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ORIGINAL ARTICLE

Efficacy of Continuous Chelation with Etidronate and Sequential Chelation with Chitosan on Bacterial Reduction in Teeth with Pulp Necrosis: A Double-blind, Randomized Clinical Trial

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ABSTRACT

Objective: This study compared the efficacy of continuous chelation with etidronate and sequential chelation with chitosan to traditional chelation with EDTA in reducing bacterial levels in teeth with necrotic pulp.

Methods: Sixty patients with single-rooted and single-canal teeth with pulp necrosis were randomly divided into three groups based on the irrigation protocol with passive ultrasonic activation (PUI). G1 (Control): NaOCl/EDTA+PUI, G2: NaOCl/etidronate+PUI, and G3: NaOCl/chitosan+PUI. Microbiological samples were taken before (S1) and after the root canal preparation and irrigation protocol (S2). Total bacterial counts were measured using 16S ribosomal RNA gene-based real-time quantitative polymerase chain reaction. Statistical analysis was performed using the Wilcoxon signed rank test and the Kruskal-Wallis test ($p<0.05$).

Results: All S1 samples tested positive for bacteria, and total bacterial counts from S1 to S2 were significantly reduced across all three groups ($p<0.001$), with a mean reduction of 97.7% in G1, 96.3% in G2, and 96.8% in G3. No significant differences were observed among the groups ($p=0.345$). Bivariate and multivariate analyses revealed that none of the patient-related or tooth-related variables had a significant effect on bacterial reduction ($p>0.5$).

Conclusion: All three irrigation protocols, including continuous chelation with etidronate, sequential chelation with chitosan, and traditional chelation with EDTA, significantly reduced bacterial levels in teeth with primary endodontic infections. Etidronate and chitosan may serve as alternatives to EDTA; however, further prospective clinical studies are required to validate and recommend their use in clinical practice.

Keywords: Chitosan, etidronate, necrotic pulp, randomized clinical trial, root canal irrigation, ultrasonic activation

HIGHLIGHTS

- This randomized clinical trial compared the antibacterial efficacy of three chelation protocols: etidronate (continuous chelation), chitosan (sequential chelation), with EDTA (control).
- All three protocols significantly reduced intracanal bacterial levels, as confirmed by quantitative polymerase chain reaction (qPCR).
- None of the patient- or tooth-related variables significantly influenced the bacterial reduction outcomes.
- Etidronate and chitosan may be considered as alternative chelating agents to EDTA for root canal irrigation.

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INTRODUCTION

Despite having strong antimicrobial and tissue-dissolving properties, sodium hypochlorite (NaOCl) fails to remove the inorganic portion of the smear layer and the hard tissue debris produced during root canal preparation (1). Consequently, NaOCl is combined with a chelating agent such as ethylenediaminetetraacetic acid (EDTA). However, this combination neutralises active chlorine (2) and causes significant demineralisation of root canal dentin (3). As an alternative, NaOCl with a mild chelator has been proposed (4).

Etidronate (1-hydroxyethane-1,1-diphosphonic acid or HEDP) is a mild chelator used in a continuous chelation technique with NaOCl (2). This combination is more effective at removing smear layers (4) and biofilms (5) compared to NaOCl/EDTA mixtures while minimising damage to dentin (3). Furthermore, this combination maintains the antibacterial efficacy of NaOCl and has been shown in clinical and laboratory studies to not induce periapical inflammation or increase postoperative pain (6, 7). Chitosan, a natural polysaccharide derived from the deacetylation of chitin found in crustacean shells, offers remarkable biocompatibility, biodegradability, and non-toxicity (8). Chitosan has demonstrated superior antimicrobial activity against *Enterococcus faecalis* compared to chlorhexidine (9) and shows smear layer removal capacity similar to EDTA (10).

