan Nanoparticles-Predisinfection (e) Saline-Post Disinfection (f) EDTA-Post disinfection (g) Phytic acid-Post disinfection (h) Chitosan nanoparticles-Post disinfection

EDTA: Ethylene diamine tetraacetic acid

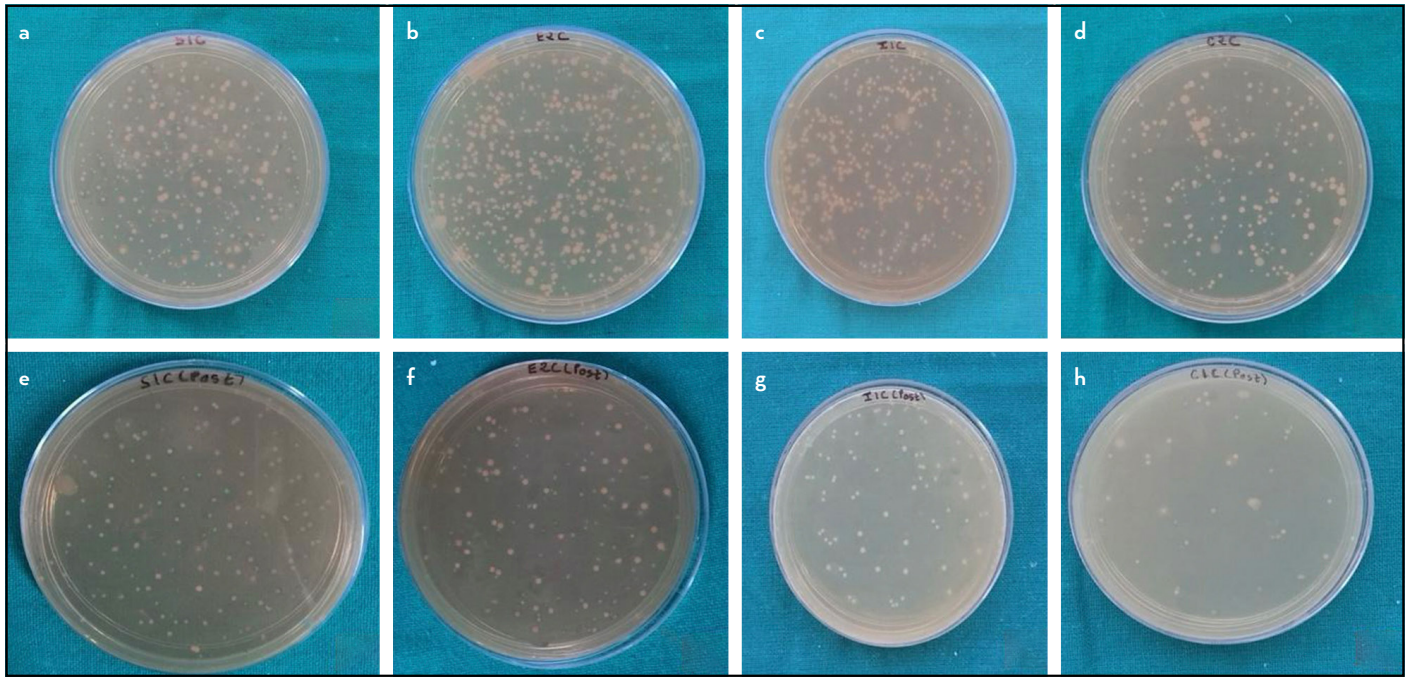

Figure 3. Calcium hydroxide medicament group. (a) Saline-Predisinfection (b) EDTA-Predisinfection (c) Phytic acid-Predisinfection (d) Chitosan Nanoparticles-Predisinfection (e) Saline-Post Disinfection (f) EDTA-Post disinfection (g) Phytic acid-Post disinfection (h) Chitosan nanoparticles -Post disinfection

sis, while the other half were used to measure the release of TGF-β1 and VEGF from the root canals after the disinfection protocols using ELISA.

Irrigation Protocol

The root segments were first irrigated with 20 mL of 1.5% NaOCl for 5 minutes, followed by 20 mL of the respective final irrigant for 5 minutes using passive ultrasonic irrigation with U files (Mani Inc, Tochigi, Japan).

