

ORIGINAL ARTICLE

Antibacterial Efficacy of N-Acetyl Cysteine Combined with Two Photodynamic Therapy Protocols Against *Enterococcus faecalis* in Infected Root Canals: An In Vitro Study

Aim: *Enterococcus faecalis* (*E. faecalis*) is a resilient endodontic pathogen associated with persistent root canal infections. The objective of this investigation was to assess and compare antibacterial efficacy of N-acetyl cysteine (NAC) alone and in combination with two photodynamic therapy (PDT) protocols utilizing methylene blue (MB) and indocyanine green (ICG) as photosensitizers.

Methodology: Seventy-eight extracted human mandibular premolars were instrumented, sterilized, and inoculated with *E. faecalis* (MTCC 439). After 14 days of incubation, samples were randomly divided into 3 groups ($n = 26$ each): Group I – NAC alone; Group II – NAC + PDT with ICG (1 mg/mL, 810 nm diode laser); Group III – NAC + PDT with MB (20 µg/mL, 660 nm diode laser). Bacterial samples were collected before and after disinfection using sterile paper points, and quantified via colony-forming unit (CFU) analysis on Brain Heart Infusion (BHI) agar. Data were $\log_{10}$ -transformed and analyzed using paired t-tests and one-way ANOVA with Tukey's post hoc test ($P < 0.05$).

Results: All groups demonstrated significant CFU reduction post-treatment ($P < 0.0001$). Mean CFU reductions were: Group I – 0.84 ± 0.29 , Group II – 1.52 ± 0.54 , and Group III – 1.35 ± 0.47 . Group II & III showed significantly greater reductions than Group I ($P < 0.0001$ and $P = 0.0003$, respectively). No significant difference was observed between Group II & Group III ($P = 0.3554$).

Conclusion: NAC combined with either MB- or ICG-mediated PDT significantly improved disinfection of *E. faecalis*-infected root canals compared to NAC alone. This combination offers a promising adjunctive approach for managing persistent endodontic infections.

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Introduction

Endodontic infections are polymicrobial in nature and are closely associated with the development of robust biofilms within the complex root canal anatomy (1). *E. faecalis* is one of most persistent and clinically significant microorganisms implicated in the failure of root canal therapy. It is frequently isolated from refractory endodontic lesions and persistent apical periodontitis, owing to its ability to survive under harsh conditions, invade dentinal tubules, and resist conventional antimicrobial strategies (2). Mechanical instrumentation and chemical irrigation are fundamental to root canal debridement. However, anatomical challenges such as lateral canals, isthmuses, and deep dentinal tubules often limit the thorough elimination of microbial biofilms. Smear layer produced during instrumentation can further block dentinal tubules, thereby reducing efficiency of irrigants as well as intracanal medications (3). *E. faecalis* was shown to penetrate dentinal tubules up to 1200 μm , while most irrigants typically reach only 60–150 μm . Scanning electron microscopy (SEM) has confirmed widespread bacterial colonization in these difficult-to-access areas, highlighting the necessity for additional disinfection strategies beyond standard protocols (4), more in particular with oval and irregular round canals (5). To improve root canal disinfection, ongoing research focuses on developing novel antimicrobial methods that can disrupt biofilm architecture and enhance bacterial removal. N-acetyl cysteine (NAC), a thiol-based derivative of L-cysteine, has gained interest due to its ability to degrade extracellular polymeric substances and interfere with bacterial protein structures through disruption of disulfide bonds. It has shown antimicrobial activity against both planktonic and biofilm forms of *Enterococcus faecalis* (*E. fae-*

calis) and *Streptococcus mutans*. Despite its potential, NAC alone may be insufficient to achieve complete biofilm eradication within complexities of root canal system (6).