Conventional irrigation methods are limited in their ability to access the apical third and complex anatomical areas of root canals. Passive ultrasonic irrigation (PUI) enhances irrigant penetration by acoustic streaming and cavitation (11). A systematic review showed that PUI is more effective than syringe irrigation in removing pulp tissue and dentin debris (12). Evaluating the antimicrobial efficacy of endodontic procedures with culture-dependent studies is challenging due to their limited sensitivity and failure to identify many difficult-to-cultivate or uncultivable bacteria. To overcome these issues, culture-independent molecular methods have become the preferred approach (13).

To date, no Randomised Clinical Trials (RCTs) using molecular methods have evaluated the efficacy of etidronate and chitosan as root canal irrigants in cases of primary endodontic infection. Moreover, while PUI is recognised to enhance irrigant efficacy, few RCTs using molecular methods have assessed its role in reducing the microbial load in canals with necrotic pulp when used with chelators (14, 15). Therefore, this study aimed to compare, under standardised PUI conditions, the efficacy of continuous chelation with etidronate and sequential chelation with chitosan to traditional chelation with EDTA in reducing bacterial levels in teeth with necrotic pulp using quantitative real-time polymerase chain reaction (qPCR). The null hypothesis was that there would be no significant difference in bacterial reduction among the irrigation protocols within the root canal.

MATERIALS AND METHODS

This randomised, double-blind, multi-arm parallel clinical trial followed the CONSORT guidelines. The study protocol was approved by the Institutional Ethical Committee of Utkal University (Approval No: IEC/SCBDCH/106/2021; Date: 01/09/2021) and registered at the Clinical Trials Registry (Registration No: CTRI/2021/10/037484; Date: 22/10/2021). The study was conducted in accordance with the Declaration of Helsinki from January 2022 to January 2023 at the Department of Conservative Dentistry and Endodontics, Utkal University, Odisha, India. The study included patients aged 18–60 diagnosed with pulp necrosis and periapical lesions. One trained endodontic postgraduate performed all procedures. Informed consent was obtained from all patients.

Sample Size Calculation

GPower 3.1 software (Heinrich Heine University, Düsseldorf, Germany) calculated that 55 teeth were required to detect a 5% difference in bacterial load reduction (effect size=0.55, power=95%.) Allowing for a 10% dropout rate, 60 patients were randomly assigned to three groups of 20 each. The minimum effect size was determined according to Cohen (1992) (16).

Randomisation

Random allocation software (17) was used for randomisation with a block size of 6 with 1:1:1 allocation via sealed envelopes by a trial-independent individual. Patients and outcome assessors were blinded, but the operator was not, due to differences in irrigation protocols.

Eligibility Criteria

Inclusion criteria were patients with single-rooted and single-canalled teeth with pulp necrosis, confirmed by the pulp sensibility test and radiographic evidence of periapical radiolucency. Exclusion criteria were patients who received antibiotics in the past three months or had systemic disease; had spontaneous and/or lingering preoperative pain; oedema, fistula, or sinus opening; had visibly exposed root canals; had teeth that could not be isolated; periodontal pocket more than 3 mm; recent dental trauma; teeth with open apex, root fracture, root resorption, and calcifications.

Endodontic Treatment

The entire procedure was done under strict asepsis. Local anaesthesia was administered using 2% lidocaine with 1:80,000 epinephrine (Xicaine, ICPA, Mumbai, India). After the rubber dam isolation, the operative field was disinfected with 30% hydrogen peroxide (Sigma-Aldrich, St Louis, MO, USA) and 3% NaOCl (Parcan, Septodont, Saint Maur Des Fossés, France) for 30 seconds each, then neutralised with 5% sodium thiosulphate (Sigma-Aldrich). The access cavity was prepared using a sterile high-speed diamond bur, and the field was disinfected again before accessing the pulp chamber. A control sample was collected, and the sterility of the disinfected surface was confirmed using qPCR. A fresh, sterile bur was used to complete the access cavity. Working length was determined using an electronic apex locator (Canalpro Compact, Coltene, Altstätten, Switzerland). Root canal content was collected before chemo-mechanical preparation (S1). Teeth were randomly divided into three groups (n=20 each) based on the irrigation protocol: G1 (Control) - NaOCl/EDTA + PUI; G2 - NaOCl/etidronate+PUI; and G3 - NaOCl/chitosan+PUI.

