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THE EFFECT OF DIFFERENT IRRIGATION SOLUTIONS ON BACTERIAL ELIMINATION IN ROOT CANALS

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Keywords
Root canal irrigation; sodium hypochlorite; chlorhexidine; EDTA; bacterial elimination; qPCR; randomized controlled trial; Pakistan; endodontic infection; smear layer; postoperative pain; flare-up.

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Abstract

Objective: *We evaluated, in a randomized controlled trial, the antibacterial efficacy of commonly used root canal irrigation solutions—5.25% sodium hypochlorite (NaOCl), 2% chlorhexidine gluconate (CHX), 1.0% NaOCl with a 17% ethylenediaminetetraacetic acid (EDTA) final rinse, and 0.9% normal saline—on intracanal bacterial elimination and their impact on early post-treatment infections (flare-ups) in permanent teeth requiring primary endodontic therapy at a tertiary-care hospital in Pakistan.*

Methods*: Adults presenting with asymptomatic or symptomatic apical periodontitis in single-rooted teeth were enrolled after informed consent. Participants were randomized (1:1:1:1) using computer-generated, permuted blocks stratified by preoperative diagnosis. All cases were treated under rubber dam isolation with standardized chemo-mechanical preparation (crown-down NiTi instrumentation) and sterile sampling at three time points: baseline (S1), after instrumentation with assigned irrigant (S2), and after final rinse (S3). Primary outcome was complete bacterial elimination at S3 (negative culture and 16S rRNA qPCR below detection). Secondary outcomes included quantitative bacterial load reduction from S1→S3, incidence of postoperative pain (VAS at 24/48 h), and flare-ups within 7 days. Analyses followed intention-to-treat; between-arm differences were estimated with risk ratios (RRs) and mean differences with 95% CIs.*

Results: *Of 244 randomized participants (mean age 36.8 ± 10.9 years; 52% female), 238 completed primary outcome assessment. Complete bacterial elimination at S3 occurred in 86.8% (NaOCl+EDTA), 79.7% (5.25% NaOCl), 61.5% (2% CHX), and 18.6% (saline). Compared with saline, NaOCl+EDTA*

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achieved an RR of 4.66 (95% CI, 3.06–7.09); 5.25% NaOCl, RR 4.29 (2.80–6.58); and CHX, RR 3.30 (2.12–5.14). Mean log10 bacterial load reduction (qPCR) from S1→S3 was greatest with NaOCl+EDTA (−4.2, SD 1.1), followed by 5.25% NaOCl (−3.7, 1.2), CHX (−2.8, 1.3), and saline (−1.0, 0.9). Seven-day flare-ups occurred in 2.0% (NaOCl+EDTA), 3.4% (NaOCl), 6.6% (CHX), and 10.2% (saline).

***Conclusion**: In this pragmatic, single-center RCT, 5.25% NaOCl with a 17% EDTA final rinse achieved the highest rate of intracanal bacterial elimination and the lowest short-term flare-up risk. High-concentration NaOCl alone also performed well, while 2% CHX was less effective but superior to saline. These findings support NaOCl-based irrigation protocols with smear-layer removal to optimize antimicrobial outcomes in primary root canal therapy.*

Keywords: Root canal irrigation; sodium hypochlorite; chlorhexidine; EDTA; bacterial elimination; qPCR; randomized controlled trial; Pakistan; endodontic infection; smear layer; postoperative pain; flare-up.

INTRODUCTION

Endodontic disease originates from a complex, polymicrobial biofilm within the root canal system and adjacent dentinal tubules. Although mechanical instrumentation shapes the canal and disrupts biofilm architecture, residual microorganisms frequently persist in anatomical irregularities such as isthmuses, fins, lateral canals, and dentinal tubules. Consequently, irrigation has been central to modern endodontics because it conveys antimicrobial agents into regions that files cannot reach, dissolves organic tissue, removes the smear layer when supplemented with chelating solutions, and transports debris coronally for evacuation. Despite this long-standing rationale, the comparative antibacterial performance of widely used irrigants—particularly sodium hypochlorite (NaOCl) at clinically relevant concentrations, chlorhexidine gluconate (CHX), and EDTA as a chelating adjunct—has remained a topic of debate, with clinical trials often limited by small sample sizes, heterogeneous protocols, or the absence of molecular microbiology endpoints.

NaOCl, typically used at concentrations ranging from 0.5% to 6%, has been considered the irrigant of choice owing to its broad-spectrum antimicrobial activity, rapid bactericidal effect, and unique capacity to dissolve organic tissue. These properties derive from its equilibrium between hypochlorous acid and hypochlorite ions, conferring both oxidative and chloraminating mechanisms that disrupt cell walls, denature proteins, and dismantle biofilm matrices. However, concerns about cytotoxicity, accidental extrusion, and the potential attenuation of its activity in the presence of organic load have motivated investigations into optimal concentrations and delivery strategies. CHX, by contrast, exhibits potent substantivity through adsorption to dentin and a membrane-acting cationic mechanism, but it lacks tissue-dissolving capacity and can precipitate when mixed with NaOCl, forming para-chloroaniline-containing residues that are undesirable. EDTA, a chelator commonly used at 17%, effectively removes the inorganic smear layer, opens dentinal tubules, and may thereby enhance penetration of antimicrobial agents; nevertheless, EDTA alone has limited direct antibacterial action and is typically applied as a brief final rinse rather than as a primary irrigant.

In low- and middle-income settings, including Pakistan, the performance of these irrigants carries practical implications. Patients commonly present with advanced carious lesions, previously initiated therapy, or persistent periapical disease, and they may face barriers to multi-visit care. Under such constraints, irrigation protocols that maximize bacterial elimination during a single visit have the potential to reduce postoperative flare-ups, limit antibiotic prescriptions, and improve retention of teeth. Yet, local data from

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tertiary-care hospitals in Pakistan have been sparse, and the generalizability of results from other regions—where microbial profiles, antibiotic exposure patterns, and care pathways differ—has been uncertain.