Photodynamic therapy (PDT) was explored as an adjunctive disinfection approach (7). PDT employs a photosensitizer that, upon activation by light of specified wavelength in presence of oxygen, generates ROS (reactive oxygen species), encompassing singlet oxygen as well as free radicals. Such ROS disrupt microbial membranes, nucleic acids, and proteins, leading to bacterial cell death. Unlike high-power laser techniques, PDT exerts a photochemical effect with minimal heat production, reducing the risk of periradicular tissue injury. Its selective antimicrobial action, absence of microbial resistance, and biocompatibility support its application in endodontic therapy (8,9). Commonly studied photosensitizers such as Methylene blue (MB) and toluidine chloride are cationic dyes known for their broad-spectrum antimicrobial effects. MB, a phenothiazine compound with an absorption peak between 500 and 700 nm, has demonstrated strong antimicrobial efficacy even under less-than-ideal light conditions (8,10). However, its use may result in undesirable staining of dental structures. To overcome this limitation, Indocyanine green (ICG), an anionic dye with peak absorption at 810 nm, has been introduced. ICG primarily functions through photothermal activity and has demonstrated a favourable safety profile along with antimicrobial potential (11).

Effective photodynamic therapy requires that the wavelength of the light source closely corresponds to the absorption spectrum of the photosensitizer in order to optimize the generation of ROS. Diode lasers are commonly employed due to their portability, optical fibre delivery, and ease of clinical use. In the current study, MB (20 $\mu\text{g}/\text{mL}$) was activated utilizing 660nm diode laser, and ICG (one mg per mL) was activated using 810nm diode laser,

both following NAC irrigation (12). The rationale for this study stemmed from the need to enhance intracanal disinfection by combining the biofilm-disrupting properties of N-acetyl cysteine (NAC) with the antimicrobial capabilities of photodynamic therapy (PDT). This in vitro investigation was designed to assess and compare the antibacterial efficacy of NAC alone and in combination with two distinct PDT protocols using methylene blue (MB) with a 660 nm diode laser and indocyanine green (ICG) with an 810 nm diode laser against *E. faecalis* in infected root canals. Bacterial viability has been evaluated using colony-forming unit (CFU) quantification to determine whether these combined approaches provide synergistic antimicrobial effects that can provide synergistic antimicrobial effects in vitro. The tested hypothesis was that NAC combined with PDT (MB or ICG) will show greater reduction in *E. faecalis* compared to NAC alone.

Materials and Methods

Ethical Approval

This in vitro study has been performed using extracted human teeth collected in accordance with ethical guidelines for research involving human biological material. Ethical approval has been obtained from the Institutional Human Ethics Committee (IHEC) of KLE VK Institute of Dental Sciences, under approval number EC/NEW/INST/2021/2435/427, before commencement of study. All specimens were anonymized, and no identifiable patient information was used. The current study was drafted according to PRILE guidelines (13).

Sample Size Calculation

Sample size has been computed utilizing G*Power version 3.0.10 software (Heinrich Heine University, Düsseldorf, Germany). According to data from earlier similar research (14), having an effect size 0.904, an alpha error probability 5 percent, and statistical power

(1- β) of 90%, the estimated sample size was 26 specimens per group. Accordingly, for three groups, the total required sample size was 78 specimens. Teeth with single straight canals and fully developed apices were included, confirmed by radiographs and canal patency with a #10 K-file. Teeth with cracks, resorption, caries, previous treatment, or open apices were excluded. All teeth have been cleaned of soft tissue and calculus with an ultrasonic scaler and preserved in a 0.1 percent thymol solution until utilized.

Specimen Preparation

Decoronation of the specimens was done utilizing a diamond disc (Komet, USA) under water cooling to obtain root segments of length 14 mm. Working length has been described by inserting size 10 K-file (MANI, Japan) until it is observable at apical foramen, followed by a subtraction of 0.5 mm. Biomechanical preparation was performed utilizing ProTaper Universal rotary system (Dentsply Sirona, USA) up to size F3. Canals were irrigated using 5 ml of 3% sodium hypochlorite (NaOCl) (Vishal Dental Care, India) and 2 ml of 17% EDTA (Canal Large, India) throughout instrumentation. Final irrigation included 2 mL of 17% EDTA for 60s, followed by 5 mL of 3% NaOCl for 60 s, and 5 mL of sterile distilled water for 60 s. All specimens were autoclaved at 121 °C, 15 psi, for 20 minutes for sterilization. The apical foramina of all specimens were sealed with composite resin (Filtek Z350 XT, 3M). Sterility was confirmed by paper-point sampling of random specimens into BHI broth, which showed no growth after 72 h.