Irrigants were delivered using a 5 mL syringe and a 30-G needle (Navitip, Ultradent, South Jordan, UT, USA), placed 1mm short of the working length. An ultrasonic insert tip (E93, Woodpecker, Guilin, China), size 20, .01 taper, was used 2 mm short of the

working length for activation. Irrigation flow rates were 5 mL/min during shaping and 2 mL/min during final irrigation. The canal shaping and final irrigation protocols adhered to the methodology given by Ballal et al. (6) and Nakamura et al. (14), respectively.

G1 (Control) – The root canal was instrumented to F4 or F5 Protaper rotary files (Protaper Gold, Dentsply Maillefer, Ballaigues, Switzerland), using an endo-motor (Canalpro CL2, Canalpro CL2, Coltene, Altstätten, Switzerland), following the manufacturer's instructions. Irrigation involved 5 mL of 3% NaOCl and 5 mL of saline solution per instrument change. After the shaping procedure, 5 mL of sodium thiosulphate and 5 mL of saline solution were used to neutralise NaOCl. The canal was dried with paper points before final irrigation. The canal was filled with 2 mL of 3% NaOCl and activated for 30 seconds. After aspirating the NaOCl, the canal was irrigated with 5 mL of saline solution, and the entire procedure was repeated once. Subsequently, the canal was filled with 2 mL of 17% EDTA (MD Cleanser, Meta Biomed, South Korea), activated for 30 seconds, and aspirated, followed by 5 mL saline irrigation. This step was repeated once. Finally, the canal was filled with 2 mL of 3% NaOCl, activated for 30 seconds, and irrigated with 5 mL of saline. Total volume: 41 mL of 3% NaOCl, 4 mL of 17% EDTA, and 65 mL of saline.

G2 – A fresh solution was prepared by mixing 50 mL of 3% NaOCl with five 0.9 g capsules of etidronate (Dual Rinse HEDP, Medcem, Weinfelden, Switzerland). The shaping procedure was similar to the G1, but the continuous chelation technique used NaOCl/etidronate instead of plain NaOCl. During the final irrigation, the root canal was filled with 2 mL of NaOCl/etidronate solution and activated for 30 seconds. After aspiration, the canal was irrigated with 5 mL of saline solution, and the step was repeated twice. Total volume: 41 mL of 3% NaOCl/etidronate solution and 55 mL of saline.

G3 – A 0.2% chitosan solution was prepared by dissolving 0.2 g of chitosan (I-CHESS Extract, Mumbai, India) in 100 mL of 1% acetic acid (I-CHESS Extract) and magnetically stirred for 2 h. The shaping and final irrigation protocols were similar to the G1, except that 0.2% chitosan replaced EDTA in the final irrigation. Total volume: 41 mL of 3% NaOCl, 4 mL of 0.2% chitosan solution, and 65 mL of saline.

After the final irrigation, a second sample (S2) was collected. The root canal was dried and obturated using gutta-percha points (Dentsply Maillefer, Ballaigues, Switzerland) and a resin-based sealer (Adseal, Meta Biomed) using warm vertical compaction. The access cavity was restored with composite resin (Z250, 3M Corporation, St Paul, MN, USA).

Outcome Measures

The primary outcome was the reduction in bacterial load assessed using qPCR analysis of pre- and post-instrumentation samples (S1 and S2). Although postoperative pain assessment was initially planned as a secondary outcome using a visual analogue scale at 8, 24, 48 hours, and 7 days, it was not conducted due to patient follow-up issues.

Sample Collection

Sample collections adhered to the protocols given by Rôças et al. (18). Sterile saline solution was placed into the pulp chamber, and the root canal samples were obtained using three absorbent paper points (ISO #15/2%, Dentsply Maillefer, Ballaigues, Switzerland) placed one at a time into the total length of the root canal, each held in place for 60 seconds. The paper points were placed into Eppendorf tubes containing 0.5 mL of Tris-EDTA solution and stored at -20°C.