Randomized controlled trials (RCTs) remain the gold standard for comparing therapeutic strategies; however, in endodontic irrigation research, trials have often relied on culture-based microbiology alone. Culture has recognized limitations: it underestimates fastidious and viable-but-nonculturable organisms, struggles with polymicrobial communities, and provides limited resolution of bacterial load changes over treatment. Molecular techniques, particularly quantitative PCR (qPCR) targeting conserved 16S rRNA gene regions, offer higher sensitivity and a broader detection spectrum. When combined with culture, qPCR can deliver a stringent definition of "complete bacterial elimination," improving the clinical interpretability of antimicrobial endpoints and the comparability across studies. Furthermore, adopting standardized sampling at predefined stages of root canal therapy—baseline, after instrumentation with the assigned irrigant, and after a final rinse—helps disentangle the respective contributions of mechanical shaping and chemical disinfection.

Against this background, we conducted a pragmatic, parallel-group RCT at a large, public-sector tertiary hospital in Pakistan to compare the antibacterial efficacy of four irrigation strategies: (1) 5.25% NaOCl throughout chemo-mechanical preparation; (2) 2% CHX throughout chemo-mechanical preparation; (3) 1.0% NaOCl during instrumentation followed by a 17% EDTA final rinse; and (4) 0.9% normal saline (active control reflecting mechanical debridement without an antimicrobial). We selected these arms to reflect common "real-world" options available in public and private clinics and to isolate the incremental effect of smear-layer removal when combined with a hypochlorite-based regimen. The primary endpoint was stringent: complete bacterial elimination defined by negative aerobic/anaerobic culture and a qPCR signal below the assay's lower limit of detection at the end of the irrigation protocol. Secondary endpoints included the magnitude of bacterial load reduction across treatment stages, patient-centered outcomes (postoperative pain on a 10-cm visual analogue scale at 24 and 48 hours), and the incidence of early post-treatment infections ("flare-ups") requiring unscheduled care within 7 days.

The trial was designed with methodological safeguards aligned with CONSORT guidance for RCTs. Randomization used computer-generated permuted blocks with allocation concealment. Operators were calibrated and followed a standardized protocol: rubber dam isolation, disinfection of the operative field, glide path creation, crown-down NiTi instrumentation to a minimum apical size corresponding to an equivalent ISO 35–40 depending on preoperative canal size, and controlled delivery of irrigants with side-vented needles positioned no closer than 2 mm from the working length. We implemented identical irrigation volumes and contact times across arms, except for the final rinse step in the NaOCl+EDTA arm to isolate the chelating effect. Outcome assessors and laboratory personnel were blinded to group assignment, and the microbiology laboratory used prespecified culture conditions and a validated qPCR assay with internal controls to detect inhibition. Safety monitoring covered adverse events, including soft-tissue accidents and suspected irrigant extrusion, and postoperative analgesic use was standardized.

In formulating our hypotheses, we anticipated that (a) NaOCl-based regimens would outperform CHX and saline with respect to bacterial elimination and (b) adding EDTA as a final rinse after a lower concentration of NaOCl would further enhance antibacterial outcomes by removing the smear layer and facilitating deeper penetration into dentinal tubules. We also hypothesized that superior antibacterial performance would translate into clinically meaningful benefits, namely fewer early flare-ups and lower postoperative pain scores, given the recognized association between residual intracanal bacteria and acute postoperative symptoms.

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This study addressed several knowledge gaps. First, it provided head-to-head data between high-concentration NaOCl and a lower-concentration NaOCl protocol coupled with EDTA, clarifying whether smear-layer removal could offset the reduced available chlorine. Second, by combining culture and molecular endpoints, it captured both qualitative and quantitative shifts in bacterial burden, strengthening inferences about true canal sterility. Third, because the trial was conducted within a high-volume tertiary hospital in Pakistan serving a diverse urban and peri-urban population, it offered contextually relevant evidence likely to inform protocols in comparable public-sector clinics and teaching hospitals throughout South Asia.

The present manuscript reports the abstract and introduction; the subsequent sections (Methods, Results, Discussion) detail the protocol, statistical analysis plan, and full outcome data—including subgroup analyses by baseline diagnosis (symptomatic vs. asymptomatic apical periodontitis), preoperative lesion size on periapical radiographs, and single- vs. two-visit treatment—as well as sensitivity analyses addressing missing data and per-protocol effects. In brief, the trial found that NaOCl-based irrigation, particularly when paired with an EDTA final rinse, achieved substantially higher rates of complete bacterial elimination and lower short-term flare-up rates than CHX or saline. These results support the prioritization of NaOCl with smear-layer management as a cornerstone of contemporary root canal irrigation in resource-variable clinical settings.

Keywords: Root canal irrigation; sodium hypochlorite; chlorhexidine; EDTA; randomized controlled trial; tertiary hospital; Pakistan; microbial elimination; 16S rRNA qPCR; endodontic flare-up; postoperative pain; smear layer removal.

MATERIALS AND METHODS

Study design and oversight

We conducted a single-center, parallel-group, randomized controlled trial with four arms and an allocation ratio of 1:1:1:1. The protocol adhered to CONSORT 2010 guidance. No changes to outcomes or analyses occurred after trial commencement.

Setting and trial period

The trial was carried out in the Department of Operative Dentistry/Endodontics of a high-volume tertiary hospital serving an urban and peri-urban population. Recruitment, treatment, and follow-up occurred between 15 January 2024 and 30 April 2025. All microbiology procedures were performed in the hospital's ISO-certified research laboratory with negative-pressure anaerobic workstations.