Contamination Protocol

The *Enterococcus faecalis* strain MTCC 439 was used for bacterial inoculation. Colonies have been suspended in Brain heart infusion broth (BHI) (TM Media, India) as well as incubated for 24 hrs to reach turbidity equivalent to 0.5 McFarland standard ($\sim 1 \times 10^8$ CFU per mL). Each root specimen has been

**RATIONALE/JUSTIFICATION**

The study explores the antibacterial efficacy of N-acetyl cysteine (NAC) alone and in combination with two photodynamic therapy (PDT) protocols using methylene blue (MB) and indocyanine green (ICG) against *Enterococcus faecalis* in infected root canals.

AIM

The study aimed at evaluating and comparing the antibacterial efficacy of NAC alone and when combined with PDT protocols using MB or ICG photosensitizers activated by diode lasers in root canals infected with *E. faecalis*.

ETHICAL APPROVAL

EC/NEW/INST/2021/2435/427

SAMPLES

Seventy-eight freshly extracted single-rooted human mandibular premolars.

EXPERIMENTAL AND CONTROL GROUPS, INCLUDE INDEPENDENT VARIABLES

Group I – NAC irrigation only (n = 26)
Group II – NAC + ICG-mediated PDT (n = 26)
Group III – NAC + MB-mediated PDT (n = 26)

OUTCOME(S) ASSESSED, INCLUDE DEPENDENT VARIABLES AND TYPE

Reduction in *E. faecalis* bacterial load quantified as colony-forming units (CFUs) before and after treatment.

METHOD USED TO ASSESS THE OUTCOME(S) AND WHO ASSESSED THE OUTCOME(S)

Microbiological sampling with sterile paper points → Culturing on BHI agar → Manual CFU counting after 72-hour incubation.

RESULTS

All groups showed significant CFU reduction post-treatment ($P < 0.0001$). NAC combined with PDT (Groups II and III) achieved significantly greater bacterial reduction than NAC alone ($P < 0.0001$ and $P = 0.0003$, respectively). No significant difference was observed between MB- and ICG-mediated PDT groups ($P = 0.3541$).

CONCLUSION(S)

Combining NAC with PDT (either MB or ICG) significantly improves antibacterial efficacy compared to NAC alone, suggesting a promising adjunctive disinfection protocol in endodontics.

FUNDING DETAILS

The research did not receive any specific grant from funding agencies.

CONFLICT OF INTEREST

None

placed in sterile microcentrifuge tube containing 1 mL of BHI broth. A 50 μ L aliquot of the bacterial suspension was inoculated into each canal under aseptic conditions in a laminar airflow hood. To confirm culture purity, 5 μ L of the bacterial broth was sub-cultured on BHI agar. The specimens have been incubated at $37 \pm 1^\circ\text{C}$ for 14 days to allow mature biofilm development.

Intervention

Subsequently, all samples were randomly allocated into three experimental groups, each corresponding to a specific disinfection strategy implemented after the contamination phase. This randomization was achieved using a computer-generated sequence with concealed allocation to ensure unbiased assignment.

Group 1: N-acetylcysteine (NAC) only (Control) [n=26]

The specimens were irrigated with 5 mL of NAC solution (200 mg/mL), prepared by dissolving 0.2 g of NAC powder (MOLYCHEM, India) in 1 mL of sterile distilled water, and adjusting the pH to 11. This was followed by a final flush with 4 mL of sterile distilled water.