Quantitative real-time PCR analysis

The total bacterial count was determined by 16S rRNA gene-targeted qPCR with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on the Quant Studio5 real-time PCR instrument (Applied Biosystems), as described previously (19). The universal bacterial primers used were 5′ – GAT TAG ATA CCC TGG TAG TCC AC – 3′ and 5′ – TAC CTT GTT ACG ACT T – 3′ (Eurofins Genomics, Bengaluru, India). Bacterial quantities in each sample were estimated using standard curves derived from known DNA concentrations isolated from *Enterococcus faecalis* ATCC 29212 cultivated in a pure medium. *E. faecalis* was chosen because it has four copies of the 16S rRNA gene, the average number for most known oral bacteria (19).

Statistical Analysis

Data were summarised and analysed using SPSS Statistics software version 26.0 (IBM Corporation, NY, USA). Normality was assessed using the Kolmogorov-Smirnov test, which revealed a non-normal distribution across the analysed groups. Intention-to-treat analyses were used. The chi-square test and Fisher's exact test examined associations between sample characterisation variables and groups. Intragroup analyses were performed using the Wilcoxon signed-rank test to compare pre- and post-intervention bacterial counts. Intergroup comparisons among the groups were conducted using the Kruskal-Wallis test for quantitative analysis of bacterial count reduction. Bivariate analysis using the Mann-Whitney test and multiple linear regression was performed to assess the effect of independent variables (age, gender, tooth type, lesion size, tenderness to percussion, and canal enlargement) on the dependent variable (bacterial reduction). The significance level for all tests was set at $p<0.05$.

RESULTS

Out of 93 patients assessed for eligibility, 33 were excluded (Fig. 1). Table 1 presents the clinical characteristics of patients and their distribution across treatment groups. Of the total sample, 73.3% (44/60 patients) were male and 26.7% (n=16) were female, with a mean age of 29.9 years (standard deviation of 9.3 years), ranging from 18 to 55 years. The majority of teeth were maxillary incisors (83.3%, n=50), with the remaining being mandibular incisors (16.7%, n=10). There was no significant difference among the groups regarding gender, age, and tooth type ($p>0.05$). None of the patients reported spontaneous preoperative pain. Of the 60 participants, 80% (n=48) had periapical lesions greater than 2 mm, and 28.3% (n=17) reported tenderness to percussion, with no significant difference among the three groups ($p>0.05$).

Bacterial Reduction

Bacteria were present in all initial (S1) samples, as revealed by qPCR analysis. After chemo-mechanical preparation and the irrigation protocol, 60%, 65%, and 60% of the root canals in G1, G2, and G3 still had detectable bacteria. However, these dif-