Medicament Protocol

The root segments were treated first with 1.5% NaOCl (20 mL for 5 minutes) followed by 17% EDTA (20 mL for 5 minutes) to simulate the American Association of Endodontists (AAE)

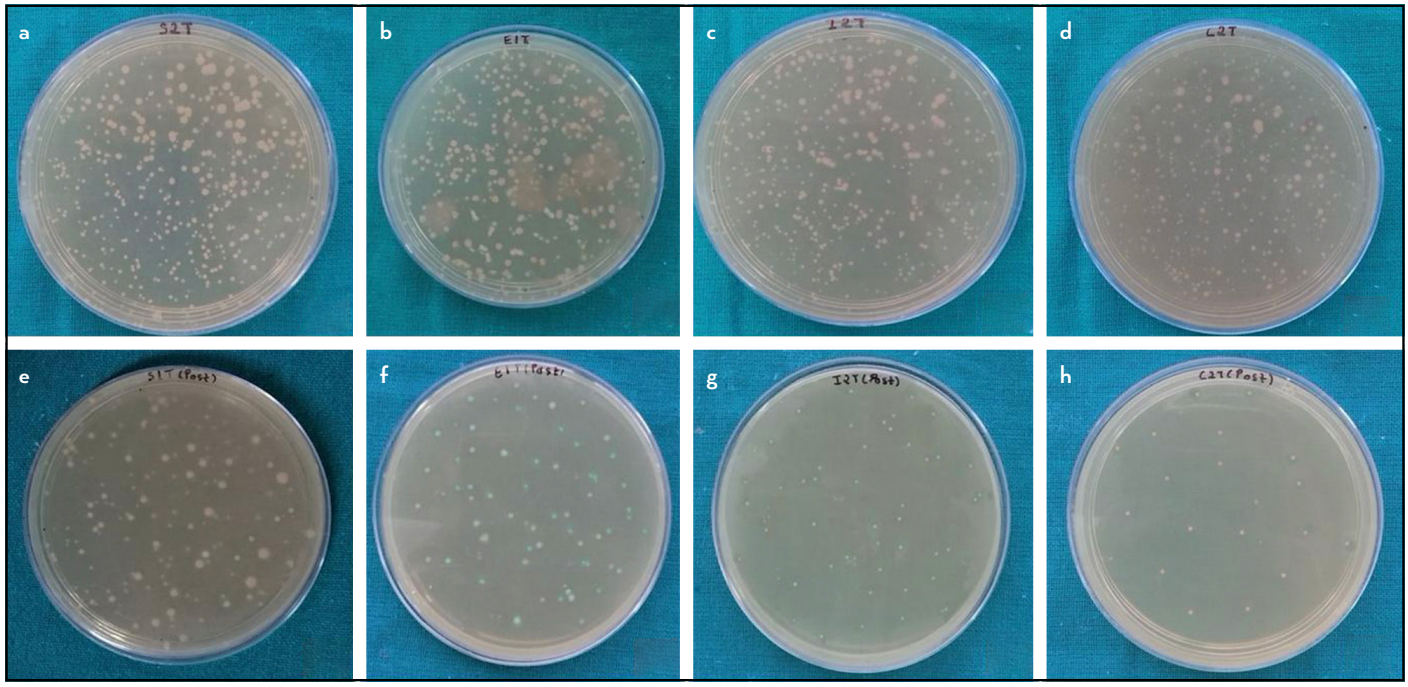

Figure 4. Triple antibiotic paste medicament group. (a) Saline-Predisinfection (b) EDTA-Predisinfection (c) Phytic acid-Predisinfection (d) Chitosan Nanoparticles-Predisinfection (e) Saline-Post Disinfection (f) EDTA-Post disinfection (g) Phytic acid-Post disinfection (h) Chitosan nanoparticles -Post disinfection

EDTA: Ethylene diamine tetraacetic acid

Regenerative Endodontic Procedures (REP) protocol. Calcium hydroxide (RC CAL, Prime Dental, Mumbai) and triple antibiotic paste (1 g each of Metronidazole, Ciprofloxacin, and Doxycycline mixed in saline) were placed into the root canals. After a 7-day incubation period, the root segments were irrigated with the final irrigants as per the subgroups using passive ultrasonic irrigation.

Biofilm Reduction

After the 21-day contamination period, a sterile ISO size 80 absorbent paper point was placed inside the canal of each specimen for 1 minute and transferred to sterile Eppendorf tubes containing 1 mL of BHI broth to create a suspension. The suspension was vortexed for 30 seconds, and serial dilutions were prepared. A 0.1 mL aliquot of the suspension was seeded onto BHI agar plates, which were incubated at 37°C for 24 hours to allow colony formation. The CFUs were then counted using colony counting methods.