Group 2: NAC + Indocyanine Green (ICG)-mediated PDT [n=26]

Following NAC irrigation, indocyanine green (ICG) solution (1 mg/mL; AUROGREEN, India) was introduced into the canal and retained for 5 minutes. Photodynamic activation was performed using an 810 nm diode laser (INDILASE, India) at a power output of 0.1 W, delivered through a 200 μ m optical fibre. Irradiation was applied for a total of 60 seconds, consisting of 10-second cycles with 10-second rest intervals after every 20 seconds. The fibre-optic tip was positioned 1 mm short of the working length and moved helically from apical to coronal

Group 3: NAC + MB-mediated PDT

[n=26]

Following NAC treatment, a 20 μ g/mL methylene blue (MB) solution (ISO-CHEM, India) was introduced into the canal and retained for 5 minutes. Photodynamic activation was performed using a 660 nm diode laser (INDILASE, India) at an output of 0.1 W, delivered through a 200 μ m optical fibre. Irradiation was applied for a total of 90 seconds in 10-second bursts with 30-second rest intervals. The fibre-optic tip was positioned 1 mm short of the working length and moved in a helical motion from apical to coronal.

Outcome Measures

Microbiological sampling was performed before and after disinfection using sterile size 20 paper points, which had been inserted into canal for one minute. Paper points have been transferred into Eppendorf tubes comprising 10 μ L of sterile saline and vortexed briefly. The resulting suspension was streaked onto BHI agar plates using sterile spreaders. Plates have been incubated for 72 hrs at 37°C . Colony forming units (CFUs) were enumerated using an automated colony counter and recorded to assess bacterial viability. Figure 1 illustrates the PRILE flowchart outlining the methodology followed in the study.

Statistical Analysis

Data were entered in Microsoft Excel 2021 and analyzed using GraphPad Prism for Windows, Version 10.1.2 (GraphPad Software, La Jolla, California, USA). CFU values were $\log_{10}$ -transformed to normalize distribution and reduce variability, aiding interpretation across groups. Normality was confirmed via the Shapiro-Wilk test and visual assessments of histograms. Descriptive statistics included mean and standard deviation. Intra-group comparisons used paired t-test; inter-group comparisons employed one-way ANOVA with Tukey's post-hoc test.

Figure 1
PRILE 2021 Flowchart

Table 1

Mean ($\pm$ standard deviation) and comparisons between pre-treatment and post-treatment colony-forming unit values for all the groups

Results

Table 1 and Figure 2 depict mean CFU values before and after treatment across the study groups. All groups showed statistically significant decrease in CFU following treatment ($P < 0.0001$), (Figure 1). Group I had the least reduction

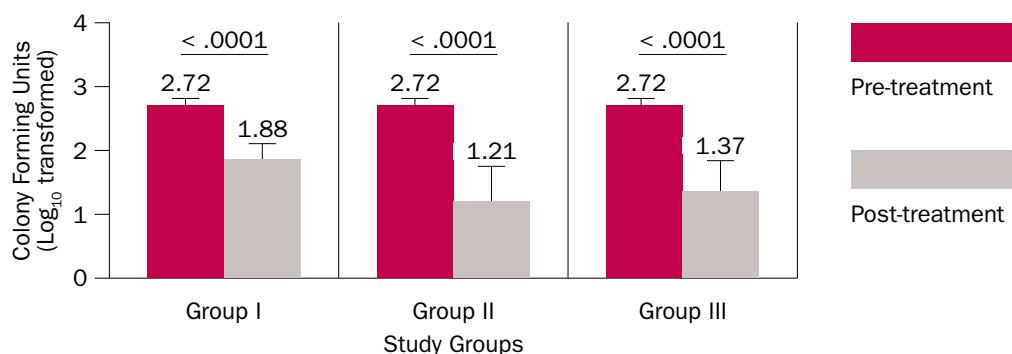
(0.84 ± 0.29), while Groups II and III demonstrated greater reductions of 1.52 ± 0.54 and 1.35 ± 0.47 , respectively. The inter-group comparison of mean CFU reductions revealed statistically significant difference ($P < 0.0001$). Pairwise comparisons showed that the reductions in Groups II & III have been