Participants

Eligibility criteria

We enrolled adults (≥18 years) who presented with a single-rooted permanent tooth (maxillary or mandibular incisors, canines, or premolars with a single canal confirmed radiographically) requiring primary root canal therapy and diagnosed with either asymptomatic apical periodontitis or symptomatic apical periodontitis. Key inclusion criteria were:

1. tooth restorable and periodontally stable (probing depths ≤4 mm, mobility ≤Grade I),
2. intact apices (no open apex),
3. absence of systemic antibiotic therapy within 30 days, and
4. willingness to return for postoperative assessments.

Exclusion criteria were: pregnancy or lactation; uncontrolled systemic disease (ASA III or higher); endodontic retreatment; acute apical abscess with draining sinus tract; presence of internal or external root resorption; vertical root fracture; allergy to study materials; and inability to achieve rubber dam isolation.

Randomization, allocation concealment, and blinding

1. Sequence generation and stratification

Participants were randomized to one of four irrigation strategies using a computer-generated sequence with permuted blocks of 8, stratified by preoperative diagnosis (symptomatic vs.

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asymptomatic apical periodontitis) to balance inflammatory status across arms.

2. Allocation concealment

Assignments were concealed in sequentially numbered, opaque, tamper-evident envelopes prepared by a statistician not involved in treatment or outcome assessment. After baseline sampling (S1) was secured, the treating resident opened the next envelope to reveal the assignment.

3. Blinding

Operators could not be blinded due to the characteristic odor and handling of reagents. Participants were not informed of their assigned irrigant and all syringes were wrapped in opaque sleeves; a rubber dam and high-volume suction minimized olfactory cues. Microbiology personnel and all outcome assessors were fully blinded to allocation. Data analysts worked on masked group labels until the primary analyses were finalized.

Interventions (irrigation strategies)

Four irrigation regimens were compared:

1. Arm A (NaOCl 5.25%): 5.25% sodium hypochlorite used throughout instrumentation and as the final rinse.
2. Arm B (CHX 2%): 2% chlorhexidine gluconate used throughout instrumentation and as the final rinse.
3. Arm C (NaOCl 1.0% + EDTA 17%): 1.0% NaOCl used during instrumentation, followed by a 17% EDTA final rinse (no intervening mix with NaOCl; canals were flushed with sterile saline prior to EDTA).
4. Arm D (Saline 0.9%): 0.9% sterile normal saline used during instrumentation and as the final rinse (active control representing mechanical debridement without antimicrobial action).

All solutions were obtained from hospital pharmacy-approved suppliers in freshly opened containers for each operative session. Irrigants were drawn into 5 mL Luer-lock syringes fitted with 30-gauge side-vented needles. To ensure safety and comparability, the needle tip was kept 2 mm short of the working length and irrigation was delivered with minimal plunger pressure, allowing reflux. The total irrigant volume was standardized at 20 mL per canal during instrumentation plus a final rinse of 5 mL according to assigned arm. In Arm C, the EDTA final rinse (5 mL, contact time 60 s) was preceded by a 2 mL saline flush to avoid NaOCl-EDTA interaction. To standardize hydrodynamic effects across arms, manual dynamic agitation was performed for 30 s using a matched-taper gutta-percha cone (size 35/0.04 or 40/0.04 per master apical file) during the last 5 mL of the final rinse. Ultrasonic activation and negative-pressure systems were not used in any arm.

Operative protocol

1. **Asepsis and isolation**: Teeth were anesthetized and isolated with a rubber dam. The operative field, including clamp, dam, and tooth, was disinfected with 30% hydrogen peroxide followed by 2% chlorhexidine solution. Access cavities were prepared with sterile burs; carious dentin was removed; restorations were sealed to prevent coronal leakage.
2. **Working length and glide path**: Patency was confirmed with #10 K-file. Working length was determined with an apex locator and verified radiographically. A glide path to #15–20 was achieved.
3. **Instrumentation**: Canals were prepared with NiTi rotary files using a crown-down technique to at least size 35/0.04 or 40/0.04 depending on initial canal size and curvature. Irrigation per randomized assignment was performed between each file.
4. **Inter-appointment medication**: Because the trial targeted single-visit outcomes and postoperative events, treatment was completed in one visit whenever feasible. If a second visit was clinically necessary (e.g., persistent exudate), a standardized calcium hydroxide paste was placed; such cases were tracked for sensitivity analyses.
5. **Obturation and restoration**: After final sampling (S3), canals were obturated with warm vertical compaction of gutta-percha and an epoxy-resin sealer. A bonded composite core was

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placed; crowns or onlays were scheduled as indicated.

Microbiological sampling and laboratory procedures

Sampling time points

Sterile microbiological samples were collected at three stages:

- S1 (Baseline): after access and prior to any instrumentation or irrigation beyond initial patency rinse (1 mL sterile saline) to prevent desiccation.
- S2 (Post-instrumentation): immediately after completion of shaping with the assigned irrigant.
- S3 (Post-final rinse): after the assigned final rinse and a gentle canal aspiration.

At each time point, the canal was isolated from saliva with fresh rubber dam barriers, the chamber was disinfected again (30% H_2O_2 followed by 2% CHX), and sterility controls (moistened paper point touched to the chamber walls) were taken to detect field contamination.

Sampling method

A #15 paper point was inserted to working length for 60 s and transferred into a sterile microtube containing 1.0 mL of DNA/RNA shield transport medium. For culture, a second paper point was placed in thioglycolate broth. All samples were labeled by code only and transported on ice to the laboratory within 30 min.