To confirm residual biofilm presence after disinfection, dentine shavings were collected from the root segments using a sterile #4 Peeso reamer (Mani Inc, Tochigi, Japan) and transferred to sterile tubes with 1 mL of BHI broth. After incubation at 37°C for 24 hours in an aerobic environment, the tubes were vortexed, and serial dilutions were performed. A 1 µL sample of each dilution was plated on agar plates, and CFUs were counted to quantify bacterial reduction.

Growth Factor Release

After each disinfection protocol, root segments were placed in 1 mL of HBSS buffer for 24 hours. The medium was then withdrawn and used to measure the release of TGF-β1 and VEGF using specific human ELISA kits, following the manufacturer's protocol.

Statistical Analysis

Data on CFU/mL and growth factor release were analyzed using SPSS software (IBM Corp, Version 22.0, New York, USA). The mean and standard deviation were calculated, and one-way ANOVA was used to compare bacterial reduction and growth factor release between groups. Post hoc Tukey's test was applied for pairwise comparisons. Statistical significance was set at $p<0.05$, with a confidence interval of 95%.

RESULTS

Reduction in *E. faecalis* Biofilm After Disinfection Protocol

Compared to bacterial levels before disinfection, the greatest reduction in *E. faecalis* biofilm was observed in the TAP group (Group III), followed by the calcium hydroxide group (Group II), and then the irrigation-only group (Group I) ($p<0.05$). Within the subgroups, significant reductions were seen in the chitosan nanoparticles group, followed by the phytic acid group, then the EDTA group, and finally the saline group for all three major groups ($p<0.05$) (Table 1).

Release of TGF-β1 and VEGF from the Root Canals After Disinfection Protocol

24 hours after irrigation, the chitosan nanoparticles subgroup in Group I (irrigation only) released the highest amounts of both TGF-β1 and VEGF, which was significantly greater than the phytic acid and EDTA subgroups ($p<0.05$). The saline subgroup released the least amount of growth factors, though this was still statistically significant ($p<0.05$). For Groups II (calcium hydroxide) and III (TAP), the chitosan nanoparticles subgroup again released the most growth factors, significantly more than the phytic acid subgroup ($p<0.05$). The phytic acid subgroup released more than the EDTA subgroup, but there was

TABLE 1. Reduction in *E.faecalis* count after disinfection (CFU/mL) of Group I, II and III for Saline (Subgroup A), 1 EDTA (Subgroup B), 1% phytic acid (Sub group C) and 0.2% CNPs (Sub group D)

Group number	Reduction in *E.faecalis* count after disinfection									
	Intra group comparison (p)									
	Sub group A (Saline)	Sub group B (17% EDTA)	Sub group C (1% phytic acid)	Sub group D (0.2% CNPs)	Saline vs. EDTA	Saline vs. phytic acid	Saline vs. CNPs	EDTA vs. phytic acid	EDTA vs. CNPs	Phytic acid vs. CNPs
					p					
Group I	196.61±4.12	209.82±5.14	223.88±6.09	234.77±4.97	0.007*	0.001*	0.001*	0.003*	0.001*	0.029*
Group II	204.36 ±5.66	217.80±6.20	232.96±5.87	244.19±3.81	0.001*	0001*	0.001*	0.001*	0.001*	0.009*
Group III	215.69 ±7.01	226.04±5.41	240.0±4.61	250.11±5.03	0.015*	0001*	0.001*	0.005*	0.001*	0.010*
Inter group comparison (p)										
Group I vs. II	0.034*	0.025*	0.010*	0.002*						
Group I vs. III	0.001*	0.001*	0.001*	0.001*						
Group II vs. III	0.002*	0.002*	0.048*	0.047*						

*: Indicates significant difference at p≤0.05. CFU: Colony-forming unit, EDTA: Ethylene diamine tetraacetic acid, CNP: Chitosan nanoparticles

no statistical difference between the two (p>0.05). The least amount of growth factors was released by the saline subgroup. Across all major groups, Group I (irrigation only) exhibited the highest release of growth factors, followed by Group II (calcium hydroxide), and then Group III (TAP) (p<0.05) (Table 2, 3).

DISCUSSION

Despite the high success rates of conventional root canal treatments, an ideal therapeutic approach for teeth with open apices involves Regenerative Endodontic Procedures (REP). REP is a form of tissue engineering that combines disinfection of infected root canals with apical enlargement to allow for revascularization. It also utilizes adult stem cells, scaffolds (matrix), and growth factors (morphogens) (22). The irrigating solutions used in REP must not only disinfect the root canal system but also promote the secretion of growth factors to support stem cell migration and differentiation, making dentine conditioning a crucial step in the procedure (23).

