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Effectiveness of N-acetylcysteine in Endodontics: Comparison and combination with Calcium Hydroxide in the inhibition of *Enterococcus faecalis*

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Abstract
The aim of this study was to evaluate the antibacterial activity of N-acetylcysteine (NAC), calcium hydroxide ($Ca(OH)_2$), and their combination against Enterococcus faecalis, a microorganism frequently associated with endodontic infections. An *in vitro* experimental study was conducted using a bile esculin agar well diffusion assay with 24 plates inoculated with a standard strain of E. faecalis (ATCC 29212). Wells were prepared in the culture medium and the experimental materials were applied. Antibacterial activity was determined by measuring inhibition zone diameters (mm) at 24, 48, and 72 h. Data were analyzed using normality tests (Kolmogorov–Smirnov and Shapiro–Wilk). Paired comparisons were performed using the Wilcoxon signed-rank test or the paired Student's t-test, as appropriate. Statistical significance was set at $p < 0.05$. Results showed that NAC exhibited moderate antibacterial activity with a slight decrease over time. The combination of NAC with calcium hydroxide did not demonstrate a synergistic effect, as inhibition zone diameters were similar to NAC and lower than calcium hydroxide alone. Under the *in vitro* agar diffusion model used, calcium hydroxide showed the highest antibacterial efficacy against E. faecalis, and NAC did not enhance its effect when used in combination.

Keywords: *Enterococcus faecalis*, n-acetylcysteine, intracanal medicament, endodontics, calcium hydroxide, inhibition zones

Introduction

The root canal system presents a highly complex anatomy, characterized by the presence of lateral canals, isthmuses, and dentinal tubules. These anatomical features hinder effective cleaning during endodontic treatment, resulting in inaccessible areas where microorganisms can remain even after instrumentation and irrigation (Zou *et al*., 2024) [24]. These anatomical complexities favor the formation of resistant biofilms, defined as organized microbial communities within an extracellular matrix that limits the penetration of antimicrobial agents and contributes to bacterial persistence (Jhajharia *et al*., 2015) [10]. In this context, intraradicular biofilms constitute protected microenvironments that increase microbial resistance to mechanical instrumentation and conventional irrigation (Jayakumar *et al*., 2024) [9], representing one of the main factors associated with endodontic failure.

Among the pathogens associated with persistent infections, *Enterococcus faecalis* stands out due to its ability to survive under adverse conditions within the root canal system, penetrate dentinal tubules, and firmly adhere to dentin, which allows it to persist after instrumentation and irrigation procedures and contribute to treatment failure (Alghamdi & Shakir, 2020) [2]. In addition, it possesses survival mechanisms that include tolerance to alkaline pH environments, resistance to nutritional deprivation, and the ability to form biofilms, which favors its adaptation and persistence in previously treated canals (Stuart *et al*., 2006) [20]. Additionally, the presence of this microorganism in complex anatomical areas may promote the persistence of periapical inflammation, by remaining in direct contact with periradicular tissues, compromising tissue repair and the prognosis of endodontic treatment (Siqueira & Rôças, 2008) [19]. Therefore, understanding its survival mechanisms is essential for the development of more effective intracanal disinfection strategies.

In clinical practice, calcium hydroxide has been widely used as an intracanal medicament due to its antimicrobial action, mainly attributed to the release of hydroxyl ions and the elevation of pH to highly alkaline levels, creating an unfavorable environment for the survival of numerous microorganisms (Siqueira & Lopes, 1999) [18]. However, studies have shown that the antimicrobial efficacy of this material may be influenced by factors such as compound solubility, ionic diffusion, and contact time, which may affect its ability to eliminate resistant bacteria such as *Enterococcus faecalis* (Kim & Kim, 2014) [11]. Likewise, its performance has been observed to vary depending on the formulation used and its combination with other agents (Teja *et al*., 2023) [21].

Due to these limitations, there is a need to explore alternative agents with greater antibacterial and antibiofilm capacity that allow optimization of the root canal system disinfection.

In this context, N-acetylcysteine is a cysteine derivative clinically used as a mucolytic agent and glutathione precursor, with recognized antioxidant properties. Although it is not an antibiotic, it has reported antimicrobial activity and has demonstrated the ability to disrupt pathogenic bacterial biofilms, which supports its potential in the oral environment (Pei *et al*., 2018) [14]. In fact, NAC has been reported to decrease biofilm formation and reduce the bacterial extracellular matrix and facilitate the penetration of antibacterial agents (Dinicola *et al*., 2014) [6].

In vitro studies have demonstrated that N-acetylcysteine inhibits biofilm formation and is capable of disrupting mature biofilms adhered to dentin surfaces, which results in a significant reduction in bacterial viability (Moon *et al*., 2016) [13]. Consistently, its effectiveness in eliminating multispecies endodontic biofilms has been reported, with a marked decrease in bacterial load and structural alterations

of the biofilm matrix observed, depending on the experimental model and the evaluation method used (Choi *et al*., 2018) [5]. In agreement, a recent scoping review synthesizes the experimental evidence supporting the antimicrobial and antibiofilm efficacy of N-acetylcysteine, reporting comparable or superior results to traditional agents used in endodontics, which highlights its potential as an alternative for root canal system disinfection (Abdulrab *et al*., 2022) [1].