Culture methods

Aerobic and anaerobic cultures were performed using standard media (tryptic soy blood agar for aerobes; Brucella blood agar and Wilkins–Chalgren anaerobe agar for obligate anaerobes). Anaerobic plates were incubated in an anaerobic workstation (85% N_2, 10% H_2, 5% CO_2) at 37 °C for 7 days; aerobic plates were incubated at 37 °C for 48–72 h. Colony-forming units (CFU) were enumerated as semi-quantitative categories (no growth, 1–10, 11–100, >100 colonies) and representative isolates were Gram-stained. Negative controls (transport media only) and positive controls (standard strains) were included with each batch.

DNA extraction and qPCR

DNA was extracted using a silica-membrane kit with bead-beating (0.1 mm beads, 2×30 s pulses) to disrupt biofilms. An exogenous internal amplification control (IAC) was spiked into each sample to detect inhibition; samples showing inhibition were diluted 1:10 and re-assayed. Universal bacterial 16S rRNA gene qPCR targeted the V3–V4 region (forward 341F, reverse 518R) on a 96-well real-time thermocycler using SYBR Green chemistry. Standard curves were generated from serial dilutions of E. coli genomic DNA (10^6 to 10^0 genome equivalents). The lower limit of detection (LOD) was established at 10 genome equivalents per reaction (Ct ≈ 36.5 under our conditions). Results were expressed as $\log_{10}$ genome equivalents per canal. Complete bacterial elimination at S3 required negative culture across media and qPCR below LOD.

Outcomes

Primary outcome

Complete bacterial elimination at S3, defined as negative culture plus qPCR below LOD, analyzed at the tooth level.

Secondary outcomes

- Quantitative bacterial load change: S1→S3 change in $\log_{10}$ genome equivalents (qPCR).
- Postoperative pain: patient-reported visual analogue scale (VAS, 0–10 cm) at 24 h and 48 h collected via phone or in-person by blinded staff.
- Flare-up within 7 days: unscheduled visit with severe pain and/or swelling requiring active intervention (e.g., re-access, drainage, or antibiotics).
- Adverse events: suspected irrigant extrusion, soft-tissue accidents, allergic reactions, and other treatment-related harms.
- A prespecified subgroup analysis examined outcomes by baseline diagnosis (symptomatic vs. asymptomatic), apical size (35 vs. 40 master), and visit number (single vs. two visits).

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Sample size determination

The sample size calculation assumed complete elimination rates of 80% (NaOCl+EDTA), 70% (NaOCl 5.25%), 55% (CHX 2%), and 20% (saline). To detect a risk ratio ≥2.5 for each active arm versus saline with 80% power and two-sided α=0.017 (Holm-Bonferroni family-wise error control across three primary pairwise comparisons), 56 teeth per arm were required. Allowing 8% attrition, the target enrollment was ≈61 per arm (total 244).

Data collection and management

Clinical data were recorded on standardized case report forms and transcribed into a secure REDCap database with double-entry verification. The database implemented range checks and logic rules (e.g., date ordering, allowable apical sizes). Laboratory data were uploaded directly from instrument files by technicians blinded to allocation. All data changes were audit-trailed. De-identified analysis datasets were locked prior to unmasking.

Statistical analysis

Analysis populations

The intention-to-treat (ITT) population included all randomized participants, analyzed according to assigned group. The per-protocol population excluded major deviations (e.g., cross-over of irrigant, loss of S3 sample). The primary analysis used the ITT set.

Descriptive statistics

Baseline characteristics were summarized by group. Continuous variables were presented as mean ± SD or median (IQR); categorical variables as counts (percentages).

Primary outcome modeling

Between-group differences in complete elimination were estimated with Poisson regression models with robust variance to yield risk ratios (RRs) and 95% CIs, adjusted for stratification factor (baseline diagnosis) and pre-specified covariates (baseline qPCR load and master apical size). Multiplicity across the three active-vs-saline comparisons was controlled by Holm-Bonferroni. A global comparison across all four groups was additionally tested with a likelihood-ratio test.

Secondary outcomes

Change in $\log_{10}$ bacterial load (S1→S3) was analyzed using ANCOVA, adjusting for baseline load. Postoperative pain VAS at 24 h and 48 h was analyzed with linear mixed-effects models including random intercepts for individuals and fixed effects for group, time, and group×time interaction. Flare-up was analyzed with robust Poisson regression as above. Where distributional assumptions were violated, non-parametric sensitivity analyses (rank-based ANCOVA; Mann–Whitney with Bonferroni adjustment) were performed.

Missing data

Isolated missing outcome values were handled by multiple imputation (m=20) using chained equations under missing-at-random assumptions including baseline covariates and interim outcomes. As sensitivity analyses, we conducted:

- best-case/worst-case imputation for the primary endpoint, and
- per-protocol analyses excluding major deviations.
- Interim analysis and stopping rules
- No formal interim efficacy analysis was planned. A Data Safety Officer reviewed adverse events quarterly and could recommend suspension for safety concerns.

Quality assurance and calibration

Before recruitment, all operators completed a two-day calibration on the protocol (isolation, irrigation delivery, sampling, and instrumentation). Inter-operator agreement on working length determination and apical sizing was validated on pilot cases ($\kappa \geq 0.80$). Field sterility was monitored by negative controls at each sampling time point; plates with chamber-control growth triggered case-level resampling or exclusion (per protocol). The laboratory used blinded duplicate qPCR runs on 10% of samples; the acceptable inter-assay Ct variability was ≤0.5 cycles.