The first part of our study assessed the effectiveness of various disinfection protocols against *Enterococcus faecalis* biofilm. Significant bacterial reduction was observed in all groups, regardless of the final irrigant used. The antibacterial efficacy of chitosan nanoparticles was found to be the highest among the final irrigants across all three groups, supporting the findings of Geetapriya et al. (24). The polycationic structure of chitosan interacts with the negatively charged bacterial surface, altering cellular permeability, causing leakage of intracellular components, and ultimately leading to bacterial cell death (11, 25).

Phytic acid exhibited stronger antibacterial activity against *E. faecalis* than EDTA and saline, which has been confirmed in previous studies (26–28). Phytic acid disrupts bacterial membrane integrity by reacting with divalent cations and increasing cellular permeability. This, in turn, affects cellular morphology and reduces ATP concentration within the bacteria, making it effective against both gram-positive and gram-negative bacteria (29). In this study, the antibacterial efficacy of EDTA was found to be less than that of chitosan nanoparticles and phytic acid. Previous research by Arias-Moliz et al. (8) and Zhang et al. (9) also reported that EDTA has weaker antibacterial properties against *E. faecalis*. This is likely due to its calcium-chelating effect, which facilitates bacterial removal but may limit its penetration into the dentinal tubules due to its small surface tension and poor permeability (9).

A significant reduction in bacterial load was also observed with saline as the final irrigating solution, likely due to the prior use of sodium hypochlorite for 5 minutes. This result is consistent with studies by Siqueira et al. (30) and Plutzer et al. (31). Sodium hypochlorite's antimicrobial effect is primarily due to its high pH, which disrupts bacterial cell integrity and interferes with enzymatic and metabolic processes (31, 32).

The use of medicaments further enhanced bacterial reduction, with the TAP group (Group III) showing the greatest bacterial reduction, followed by the calcium hydroxide group (Group II), regardless of the final irrigant used. Our findings align with those of Adl et al. (33) and Mozayeni et al. (34), who demon-

TABLE 3. VEGF release after disinfection (pg/mL) of Group I, II and III for Saline (Subgroup A), 1 EDTA (Subgroup B), 1% phytic acid (Sub group C) and 0.2% CNPs (Sub group D)

Group number	VEGF release after disinfection									
	Intra group comparison (p)									
	Sub group A (Saline)	Sub group B (17% EDTA)	Sub group C (1% phytic cid)	Sub group D (0.2% CNPs)	Saline vs. EDTA	Saline vs. phytic acid	Saline vs. CNPs	EDTA vs. phytic acid	EDTA vs. CNPs	Phytic acid vs. CNPs
					p					
Group I	4.33±2.62	53.78±8.69	73.75±15.63	93.76±22.35	0.001*	0.001*	0.001*	0.045*	0.001*	0.044*
Group II	2.68±3.00	40.79±8.67	49.07±6.68	89.07±6.68	0.001*	0.001*	0.001*	0.080	0.001*	0.001*
Group III	1.32±1.17	28.53±5.71	32.20±4.30	83.30±5.04	0.001*	0.001*	0.001*	0.361	0.001*	0.001*
Inter group comparison (p)										
Group I vs. II	0.03*	0.009*	0.001*	0.535						
Group I vs. III	0.050*	0.001*	0.001*	0.062						
Group II vs. III	0.504	0.013*	0.009*	0.395						

*: Indicates significant difference at p≤0.05. VEGF: Vascular endothelial growth factor, EDTA: Ethylene diamine tetraacetic acid, CNP: Chitosan nanoparticles

TABLE 2. TGF-β1 release after disinfection (pg/mL) of Group I, II and III for Saline (Subgroup A), 1 EDTA (Subgroup B), 1% phytic acid (Sub group C) and 0.2% CNPs (Sub group D)

Group number	TGF-β1 release after disinfection									
	Intra group comparison (p)									
	Sub group A (Saline)	Sub group B (17% EDTA)	Sub group C (1% phytic acid)	Sub group D (0.2% CNPs)	Saline vs. EDTA	Saline vs. phytic acid	Saline vs. CNPs	EDTA vs. phytic acid	EDTA vs. CNPs	Phytic acid vs. CNPs
					p					
Group I	84.38±34.91	478.85±51.38	746.63±74.37	889.63±147.93	0.001*	0.001*	0.001*	0.001*	0.001*	0.016*
Group II	51.00±5.53	464.63±24.65	464.63±24.65	830.63±49.38	0.001*	0.001*	0.001*	1.000	0.001*	0.001*
Group III	23.63±10.45	330.75±19.07	345.25±11.64	795.63±27.69	0.001*	0.001*	0.001*	0.630	0.001*	0.001*
Inter group comparison (p)										
Group I vs. II	0.013*	0.001*	0.001*	0.417						
Group I vs. III	0.001*	0.001*	0.001*	0.124						
Group II vs. III	0.045*	0.001*	0.001*	0.728						

*: Indicates significant difference at p≤0.05. TGF-β1: Transforming growth factor beta 1, EDTA: Ethylene diamine tetraacetic acid, CNP: Chitosan nanoparticles

strated that TAP exhibited superior antibacterial activity against *E. faecalis*. Calcium hydroxide's effectiveness depends on its ability to diffuse through the dentine into the tubules, where it can act against *E. faecalis*. However, dentine buffers the alkaline environment, limiting its effectiveness in some cases.