Study Groups/Time Points	Pre-treatment	Post-treatment	Difference	P value‡
Group I (n=26)	2.72 $\pm$ 0.08	1.88 $\pm$ 0.25	0.84 $\pm$ 0.29	<0.0001*
Group II (n=26)	2.72 $\pm$ 0.08	1.21 $\pm$ 0.56	1.52 $\pm$ 0.54	<0.0001*
Group III (n=26)	2.72 $\pm$ 0.07	1.37 $\pm$ 0.48	1.35 $\pm$ 0.47	<0.0001*
P value§			<0.0001*	

n: sample size per study group
SD: Standard deviation
§: Inter-group comparisons (between the study groups for the mean difference/reduction); ‡: intra-group comparisons (pre-treatment versus post-treatment within each study group)
*: statistically significant ($P \leq 0.05$)

Figure 2

Mean and standard deviation of the pre-treatment and post-treatment colony-forming unit values for all the groups. Horizontal bars above the columns represent intra-group comparisons that were statistically significant as determined by a paired t-test.

**Figure 3**

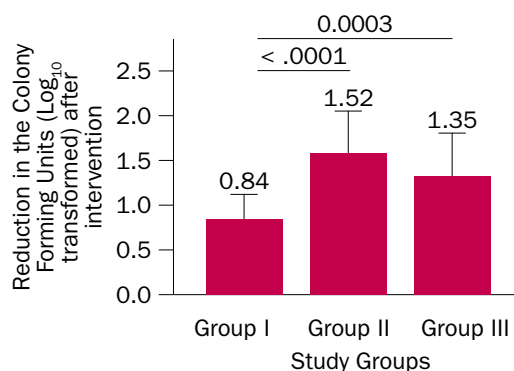
Mean and standard deviation of the reduction in colony-forming unit (CFU) values following treatment across the study groups. Horizontal bars above the columns represent pairwise group comparisons that were statistically significant as determined by Tukey's post-hoc test.

significantly greater than in Group I ($P < 0.0001$ and $P=0.0003$, respectively), whereas difference between Groups II & III have not been statistically significant ($P = 0.3554$). These findings

indicate that although all interventions were effective, those in Groups II and III produced a more substantial reduction in CFU than Group I (Table 2 and Figure 3).

Table 2

Pairwise comparisons of colony-forming unit (CFU) reduction between groups post-treatment relative to pre-treatment



Pairs	Mean Difference	Adjusted P value
Group I vs. Group II	-0.6807	<0.0001*
Group I vs. Group III	-0.5100	0.0003*
Group II vs. Group III	0.1707	0.3554NS
Group III (n=26)	2.72 $\pm$ 0.07	1.37 $\pm$ 0.48

NS: not significant ($P > 0.05$),
*: statistically significant ($P < 0.05$)

Discussion

Effective disinfection of root canal system remains cornerstone of successful endodontic therapy. *E. faecalis*, known for its ability to penetrate dentinal tubules and form resilient biofilms, is frequently implicated in persistent infections and endodontic treatment (15). The present in vitro study assessed antibacterial efficacy of NAC, both alone and in combination with PDT, using MB and ICG as photosensitizers, against *E. faecalis* in contaminated root canals. The results demonstrated that NAC alone exhibited limited antimicrobial activity, while its combination with either PDT protocol significantly enhanced bacterial reduction (16). Hence, the aim of this study was to evaluate whether the combination of NAC with PDT (using either MB or ICG) would result in a greater reduction of *E. faecalis* compared to NAC alone.

NAC has recently gained attention as potential endodontic irrigant because of its biofilm-disrupting capabilities, attributed to the cleavage of disulfide bonds in the extracellular polymeric substance (EPS) matrix. Compared to traditional agents like NaOCl, NAC is biocompatible, anti-inflammatory, and safer for use near periapical tissues, especially in open apices or immature teeth (17,18,19). However, in our study, NAC alone at 200 mg/mL, the concentration recommended in earlier studies (Group I), showed the least bacterial reduction (mean CFU reduction: 0.84 ± 0.29). This is consistent with previous reports indicating that NAC monotherapy is insufficient for the complete eradication of mature *E. faecalis* biofilms (20,21).