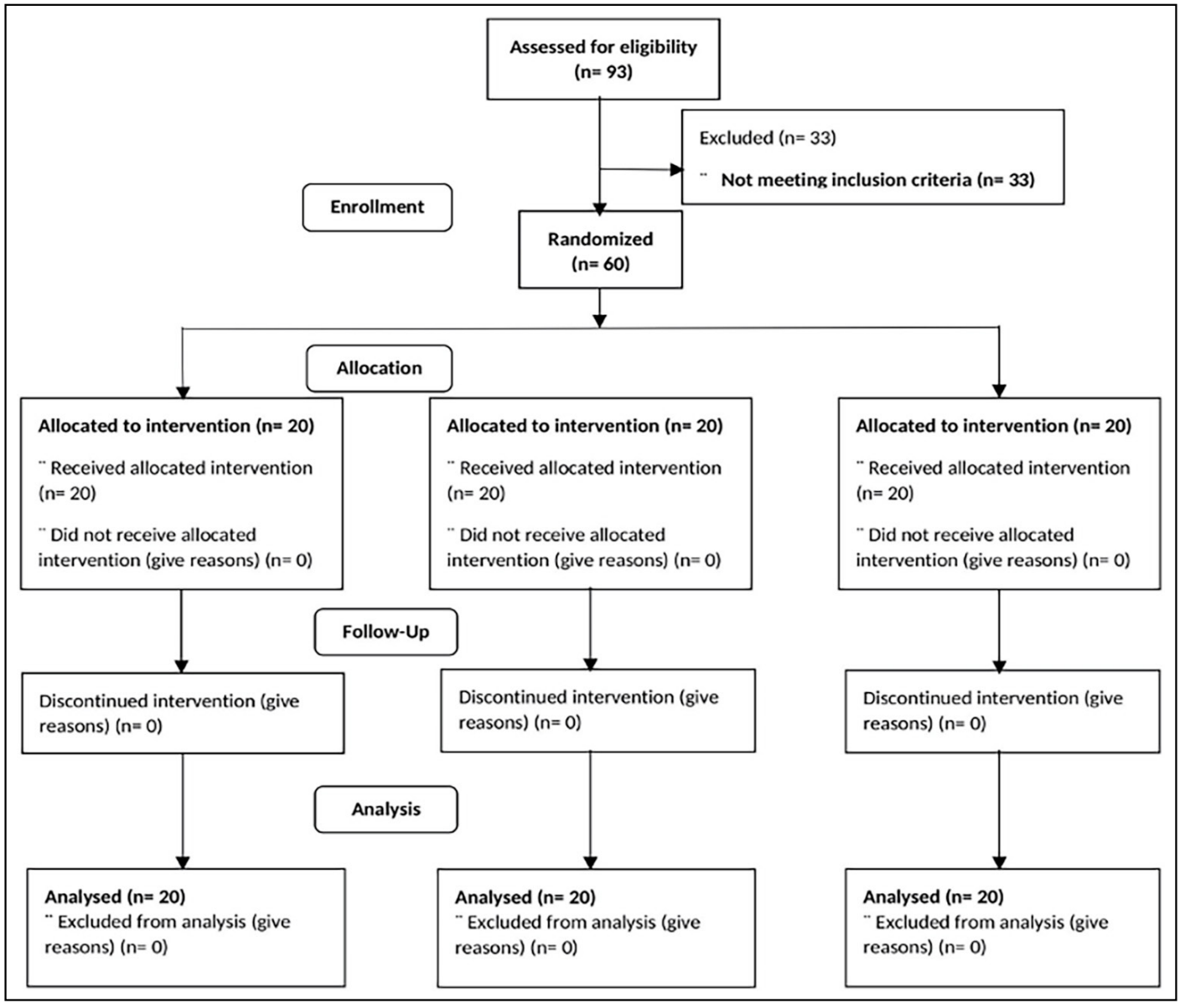

Figure 1. CONSORT flow chart.

ferences were not statistically significant (p>.05). Quantitative data are presented in Table 2. In G1, the median bacterial levels in S1 samples were 5.85×10^6 cells, decreasing significantly in S2 to a median of 3.90×10^3 cells (p<0.001). The mean reduction from S1 to S2 was 97.7% (median, 99.6%). In G2, the median bacterial levels in S1 samples were 3.09×10^5 cells, decreasing significantly in S2 to a median of 2.42×10^3 cells (p<0.001). The mean reduction from S1 to S2 was 96.3% (median, 98.4%). In G3, the median bacterial levels in S1 samples were 8.21×10^5 cells, decreasing significantly in S2 to a median of 6.28×10^3 cells (p<0.001). The mean reduction from S1 to S2 was 96.8% (median, 98.6%). No significant difference was observed in bacterial reduction among the three irrigation protocols (p=0.345).

Bivariate analyses (Table 3) and multiple linear regression (Table 4) performed on various independent variables, such as age, gender, tooth type, canal enlargement, tenderness to percussion, and periapical lesion size, revealed that none significantly affected bacterial reduction (p>0.05).