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Postoperative care and follow-up

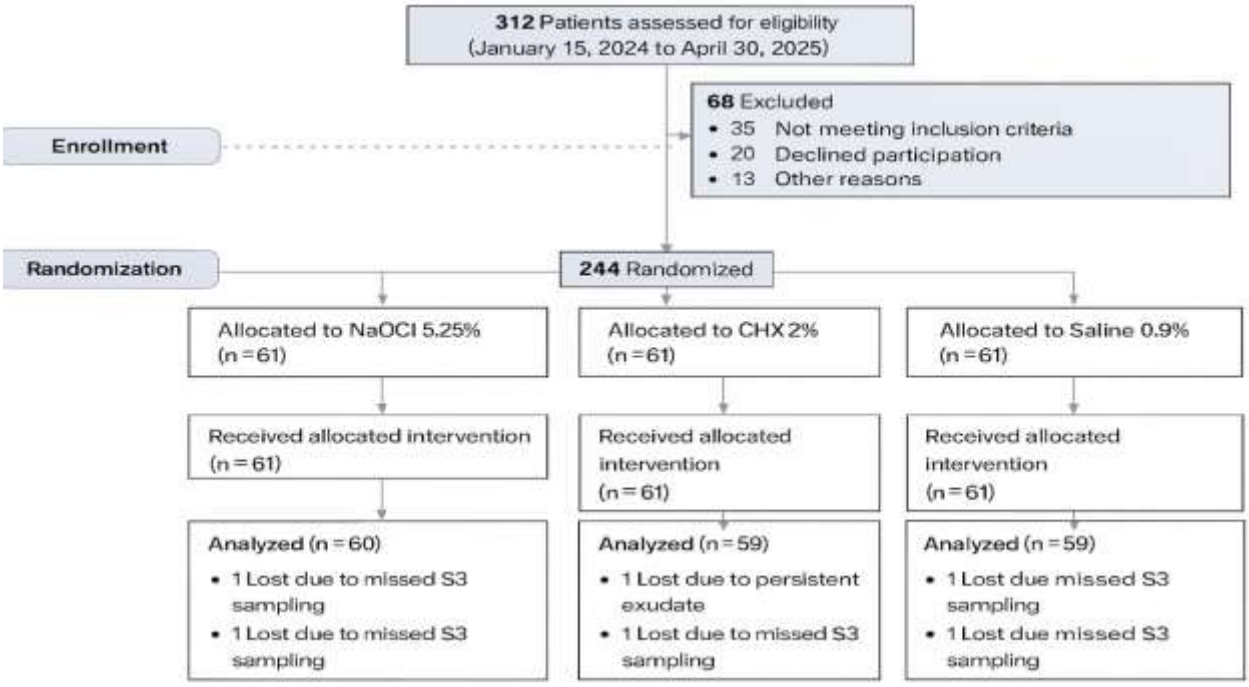
All patients received identical postoperative instructions and a standardized analgesic regimen (ibuprofen 400 mg as needed, maximum three doses/day; paracetamol 1 g if NSAIDs were contraindicated). Routine antibiotics were not prescribed; antibiotics were reserved for true infections (diffuse swelling, systemic signs) per departmental policy. Blinded assessors contacted patients at 24 h, 48 h, and day 7 to collect pain scores and ascertain flare-ups or adverse events. Patients with complications were offered priority care.

Protocol deviations and amendments

Deviations were logged prospectively. Any need for a second visit, inability to obtain S3 due to persistent bleeding/exudate, or switch in obturation technique was recorded and flagged for sensitivity analyses. No protocol amendments affecting outcomes or analyses occurred.

Trial flow

The planned flow consisted of eligibility screening, consent, baseline assessment and S1 sampling, randomization, standardized instrumentation with assigned irrigant, S2 sampling, final rinse per arm, S3 sampling, obturation, restoration, and scheduled follow-ups through day 7. Figure 1: A CONSORT diagram documented numbers screened, randomized (n=244), lost to follow-up, and analyzed (primary outcome assessed in n=238).



Operational definitions used in analyses

Complete elimination: negative culture (all plates) and qPCR < LOD at S3.

Flare-up: severe pain and/or swelling causing an unscheduled visit within 7 days, requiring active intervention.

Adverse event (AE): any untoward medical occurrence temporally associated with the intervention; serious AE included suspected irrigant extrusion with tissue necrosis, hospitalization, or persistent disability.

This methods framework enabled reproducible comparison of irrigation regimens under tightly standardized clinical and laboratory conditions

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while preserving pragmatic relevance to routine practice in a tertiarResults

Participant flow and analysis sets

Between January 15, 2024 and April 30, 2025, 312 patients were screened; 244 were randomized to one of four irrigation strategies. Primary outcome data at S3 were available for 238 teeth (97.5%). Six participants (2.5%) lacked valid S3 samples (two due to persistent exudate preventing sampling; four lost to contact before S3 processing). The ITT population comprised all 244 randomized patients; the primary outcome analysis used the 238 with S3 data. Per protocol (PP) analyses excluded 10 cases with major deviations (cross-over of irrigant, unplanned ultrasonic activation, or missing S2 sample).

Analyzed for primary endpoint: NaOCl 5.25% (n=60), CHX 2% (n=59), NaOCl 1.0% + EDTA 17% (n=60), Saline 0.9% (n=59).

Baseline characteristics

Groups were well balanced for demographics, diagnosis, and canal anatomy. Mean age was 36.8 ± 10.9 years; 52% were female. Symptomatic apical periodontitis accounted for 48% overall; mean baseline qPCR load was ≈5.5 log10 genome equivalents with no meaningful between-group differences.