We also examined the effect of various disinfection protocols on the release of TGF-β1 and VEGF from radicular dentine. The presence of a smear layer, residual bacteria, and leftover medicament can prevent growth factor release. As a result, successful regenerative endodontic treatment depends on using irrigants that offer superior chelating and antibiofilm actions, in addition to removing residual medicaments (19). Our findings align with those of previous studies that reported the maximum release of growth factors in the irrigation-only group, where medicaments could not interfere with the release due to their incomplete removal from the canals following irrigation (19, 20, 35).

Chitosan nanoparticles promoted the highest release of growth factors, significantly higher than other subgroups in Group I (irrigation only group). This finding supports studies by del Carpio-Perochena et al. (11) and Ratih et al. (36), which attribute these results to the nanoparticles' efficient smear layer removal capability. Phytic acid also showed better growth factor release than EDTA but was less effective than chitosan nanoparticles in Group I. Phytic acid's high negative charge and affinity for calcium ions, combined with its acidic nature, help in removing the smear layer and facilitating growth factor release (20). Its antibacterial action also contributes to enhanced growth factor release, as confirmed by Deniz Sungur et al. (10).

However, these results contradict studies by Jagzap et al. (37) and Afshan et al. (38), who found 17% EDTA to be more effective than 1% phytic acid in smear layer removal. EDTA, a potent chelator, binds to calcium ions, breaking down hydroxyapatite crystals and releasing growth factors. Yet, when used for extended periods, EDTA can erode dentine, negatively affecting the dentine matrix integrity (16, 39). Some studies, such as those by Sadaghiani et al. (40) and Chae et al. (21), reported less growth factor release with EDTA than with citric acid, suggesting the effect may vary with the concentration and exposure time.

The chelating action of chitosan nanoparticles was also found to effectively remove calcium hydroxide from the root canals, leading to growth factor release comparable to that in Group I. This aligns with the study by Raghu et al. (41), which found 0.2% chitosan to be more effective in removing calcium hydroxide than EDTA or citric acid.

For Group III (TAP), chitosan nanoparticles again showed a performance similar to Group I, which can be attributed to the nanoparticles' small size and large surface area/mass ratio, allowing them to reach and remove TAP from the intricate anatomy and dentinal tubules (42). Phytic acid, although less effective than chitosan nanoparticles, likely contributes to some degree of medicament removal due to its acidic nature.

Even with EDTA, the removal of TAP was less efficient, but significant growth factor release was still observed in the medicament groups. This can be explained by the use of passive ultrasonic irrigation (PUI), which has been shown to enhance medicament removal when combined with NaOCl/EDTA (43, 44).

Saline, though less effective, also led to detectable quantities of growth factor release in all groups. This can be attributed to the use of PUI, which, through its higher irrigant flow velocity and slight mechanical action, enhances penetration and removal of medicaments (45).

The use of medicaments like calcium hydroxide and TAP can reduce growth factor release, as they cannot be entirely removed from the root canals. TAP, in particular, binds to dentine, making its removal more difficult compared to calcium hydroxide, which is water-soluble and more easily removed (46–48). This may explain why calcium hydroxide resulted in less growth factor release compared to TAP.

In our study, more TGF-β1 was released than VEGF across all groups, which is consistent with previous research showing that TGF-β1 is more readily released from demineralized dentine compared to other growth factors (16, 19, 40). TGF-β1 is considered a key growth factor in regenerative endodontics due to its role in tissue repair and stem cell differentiation.

Our study provides quantitative data on VEGF release from the apical thirds of root segments, as VEGF is involved in angiogenesis and is more abundant near the vascularization regions of the root canal. This is one of the first studies to measure VEGF release quantitatively after disinfection, adding valuable insights to the field.