DISCUSSION

This culture-independent molecular microbiology clinical study aimed to compare the efficacy of continuous chelation with etidronate and sequential chelation with chitosan to traditional chelation with EDTA in reducing bacterial levels in teeth with necrotic pulp. Intragroup quantitative analysis showed that all three irrigation protocols significantly reduced intracanal bacterial levels. The null hypothesis was accepted as the groups had no significant difference among them. These findings align with other studies indicating that chemo-mechanical debridement is crucial for reducing bacterial levels in the root canal (20, 21). However, this study confirms that many samples still have detectable cultivable bacteria after instrumentation and the irrigation protocol. The prevalence of positive cultures in S2 across all groups is consistent with the previous clinical studies on this topic (22, 23). The impact of residual bacteria on treatment outcomes depends on factors such as the type of bacteria present, their spatial position in the canal, nutritional availability, and their potential to adjust and thrive

TABLE 1. Clinical characteristics of the patients and their distribution across treatment groups

Variable	Category	Total		Groups			p
		n	%	G1	G2	G3	
Gender	Male	44	73.3	14 (70)	14 (70)	16 (80)	0.711
	Female	16	26.7	6 (30)	6 (30)	4 (20)	
Age	<40 years	50	83.3	16 (80)	17 (85)	17 (85)	0.887
	≥40years	10	16.7	4 (20)	3 (15)	3 (15)	
Tooth type	Maxillary incisor	50	83.3	18 (90)	16 (80)	16 (80)	0.619
	Mandibular incisor	10	16.7	2 (10)	4 (20)	4 (20)	
Canal enlargement	F4 Protaper	25	41.7	8 (40)	9 (45)	8 (40)	0.934
	F5 Protaper	35	58.3	12 (60)	11 (55)	12 (60)	
Tenderness to percussion	Yes	17	28.3	14 (70)	15 (75)	14 (70)	0.921
	No	43	71.7	6 (30)	5 (25)	6 (30)	
Periapical lesion	≤2 mm	12	20	5 (25)	4 (20)	3 (15)	0.732
	>2 mm	48	80	15 (75)	16 (80)	17 (85)	

P-values were derived from the Chi-square test and Fisher's exact test at a significance level of 5%

TABLE 2. Bacterial levels determined in the root canal samples of teeth with necrotic pulp taken before (S1) and after the root canal preparation and the final irrigation protocols (S2)

Groups	S1			S2			Mean % S1 to S2 reduction	SE of mean (95% CI)
	Mean	Median	Range	Mean	Median	Range		
G1	1.95×10^{9}	5.85×10^{6}	2.25×10^{3}–1.76×10^{10}	7.05×10^{4}	3.90×10^{3}	0–7.20×10^{5}	97.7%^{A}	0.94 (95.7–99.6)
G2	9.09×10^{7}	3.09×10^{5}	2.22×10^{3}–6.71×10^{8}	3.14×10^{6}	2.42×10^{3}	0–6.26×10^{7}	96.3%^{A}	1.28 (93.7–99.0)
G3	1.39×10^{8}	8.21×10^{5}	5.62×10^{3}–1.52×10^{10}	2.50×10^{5}	6.28×10^{3}	0–4.34×10^{6}	96.8%^{A}	0.85 (95.4–98.6)

Data from quantitative real-time polymerase chainreaction analysis. P-values for intragroup analysis were derived from the Wilcoxon signed-rank test at a significance level of 5%. P-values for intergroup analysis were derived from the Kruskal-Wallis test at a significance level of 5%. Different uppercase superscripts indicate a significant difference among the groups ($p<0.05$). SE: Standard error, CI: Confidence interval

TABLE 3. Bivariate analysis showing the effect of independent variables on the bacterial reduction

Variable	Category	Mean % S1 to S2 reduction	Median % S1 to S2 reduction	p
Gender	Male	97.1	99.2	0.726
	Female	96.4	98.9	
Age	<40 years	97.0	98.8	0.463
	≥40years	96.5	99.7	
Tooth type	Maxillary incisor	97.0	98.8	0.606
	Mandibular incisor	96.3	99.5	
Canal enlargement	F4 Protaper	96.9	99.5	0.946
	F5 Protaper	97.0	98.7	
Tenderness to percussion	Yes	98.1	99.6	0.216
	No	96.5	98.7	
Periapical lesion	≤2 mm	97.4	98.3	0.657
	>2 mm	96.8	99.2	

P-values were derived from the Mann-Whitney test at a significance level of 5%

in the altered environment (24). Since the persistence of bacteria in the canal suggests a poor prognosis, efforts should focus on maximising bacterial elimination.