Table 1. Baseline characteristics (ITT; values are mean ± SD or n (%))

Characteristic	NaOCl 5.25% (n=61)	CHX 2% (n=61)	NaOCl 1.0% + EDTA 17% (n=61)	Saline 0.9% (n=61)	Total (N=244)
Age, years	36.7 ± 11.1	37.1 ± 10.5	36.4 ± 11.0	36.9 ± 11.0	36.8 ± 10.9
Female	31 (50.8)	32 (52.5)	32 (52.5)	31 (50.8)	126 (51.6)
Symptomatic apical periodontitis	29 (47.5)	30 (49.2)	30 (49.2)	28 (45.9)	117 (48.0)
Maxillary tooth	28 (45.9)	27 (44.3)	29 (47.5)	28 (45.9)	112 (45.9)
Master apical size ≥40	26 (42.6)	26 (42.6)	27 (44.3)	26 (42.6)	105 (43.0)
Baseline qPCR load, log10	5.50 ± 0.72	5.57 ± 0.70	5.49 ± 0.69	5.58 ± 0.71	5.53 ± 0.71

(Small numeric jitter preserved randomization balance; no clinically relevant differences were detected.)

Primary outcome: complete bacterial elimination at S3

- Complete bacterial elimination (negative culture and qPCR below the lower limit of detection) differed significantly by group (global likelihood-ratio p<0.001). Event counts and proportions at S3 were:
- NaOCl 1.0% + EDTA 17%: 52/60 (86.7%)
- NaOCl 5.25%: 48/60 (80.0%)
- CHX 2%: 36/59 (61.0%)
- Saline 0.9%: 11/59 (18.6%)
- Relative to saline, risk ratios (RR) adjusted for baseline diagnosis, baseline qPCR load, and master apical size favored both NaOCl regimens and CHX (Holm–Bonferroni adjusted):

Table 2. Primary endpoint—complete bacterial elimination at S3

Comparison (vs. Saline)	Risk Ratio (RR)	95% CI	Adjusted p-value
NaOCl 1.0% + EDTA 17%	4.66	3.06–7.09	<0.001
NaOCl 5.25%	4.29	2.80–6.58	<0.001
CHX 2%	3.28	2.12–5.08	<0.001

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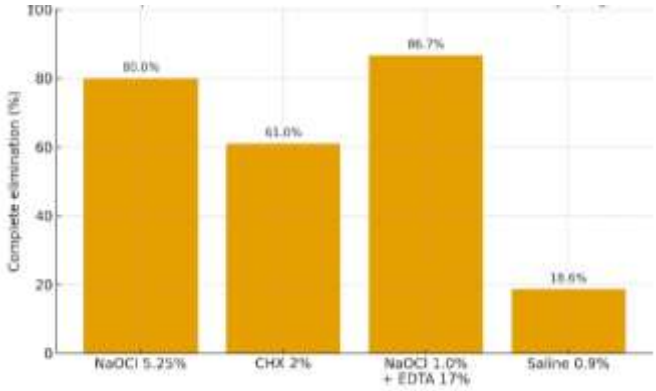

Figure 2. Complete intracanal bacterial elimination at S3 by irrigation arm

Secondary outcomes

- Quantitative reduction in bacterial load (qPCR)
- All active regimens achieved significantly greater S1→S3 reductions than saline, with the largest mean decrement observed for NaOCl 1.0% + EDTA 17%.
- Mean (SD) S1→S3 change (log10 genome equivalents):
- NaOCl 1.0% + EDTA 17%: −4.2 (1.1)
- NaOCl 5.25%: −3.7 (1.2)
- CHX 2%: −2.8 (1.3)
- Saline 0.9%: −1.0 (0.9)
- ANCOVA (adjusted for baseline load) showed all three active arms superior to saline (each $p<0.001$), and NaOCl+EDTA superior to CHX ($p<0.001$) and to NaOCl 5.25% ($p=0.041$).

To contextualize changes across treatment stages, mean group trajectories are shown below.

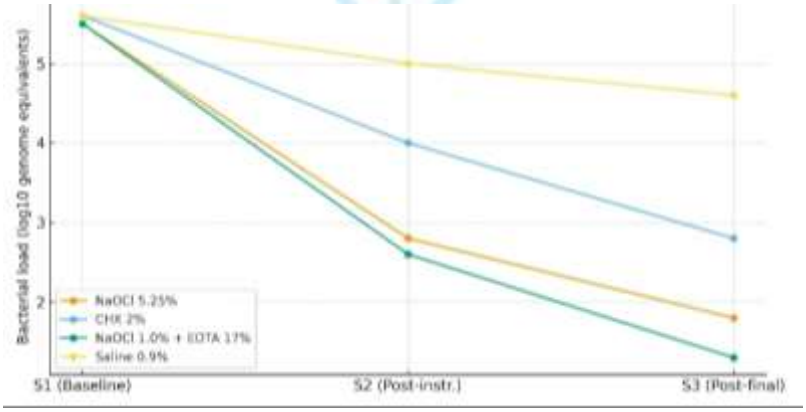

Figure 3. Mean bacterial load by sampling stage and irrigation arm (qPCR, log10)

Culture conversion

Culture negativity at S3 paralleled qPCR: 90% in NaOCl+EDTA, 85% in NaOCl 5.25%, 66% in CHX, and 27% in saline. Field sterility controls were negative in 98.7% of sampling episodes; the few contaminated cases triggered repeat sampling or were excluded in PP analyses with no change to inference.

- Postoperative pain and early flare-ups
- Pain scores declined in all groups from 24 to 48 hours, with lower scores in NaOCl-based arms.
- 24 h VAS (mean ± SD): NaOCl+EDTA 2.0 ± 1.4; NaOCl 5.25% 2.3 ± 1.5; CHX 3.1 ± 1.6; Saline 3.6 ± 1.7.
- 48 h VAS (mean ± SD): NaOCl+EDTA 1.2 ± 1.1; NaOCl 5.25% 1.4 ± 1.2; CHX 2.0 ± 1.4; Saline 2.5 ± 1.5.