Overall, the mobilization of growth factors from the dentinal matrix is a multifactorial process, influenced by the irrigants' ability to remove the smear layer, their antibacterial effectiveness, and their capacity to eliminate residual medicaments. A major strength of our study is that it evaluates the release of growth factors after biofilm formation using phytic acid and chitosan nanoparticles as final irrigants. Additionally, we are the first to provide quantitative data on VEGF release from radicular dentine after disinfection.

There are limitations to our study. Firstly, we did not evaluate bacterial load before the procedure. The decoronation of teeth does not fully represent clinical conditions, and the study was limited to simulating open apices. Furthermore, as with all *in vitro* studies, the findings cannot fully replicate clinical scenarios, so further long-term clinical studies are needed. Additionally, the use of a monospecies biofilm is a limitation, as the root canal system is typically inhabited by a multispecies biofilm.

CONCLUSION

Considering the limitations of this study, we conclude that growth factor release from the root canal is multifactorial, with residual biofilm and medicaments negatively impacting release. Chitosan nanoparticles, as the final irrigant, exhibited superior smear layer removal, antibiofilm action, and growth factor release from radicular dentine. TAP treatment resulted in the greatest bacterial reduction, regardless of the final irrigant used. Furthermore, TGF-β1 release was significantly higher than VEGF release across all groups and subgroups.

Disclosures

Ethics Committee Approval: The study was approved by the ITS Institutional Ethics Committee (no: ITS-CDSR /IIEC/2019-22, date: 14/10/2019).

Authorship Contributions: Concept – S.T.; Design – S.T., D.C.S.; Supervision – S.T., D.C.S.; Funding – A.G.; Materials – A.G.; Data collection and/or processing – A.G., V.K.B.; Data analysis and/or interpretation – S.T., D.C.S.; Literature search – A.G.; Writing – A.G., V.K.B.; Critical review – S.T., D.C.S., V.K.B.

Conflict of Interest: All authors declared no conflict of interest.

Use of AI for Writing Assistance: The authors declared that they did not use any artificial intelligence (AI) assisted technologies (such as Large Language Models [LLMs], chatbots or image creators in the production of submitted work.

Financial Disclosure: The authors declared that this study received no financial support.

Peer-review: Externally peer-reviewed.