For the other groups, the concentrations of MB (20 µg/mL) and ICG (1 mg/mL) were chosen in accordance with established PDT protocols reported in the endodontic literature (22,23). The results show that the addition of PDT significantly improved disinfection outcomes both MB- and ICG-mediated

PDT (Groups II and III) showed superior antibacterial efficacy (1.52 ± 0.54 and 1.35 ± 0.47 , respectively), with statistically significant differences compared to NAC alone ($P < 0.0001$ and $P = 0.0003$). This supports existing literature suggesting that PDT enhances antimicrobial activity when used adjunctively with irrigants, with no superiority among them (8,11). The enhanced antibacterial action seen with PDT can be explained by its photochemical mechanism. PDT involves activation of a photosensitizer by a specific light wavelength in presence of oxygen, leading to ROS generation, including singlet oxygen and free radicals. Such ROS damage to microbial membranes, nucleic acids, and proteins leads to bacterial death (24). MB, a phenothiazine dye, binds well to bacterial biofilms and is efficiently activated by red light (660 nm), while ICG, an anionic dye, absorbs light in the near-infrared spectrum (810 nm) and primarily acts via photothermal mechanisms. Although MB may stain dental tissues, it was included due to its well-established photochemical efficacy and to allow direct comparison with ICG, which has less staining potential. In the current study both photosensitizers achieved comparable antimicrobial efficacy, underscoring their suitability for endodontic disinfection protocols (25). The observed synergistic effect between NAC and PDT is likely due to NAC's ability to weaken the biofilm matrix, allowing deeper photosensitizer penetration and enhanced ROS-mediated killing. This aligns with previous evidence supporting the combination of biofilm-disrupting agents with antimicrobial therapies for improved efficacy (26).

A key strength of this study is the use of a standardized and clinically relevant *E. faecalis* biofilm model with a 14-day incubation period, which simulates persistent root canal infections (27). Furthermore, diode lasers were chosen for PDT activation due to their clinical accessibility and fiber-optic

compatibility, enabling precise delivery within root canals (28). Nonetheless, this study has limitations. The colony-forming unit (CFU) quantification method, while widely accepted, primarily reflects planktonic or loosely adherent bacteria from the main canal. This may underestimate the actual residual bacterial load, especially within dentinal tubules.

Advanced techniques, including CLSM (confocal laser scanning microscopy) or SEM, could provide more comprehensive insights into biofilm disruption and residual intratubular bacteria. More research should also focus on understanding the role of effective root canal disinfection strategies (29,30), improvising the fluid dynamics (31) without altering the instrument surface (32) and thereby focussing on effective pain control strategies (33,34). Future focus is needed more on understanding the complex root canal anatomy (35) and its intricate relation to the periodontium (36) and also on bioactive strategies (37,38,39) for improvising the overall outcomes.

The findings of this study support the adjunctive use of PDT with NAC as an effective disinfection strategy in endodontics. Given the comparable antibacterial performance of MB and ICG, clinicians may consider other factors such as staining potential, cost, and laser compatibility when selecting a photosensitizer. Further studies using polymicrobial biofilms, digital devices (40), regenerative models (41), or immature teeth and its application in vital pulp therapies (42) are needed to validate the clinical applicability of these protocols and assess their long-term effects on root dentin (43) and periapical healing (44,45).

Conclusion

Within the limitations of this in vitro study, NAC + PDT (MB or ICG) showed greater antibacterial activity than NAC alone. Further studies comparing these

protocols to NaOCl + EDTA are required before clinical recommendations can be made. Both MB and indocyanine green performed similarly when used with PDT, suggesting either photosensitizer may be effective. This combination may thus serve as a biocompatible adjunct for enhanced root canal disinfection in persistent infections.

Clinical Relevance

This study shows that combining N-acetyl cysteine (NAC) with photodynamic therapy (PDT) using either methylene blue or indocyanine green significantly enhances disinfection of *E. faecalis* compared to NAC alone. The synergistic effect suggests that NAC + PDT may represent a safe and effective adjunct for managing persistent root canal infections, with the choice of photosensitizer guided by clinical considerations such as staining potential and laser availability.

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