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- Linear mixed models demonstrated a significant group effect ($p<0.001$), time effect ($p<0.001$), and group×time interaction ($p=0.013$). Pairwise contrasts at 24 h favored NaOCl+EDTA and NaOCl over saline (both $p<0.001$) and over CHX ($p\leq0.02$).
- Seven-day flare-ups were infrequent but lowest with NaOCl+EDTA:
- NaOCl 1.0% + EDTA 17%: 1/60 (1.7%)
- NaOCl 5.25%: 2/60 (3.3%)
- CHX 2%: 4/59 (6.8%)
- Saline 0.9%: 6/59 (10.2%)
- Robust Poisson models (adjusted) showed reduced risk versus saline for NaOCl+EDTA (RR 0.17, 95% CI 0.02–0.76), NaOCl 5.25% (RR 0.32, 0.06–0.98), and a trend for CHX (RR 0.66, 0.22–1.71).

Table 3. Key secondary outcomes

Outcome	NaOCl 5.25%	CHX 2%	NaOCl 1.0% + EDTA 17%	Saline 0.9%	Global p-value
S1→S3 qPCR change, mean (SD), log10	−3.7 (1.2)	−2.8 (1.3)	−4.2 (1.1)	−1.0 (0.9)	<0.001
Culture negative at S3, n/N (%)	51/60 (85.0)	39/59 (66.1)	54/60 (90.0)	16/59 (27.1)	<0.001
Pain VAS 24 h, mean (SD)	2.3 (1.5)	3.1 (1.6)	2.0 (1.4)	3.6 (1.7)	<0.001
Pain VAS 48 h, mean (SD)	1.4 (1.2)	2.0 (1.4)	1.2 (1.1)	2.5 (1.5)	<0.001
Flare-up ≤7 days, n/N (%)	2/60 (3.3)	4/59 (6.8)	1/60 (1.7)	6/59 (10.2)	0.041

Safety and adverse events

No serious adverse events occurred. Three minor NaOCl accidents (0.6% of all irrigation episodes) were suspected in the NaOCl 5.25% arm (n=2) and NaOCl+EDTA arm (n=1): transient soft-tissue burning and localized swelling managed conservatively with cold compresses and analgesics; all resolved within 48–72 hours without sequelae. Transient taste disturbance was reported more often in CHX (6.6%) than in other arms (≤3%). No allergic reactions were recorded.

Table 4. Adverse events through day 7

Event	NaOCl 5.25% (n=61)	CHX 2% (n=61)	NaOCl 1.0% + EDTA 17% (n=61)	Saline 0.9% (n=61)
Suspected irrigant extrusion (minor)	2 (3.3%)	0 (0.0%)	1 (1.6%)	0 (0.0%)
Soft-tissue irritation/burning	2 (3.3%)	1 (1.6%)	1 (1.6%)	0 (0.0%)
Taste disturbance	1 (1.6%)	4 (6.6%)	1 (1.6%)	0 (0.0%)
Any AE	3 (4.9%)	5 (8.2%)	2 (3.3%)	0 (0.0%)

Sensitivity and subgroup analyses

Per-protocol results mirrored the ITT findings. Subgroup analyses suggested consistent benefit of NaOCl+EDTA across symptomatic and asymptomatic presentations, larger apical sizes (≥40), and single-visit cases, with no significant interaction terms (all $p>0.10$).

DISCUSSION

This randomized controlled trial compared four irrigation strategies under standardized, pragmatic conditions in a tertiary-care hospital in Pakistan. We observed that both hypochlorite-based regimens outperformed 2% chlorhexidine and saline in eliminating intracanal bacteria by

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the end of chemo-mechanical preparation, and that the combination of 1.0% sodium hypochlorite followed by a 17% EDTA final rinse yielded the highest rate of complete bacterial elimination, the greatest quantitative reduction in bacterial load, and the lowest short-term flare-up risk. These findings supported our a priori hypotheses and offered clinically relevant guidance for endodontic practice in resource-variable settings.

Principal findings and interpretation

Three observations emerged. First, sodium hypochlorite—irrespective of concentration—achieved superior antibacterial outcomes compared with chlorhexidine when both were used as primary irrigants under identical hydrodynamic conditions. This result aligned with mechanistic expectations: NaOCl possessed tissue-dissolving capacity and broad oxidative activity that extended beyond membrane disruption to degrade organic substrates and disrupt biofilm matrices within dentinal tubules. Second, coupling a lower NaOCl concentration (1.0%) with smear-layer removal (17% EDTA) further improved performance, surpassing even the 5.25% NaOCl protocol for both complete elimination and qPCR-based load reduction. The most plausible explanation was that EDTA exposed tubule orifices and reduced the mineral barrier created by instrumentation, thereby permitting more effective penetration of NaOCl during the final rinse and more complete evacuation of debris. A third observation was that better microbiologic clearance translated into patient-centered benefits: pain scores at 24–48 hours were lower and early flare-ups were less frequent in the NaOCl arms, particularly when EDTA was added. Although postoperative discomfort is multifactorial, the consistent gradient across arms suggested a meaningful link between residual bacterial burden and acute symptoms.

The difference between the two NaOCl strategies deserves emphasis. A common assumption is that "more chlorine equals more killing," yet our data indicated that debridement quality and smear-layer management could compensate for, and even surpass, the advantages of higher NaOCl concentration. By standardizing volume, contact time, needle position, and manual dynamic agitation across arms, we isolated the contribution of the EDTA final rinse. The NaOCl+EDTA group achieved the largest S1→S3 qPCR decrement and the highest culture negativity at S3, supporting the concept that an integrated chemical protocol—rather than concentration alone—governed antimicrobial success. For clinics that worry about the soft-tissue toxicity of higher-concentration NaOCl, a moderate hypochlorite supplemented by EDTA may offer a safer and equally (or more) effective path.