REFERENCES

1. Nygaard Ostby B. The role of the blood clot in endodontic therapy. An experimental histologic study. Acta Odontol Scand 1961; 19:324–53. [CrossRef]
2. Nygaard Ostby B, Hjortdal O. Tissue formation in the root canal following pulp removal. Scand J Dent Res 1971; 79(5):333–49. [CrossRef]
3. Bansal R, Bansal R. Regenerative endodontics: a state of the art. Indian J Dent Res 2011; 22(1):122–31. [CrossRef]
4. Smith, AJ, Duncan HF, Diogenes A, Simon S, Cooper PR. Exploiting the bioactive properties of the dentin-pulp complex in regenerative endodontics. J Endod 2016; 42(1):47–56. [CrossRef]
5. Namour M, Theys S. Pulp revascularization of immature permanent teeth: a review of the literature and a proposal of a new clinical protocol. Sci World J 2014; 14;2014. [CrossRef]
6. Kim NH, Rhee MS. Synergistic bactericidal action of phytic acid and sodium chloride against Escherichia coli O157: H7 cells protected by a biofilm. Int J Food Microbiol 2016; 227:17–21. [CrossRef]
7. Kim SG, Malek M, Sigurdsson A, Lin LM, Kahler B. Regenerative endodontics: a comprehensive review. Int Endod J 2018; 51(12):1367–88. [CrossRef]
8. Arias-Moliz MT, Ferrer-Luque CM, Espigares-Rodríguez E, Liébana-Ureña J, Espigares-García M. Bactericidal activity of phosphoric acid, citric acid, and EDTA solutions against *Enterococcus faecalis*. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 106(2):e84–9. [CrossRef]
9. Zhang R, Chen M, Lu Y, Guo X, Qiao F, Wu L. Antibacterial and residual antimicrobial activities against *Enterococcus faecalis* biofilm: a comparison between EDTA, chlorhexidine, cetrimide, MTAD and QMix. Sci Rep 2015; 5:12944. [CrossRef]
10. Deniz Sungur D, Aksel H, Ozturk S, Yılmaz Z, Ulubayram KE. Effect of dentine conditioning with phytic acid or etidronic acid on growth factor release, dental pulp stem cell migration and viability. Int Endod J 2019; 52(6):838–46. [CrossRef]
11. del Carpio-Perochena A, Bramante CM, Duarte MA, de Moura MR, Aouada FA, Kishen A. Chelating and antibacterial properties of chitosan nanoparticles on dentin. Restor Dent Endod 2015; 40(3):195–201. [CrossRef]
12. AAE. Regenerative Endodontics. Available at: https://www.aae.org/specialty/clinical-resources/regenerative-endodontics/. Accessed Jan 17, 2025.
13. Mohammadi Z, Jafarzadeh H, Shalavi S, Yaripour S, Sharifi F, Kinoshita JI. A review on triple antibiotic paste as a suitable material used in regenerative endodontics. Iran Endod J 2018; 13(1):1–6.
14. Estrela C, Sydney GB, Figueiredo JA, Estrela CR. Antibacterial efficacy of intracanal medicaments on bacterial biofilm: a critical review. Appl Oral Sci 2009; 17(1):1–7. [CrossRef]
15. Baca P, Junco P, Arias-Moliz MT, González-Rodríguez MP, Ferrer-Luque CM. Residual and antimicrobial activity of final irrigation protocols on *Enterococcus faecalis* biofilm in dentin. J Endod 2011; 37(3):363–6. [CrossRef]
16. Galler KM, Buchalla W, Hiller KA, Federlin M, Eidt A, Schiefersteiner M, et al. Influence of root canal disinfectants on growth factor release from dentin. J Endod 2015; 41(3):363–8. [CrossRef]
17. Zeng Q, Nguyen S, Zhang H, Chebrolu HP, Alzebdeh D, Badi MA, et al. Release of growth factors into root canal by irrigations in regenerative endodontics. J Endod 2016; 42(12):1760–6. [CrossRef]
18. Ordinola-Zapata R, Bramante CM, Cavenago B, Graeff MSZ, Gomes de Moraes I, Marciano M, et al. Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod J 2011; 45(2):162–8. [CrossRef]
19. Cameron R, Claudia E, Ping W, Erin S, Ruparel NB. Effect of a residual biofilm on release of transforming growth factor β1 from dentin. J Endod. 2019; 45(9):1119–25. [CrossRef]
20. Atesci AA, Avci CB, Tuglu MI, Ay NPO, Eronat AC. Effect of different dentin conditioning agents on growth factor release, mesenchymal stem cell attachment and morphology. J Endod 2020; 46(2):200–8. [CrossRef]
21. Chae Y, Yang M, Kim J. Release of TGF-β1 into root canals with various final irrigants in regenerative endodontics: an *in vitro* analysis. Int Endod J 2018; 51(12):1389–97. [CrossRef]
22. Nakashima M, Akamine A. The application of tissue engineering to regeneration of pulp and dentin in endodontics. J Endod 2005; 31:711–8. [CrossRef]
23. Smith A, Scheven B, Takahashi Y, Ferracane J, Shelton R, Cooper P. Dentine as a bioactive extracellular matrix. Arch Oral Biol 2012; 57(2):109–21. [CrossRef]
24. Geethapriya N, Subbiya A, Padmavathy K, Mahalakshmi K, Vivekanandan P, Sukumaran VG. Effect of chitosan-ethylenediamine tetraacetic acid on *Enterococcus faecalis* dentinal biofilm and smear layer removal. J Conserv Dent 2016: 19(5):472. [CrossRef]
25. Silva PV, Guedes DFC, Nakadi FV, Pécora JD, Cruz-Filho AM. Chitosan: a new solution for removal of smear layer after root canal instrumentation. Int Endod J 2013; 46(4):332–8. [CrossRef]
26. Nassar RI, Nassar M. Antimicrobial effect of phytic acid on *Enterococcus faecalis*. Int Arab J Antimicrob Agents 2017; 6(4):796. [CrossRef]
27. Puvvada S, Latha P, Jayalakshmi KB, Arul SK. Comparative assessment of chelating and antimicrobial efficacy of phytic acid alone and in combination with other irrigants. Int J Appl Dent Sci 2017; 3(2):19–22.
28. Sowjanya T, Naidu S, Nadimpalli MV, Dondapati GD, Raju TBVG, Prasad PK. Antimicrobial efficacy of synthetic and natural-derived novel endodontic irrigating solution– An *In vitro* study. J Int Clin Dent Res Organ 2020; 12(1):21. [CrossRef]
29. Zhou QI, Zhao YU, Dang H, Tang Y, Zhang B. Antibacterial effects of phytic acid against foodborne pathogens and investigation of its mode of action. J Food Prot 2019; 82(5):826–33. [CrossRef]
30. Siqueira JF Jr, Machado AG, Silveira RM, Lopes HP, De Uzeda M. Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of *Enterococcus faecalis* from the root canal, *in vitro*. Int Endod J 1997; 30(4): 279–82. [CrossRef]
31. Plutzer B, Zilm P, Ratnayake J, Cathro P. Comparative efficacy of endodontic medicaments and sodium hypochlorite against *Enterococcus faecalis* biofilms. Aust Dent J 2018; 63(2):208–16. [CrossRef]
32. Bystrom A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J 1985; 18(1):35–40. [CrossRef]
33. Adl A, Hamedi S, Shams MS, Motamedifar M, Sobhnamayan F. The ability of triple antibiotic paste and calcium hydroxide in disinfection of dentinal tubules. Iran Endod J 2014; 9(2):123.
34. Mozayeni MA, Haeri A, Dianat O, Jafari AR. Antimicrobial effects of four intracanal medicaments on *enterococcus faecalis*: an *in vitro* study. Iran Endod J 2014; 9(3):195–8.
35. Ferreira LN, Puppin-Rontani RM, Pascon FM. Effect of intracanal medicaments and irrigants on the release of transforming growth factor beta 1 and vascular endothelial growth factor from cervical root dentin. J Endod 2020; 46(11):1616–22. [CrossRef]
36. Ratih DN, Enggardipta RA, Kartikaningtyas AT. The effect of chitosan nanoparticle as a final irrigation solution on the smear layer removal, micro-hardness and surface roughness of root canal dentin. Open Dent J, 14(1):1874210602014010019. [CrossRef]
37. Jagzap JB, Patil SS, Gade VJ, Chandhok DJ, Upagade MA, Thakur DA. Effectiveness of three different irrigants-17% ethylenediaminetetraacetic acid, Q-MIX, and phytic acid in smear layer removal: a comparative scanning electron microscope study. Contemp Clin Dent 2017; 8(3):459. [CrossRef]
38. Afshan Z, Jat SA, Khan JA, Hasan A, Qazi FUR. erosive potential of 1% phytic acid on radicular dentine at different time intervals. Eur Endod J 2020; 5(1):28. [CrossRef]