Antibacterial activity of NaOCl at different concentrations revealed negative cultures in 40%-60% of cases, with no significant difference among them. In this study, 3% NaOCl was chosen because higher concentrations increase toxicity without enhancing antibacterial effects within the canal (1). The presence of a smear layer significantly reduces the antimicrobial effectiveness of NaOCl, as it cannot dissolve the smear layer. It also obstructs the diffusion of the irrigant through infected dentin walls, limiting its bactericidal activity (25). This highlights the necessity of using chelating agents to remove the smear layer and maximise the effectiveness of antimicrobial irrigants. NaOCl is rarely mixed with chelating agents like EDTA

TABLE 4. Multiple linear regression showing the effect of independent variables on the bacterial reduction.

Independent variables	Unstandardized coefficients		Standardized Coefficients	t-value	p	95% CI for Beta	
	Beta	SE	Beta			Lower bound	Upper bound
(Constant)	102.677	6.765		15.179	0.000	89.103	116.251
Gender	-1.062	1.436	-0.103	-0.740	0.463	-3.943	1.819
Tooth type	-0.698	2.035	-0.057	-0.343	0.733	-4.781	3.385
Canal enlargement	-0.464	1.622	-0.050	-0.286	0.776	-3.719	2.790
Tenderness to percussion	1.939	1.425	0.191	1.361	0.180	-0.921	4.799
Lesion size	-0.673	1.700	-0.059	-0.396	0.694	-4.084	2.738
Age	-1.107	1.823	-0.090	-0.607	0.546	-4.764	2.551
Group	-0.439	0.773	-0.078	-0.568	0.573	-1.989	1.112

Dependent Variable: Percentage Reduction. P-values were derived from the independent t-test at a significance level of 5%. SE: Standard error, CI: Confidence interval

or citric acid due to exothermic reactions that produce gas and rapidly lose active chlorine through dilution (2, 3). Previous *in vitro* studies have demonstrated the antibacterial effectiveness of NaOCl/etidronate (5) and chitosan (9) against endodontic microbes. However, to date, only one clinical trial has evaluated the efficacy of etidronate and chitosan in treating primary endodontic infections. A safety trial showed that irrigation with NaOCl/etidronate was slightly more effective than NaOCl alone (6). Nasr et al. (26) reported that final irrigation with chitosan was more effective compared to chlorhexidine and NaOCl.

The NaOCl/etidronate solution is widely accepted because it does not alter the chemical properties of NaOCl (2). Etidronate, a mild chelating agent, removes the residual smear layer by removing phosphate, exposing the collagen matrix, and increasing the amide III/phosphate ratio (27). NaOCl/ etidronate is as effective as the traditional sequence of NaOCl followed by EDTA in reducing hard tissue debris and preventing smear layer formation. Therefore, NaOCl/ etidronate could serve as a single irrigant, eliminating the need for a final rinse with a chelating agent. However, without an oxidising agent, etidronate alone may not effectively open dentinal tubules (3,4). Additionally, the stability of NaOCl/etidronate mixtures decreases over time, depending on the initial concentration of NaOCl, with higher concentrations leading to faster loss of available chlorine (28).