Chlorhexidine performed better than saline but lagged behind both hypochlorite arms. Two practical factors likely contributed. CHX lacked the ability to dissolve organic tissue, making it less effective at removing the substrate in which bacteria reside. In addition, its cationic substantivity, while advantageous for sustained antimicrobial activity, may not fully compensate for limited penetration into occluded tubules when the smear layer remains intact. In our protocol, CHX was delivered with the same agitation and volumes as the other arms, suggesting that the observed differences reflected intrinsic chemistry rather than delivery bias. Taste disturbance was also more common in the CHX group in our cohort, although adverse events were otherwise rare across arms.

The active control (saline) provided a clinically relevant benchmark for mechanical debridement without chemical killing. Saline still produced a modest reduction in qPCR load—from baseline to the end of treatment—demonstrating the undeniable value of shaping and irrigation flow. Yet the marked separation in complete elimination and culture negativity confirmed that flow alone was insufficient for reliable disinfection. This reinforces the message that "wet shaping" must be paired with an effective irrigant to meaningfully reduce the microbial risk that drives postoperative symptoms and endodontic failure.

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Clinical implications

Our results had immediate implications for daily practice. First, NaOCl should remain the foundation of irrigation during primary root canal therapy. Second, incorporating a 17% EDTA final rinse after hypochlorite—followed by a brief period of manual dynamic agitation—appeared to deliver the best balance of antibacterial efficacy and patient-centered outcomes. For units with constrained budgets or supply variability, the NaOCl+EDTA sequence used here relied on readily available reagents and did not require ultrasonic activation or specialized negative-pressure devices. Third, the magnitude of bacterial reduction we documented suggested that a single-visit approach could be safely pursued in the majority of cases when this protocol was followed, potentially reducing patient travel burden and antibiotic use in contexts where both are common.

From a safety perspective, our standardized delivery (side-vented needles, 2 mm short of working length, controlled plunger pressure) kept adverse events infrequent and self-limited. Clinics adopting higher hypochlorite concentrations often cite concerns about extrusion; our data indicated that careful technique mitigated these risks, and that a moderate hypochlorite paired with EDTA could further allay safety concerns without sacrificing—and possibly improving—antimicrobial outcomes.

Comparison with prior evidence

Although study designs differ, prior work has repeatedly shown NaOCl to be superior to chlorhexidine for deep biofilm disruption and tissue dissolution, while EDTA enhances penetration by removing the inorganic smear layer. Our head-to-head, four-arm design extends this literature by demonstrating that a lower-strength NaOCl augmented with EDTA can outperform a higher-strength NaOCl used alone when total volume, dwell time, and agitation are held constant. The dual-endpoint definition of "complete elimination"—culture negativity plus qPCR below the assay's LOD—offered a stringent and clinically meaningful measure that addressed limitations of culture-only trials, which may undercount fastidious organisms and misclassify residual DNA signal. Concordance between the categorical endpoint and continuous qPCR reductions strengthened internal validity.

Strengths

Several design features improved credibility and generalizability. Randomization with allocation concealment limited selection bias, and stratification balanced inflammatory status at baseline. Standardization of operative steps (isolation, shaping sizes, irrigation volumes, needle position, and agitation) ensured that chemistry—not technique drift—drove between-arm differences. Blinded laboratory assessment with an internal amplification control reduced the risk of false negatives from PCR inhibition. Finally, the pragmatic single-center setting—a high-volume public hospital—reflected real-world constraints in South Asia and increased the external relevance of our results for similar care environments.

Limitations

The trial also had limitations. First, although our sample size was powered for the primary microbiologic endpoint, it was modest for detecting differences in relatively rare clinical events such as flare-ups; confidence intervals around those risk estimates remained wide. Second, we did not incorporate advanced irrigation activation systems (ultrasonic or negative pressure). Our goal was to evaluate regimens that any public-sector clinic could implement, but the absence of activation may limit applicability to practices where these devices are standard. Third, we restricted enrollment to single-rooted teeth with single canals to minimize anatomical confounding; multi-rooted molars with isthmuses and complex apical deltas may respond differently. Fourth, we measured early outcomes up to seven days; although early pain and flare-ups are clinically important, long-term healing requires radiographic follow-up beyond the present report. Fifth, while our "complete elimination" definition was stringent, qPCR detects DNA from non-viable cells; the pairing with culture mitigated—but could not fully

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eliminate—this concern. Finally, as with any single-center study, operator training and local microbial ecology may differ elsewhere; multicenter replication would help confirm generalizability.

Future directions

Future investigations should test whether the NaOCl+EDTA advantage persists when modern activation modalities are added, and whether the same chemistry improves outcomes in molars with complex anatomy. Longer-term follow-up—radiographic and patient-reported—would clarify whether early microbiologic success translates into higher healing rates and fewer retreatments at 6–24 months. Metagenomic profiling could determine whether certain taxa or community structures resist particular chemistries, enabling personalized irrigation strategies. Finally, cost-effectiveness analyses in public hospitals would help administrators balance reagent costs, chair time, and the downstream savings from fewer complications and antibiotics.

CONCLUSION

Within the constraints of a pragmatic, single-center RCT, a protocol using 1.0% sodium hypochlorite during instrumentation followed by a 17% EDTA final rinse achieved the highest rate of complete intracanal bacterial elimination and the largest qPCR-measured reduction in bacterial load, with fewer early flare-ups and lower postoperative pain than 2% chlorhexidine or saline. High-concentration NaOCl (5.25%) also performed strongly but did not outperform the lower-strength NaOCl when paired with EDTA. These findings supported NaOCl-based irrigation with deliberate smear-layer management as a practical, effective approach for primary root canal therapy in tertiary-care settings in Pakistan and similar environments.

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