39. Gonçalves LF, Fernandes AP, Cosme-Silva L, Colombo FA, Martins NS, Oliveira TM, et al. Effect of EDTA on TGF β1 released from the dentin matrix and its influence on dental pulp stem cell migration. Braz Oral Res 2016; 30(1):e131. [CrossRef]
40. Sadaghiani L, Gleeson HB, Youde S, Waddington RJ, Lynch CD, Sloan AJ. Growth factor liberation and DPSC response following dentine conditioning. J Dent Res 2016; 95(11):1298–307. [CrossRef]
41. Raghu R, Pradeep G, Shetty A, Gautham PM, Puneetha PG, Reddy TVS. Retrievability of calcium hydroxide intracanal medicament with three calcium chelators, ethylenediaminetetraacetic acid, citric acid, and chitosan from root canals: an *in vitro* cone beam computed tomography volumetric analysis. J Conserv Dent 2017; 20(1):25–9. [CrossRef]
42. Parolia A, Kumar H, Ramamurthy S, Davamani F, Pau A. Effectiveness of chitosan-propolis nanoparticle against *Enterococcus faecalis* biofilms in the root canal. BMC Oral Health 2020; 20(1):33. [CrossRef]
43. Arslan H, Gok T, Saygili G, Altintop H, Akçay M, Çapar ID. Evaluation of effectiveness of various irrigating solutions on removal of calcium hydroxide mixed with 2% chlorhexidine gel and detection of orange-brown precipitate after removal. J Endod 2014; 40(11):1820–3. [CrossRef]
44. Topçuoğlu HS, Topçuoğlu G, Arslan H. The effect of different irrigation agitation techniques on postoperative pain in mandibular molar teeth with symptomatic irreversible pulpitis: a randomized clinical trial. J Endod 2018; 44(10):1451–6. [CrossRef]
45. de Gregorio C, Arias A, Navarrete N, Del Rio V, Oltra E, Cohenca N. Effect of apical size and taper on volume of irrigant delivered at working length with apical negative pressure at different root curvatures. J Endod 2013; 39(1):119–24. [CrossRef]
46. Nandini S, Velmurugan N, Kandaswamy D. Removal efficiency of calcium hydroxide intracanal medicament with two calcium chelators: volumetric analysis using spiral CT, an *in vitro* study. J Endod 2006; 32(11):1097–101. [CrossRef]
47. Berkhoff JA, Chen PB, Teixeira FB, Diogenes A. Evaluation of triple antibiotic paste removal by different irrigation procedures. J Endod 2014; 40(8):1172–7. [CrossRef]
48. Kim D, Kim E. Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: a literature review - part II. *In vivo* studies. Restor Dent Endod 2015; 40(2):97–103. [CrossRef]