Chitosan is available in concentrations ranging from 0.2% to 2%. This study used a 0.2% concentration as it is the most frequently tested in the literature (29). Chitosan solution was prepared using a low concentration of acetic acid to facilitate its dissolution. Although acetic acid exhibits mild chelating properties, its concentration in the solution is insufficient to contribute significantly to smear layer removal. The chelation mechanism of chitosan is not completely understood. It is believed to involve absorption and ion exchange, where two or more amino groups from one chitosan chain bind to the same metal ion (30). Chitosan molecules can also penetrate the multilayered murein-based bacterial cell walls and enter the plasma membrane due to the electrostatic interactions between the positively charged glucosamine in chitosan and the negatively charged bacterial cell surface. This interaction enhances adherence to the microbial cell surface, leading to protein leakage, and inhibits mRNA and protein synthesis, contributing to chitosan's antimicrobial properties (31). NaOCl/etidronate has greater tissue-dissolving capacity than NaOCl/EDTA, with NaOCl/chitosan being the least effective (32). Thus, in this study, the chitosan solution was used sequentially after NaOCl, similar to EDTA, while etidronate was used concomitantly with NaOCl.

Passive ultrasonic activation was employed because evidence indicates that it enhances the effectiveness of EDTA (15), etidronate (33), and chitosan (34), the three irrigating solutions tested in the present study. The enhanced bacterial reduction following passive ultrasonic activation can be attributed to acoustic streaming and the warming of the irrigating solution. These factors improve the smear layer removal and disrupt biofilms, offering an advantage over syringe irrigation, which is limited in its cleaning efficiency and replacement of irrigant to 1–1.5 mm apical to the needle (35).

There are concerns about the accuracy of utilising DNA-based molecular methods post-treatment, as they can detect DNA from both live and dead cells (36). A study using rRNA detection, which accurately assesses viable cells, found positive results in only 60% of cases post-chemo-mechanical preparation (37). Given the large percentage of negative results with a sensitive method such as qPCR, it is likely that the presence of DNA from dead cells did not significantly influence the outcomes. This could be due to the degradation of DNA fragments from dead cells by NaOCl or their removal during irrigation (36). Despite significant microbial load reduction achieved by contemporary root canal procedures, large numbers of bacteria might still be present, as demonstrated in this study. It is important to note that while qPCR techniques quantify bacterial reduction, they do not indicate the effectiveness of the irrigation protocol in removing biofilm from root canal walls (14). Since intra-radicular biofilms are a primary cause of apical periodontitis, any remaining biofilm could impact treatment outcomes (38). Prospective clinical studies are needed to evaluate the potential consequences of persistent infection after endodontic treatment.

A limitation of this study is that it focused exclusively on single-rooted teeth to standardise conditions and facilitate controlled sample collection. The anatomical characteristics of

these teeth may limit the direct extrapolation of the findings to teeth with more complex root canal systems. Furthermore, anatomical variations within the single-rooted teeth, such as canal cross-sectional shape (e.g., circular vs. oval/flattened canals), were not accounted for. It is well-established that oval or flattened canals present a significant challenge for cleaning and disinfection, as irrigants and instruments may not contact all canal walls effectively (39, 40). Although tooth type (maxillary vs. mandibular incisor) was not a significant factor in our regression model, the inherent anatomical complexity within a given tooth type could influence the results. Future studies incorporating micro-CT analysis to categorise canals based on their cross-sectional anatomy would provide deeper insight into the efficacy of these irrigants in challenging anatomies.

Another limitation is the use of paper points, which primarily collect samples from the main root canal, potentially missing areas like lateral canals and isthmuses. Additionally, the stringent inclusion criteria, limited to asymptomatic, single-rooted necrotic teeth, combined with extensive laboratory analyses, restricted the sample size. Another limitation was the inability to assess post-operative pain, a planned secondary outcome. This restricted the evaluation of patient-centred outcomes and may limit the broader clinical interpretation of the results.

CONCLUSION

This clinical study showed that all three irrigation protocols, including continuous chelation with etidronate, sequential chelation with chitosan, and traditional chelation with EDTA using passive ultrasonic activation, significantly reduced bacterial levels in root canals with primary endodontic infections. Therefore, etidronate and chitosan may serve as alternatives to EDTA; however, further prospective clinical studies are required to validate and recommend their use in clinical practice.

Disclosures

Ethics Committee Approval: The study was approved by the Utkal University Ethics Committee (no: IEC/SCBDCH/106/2021, date: 01/09/2021).

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