Under this theoretical framework, evaluating N-acetylcysteine (NAC), calcium hydroxide, and their combination could represent a strategy of interest to overcome bacterial resistance and improve the prognosis of endodontic treatments. Because agar diffusion assays reflect both antimicrobial activity and compound diffusibility in the medium, the present findings should be interpreted within the methodological scope of this model and should not be directly extrapolated to mature biofilms or clinical intracanal conditions.

The objective of this study was to evaluate antibacterial activity through the measurement of inhibition zone diameters in *in vitro* assays, in order to compare the efficacy of N-acetylcysteine (NAC), calcium hydroxide, and their combination against *Enterococcus faecalis*. Based on this approach, the following research question arises: Can N-acetylcysteine, alone or in combination with calcium hydroxide, present a greater inhibitory effect against *Enterococcus faecalis* than calcium hydroxide, in an *in vitro* model?

Materials and Methods

Study Design

A comparative *in vitro* experimental study was conducted to evaluate the efficacy of different compounds against the standard strain of *Enterococcus faecalis* (ATCC 29212). The experiment was carried out at the laboratories of Universidad de los Hemisferios, following approval by the Institutional Ethics Committee and formal authorization for laboratory use.

Sample Size Determination

The study was conducted using 24 plates, evaluated at three experimental time points: 24, 48, and 72 h. The same wells were analyzed at each interval to observe the temporal evolution of the compounds' effects. Of the 24 plates, four contained four wells each (three corresponding to the experimental treatments and one assigned to the negative control with glycerin), while the remaining 20 plates contained three wells per plate. A total of 76 wells were obtained, of which 72 corresponded to the experimental treatments and four to the negative controls. Each well was initially recorded individually; however, for statistical analysis, the plate was considered the experimental unit. When more than one well of the same treatment was present in a plate, inhibition zone diameters were averaged to obtain a single value per plate, resulting in 24 paired observations per treatment at each time point (one value per plate per treatment). The selection of 24 plates was based on previous studies using similar agar diffusion methodologies and on the expected variability in this type of microbiological analysis, in order to obtain an adequate number of replicates for experimental comparison.

Agar Preparation

The culture medium used was bile esculin agar (BEA) (HiMedia, Mumbai, Maharashtra, India). For plate preparation, 30 mL of medium were dispensed per plate, obtaining an initial volume of 180 mL for six plates, which was adjusted to 200 mL for the initial culture medium preparation.

The culture medium was prepared following the manufacturer's instructions, which recommend a concentration of 63.5 g of BEA per 1000 mL of distilled water. Based on this proportion, 12.7 g of BEA were used for the prepared volume.

Before weighing, the electronic balance (Traveler, Ohaus, USA) was verified to be level on a stable surface. A disposable plastic weighing tray was then used to accurately measure the agar powder.

The required volume of distilled water was measured using a graduated cylinder and transferred to a previously labeled screw-cap glass flask. The agar powder was then added and mixed using gentle swirling motions until a homogeneous preparation was obtained. The culture medium was sterilized in a vertical autoclave at 121 °C according to the laboratory operating protocol. It was then allowed to cool to handling temperature before being dispensed into the plates. This procedure was repeated at different stages of the study under constant preparation and sterilization conditions until the 24 required plates were completed.

Preparation of the Bacterial Suspension According to the McFarland Standard

A 0.5 McFarland visual standard was used to standardize the bacterial inoculum concentration, ensuring a suspension with adequate optical density for bacterial seeding.

A sterile 10 mL test tube was labeled with the microorganism name (*Enterococcus faecalis*), the operator's initials, the date, and the McFarland 0.5 reference, and placed on a rack. Subsequently, 6 mL (6000 µL) of sterile distilled water were added to the tube, which remained on the rack.

Using a metal loop previously heated to red hot and allowed to cool, seven previously grown *Enterococcus faecalis* colonies were collected and carefully introduced into the tube. The loop was gently agitated in the liquid to release the colonies, then removed and flamed again to red hot to ensure disinfection.

Finally, the turbidity of the suspension was carefully adjusted by visual comparison against a lined background, allowing identification of whether the suspension was too clear (indicating insufficient concentration) or too turbid (indicating excessive concentration).

Once turbidity equivalent to the 0.5 McFarland standard was achieved, the suspension was considered standardized and ready for use in the experimental assays, maintaining aseptic conditions throughout the procedure.

Agar Dispensing

Disposable Petri dishes were used and placed in a laminar flow chamber, where the work area was previously decontaminated with ultraviolet light for 20 minutes. The agar was dispensed carefully inside the chamber using a 100 mL syringe to ensure volume accuracy, distributing approximately 30 mL per plate.

Once the medium had solidified, the dishes were labeled indicating the type of agar, the date, and the person responsible for the procedure. The dishes were stored in a labeled hermetically sealed container and kept under refrigeration until use.

Inoculation of the Bacterial Suspension onto Agar

Once the McFarland 0.5 standard was adjusted and each plate was labeled, the procedure was performed in front of a lit Bunsen burner to ensure sterile conditions. Using a micropipette, 100 µL of the previously prepared suspension were collected.

The suspension was deposited at the center of the agar surface, and the disposable tip used was immediately discarded in a designated waste container.

Subsequently, using a Drigalski spatula previously sterilized by immersion in alcohol, flame exposure, and cooling, the suspension was evenly spread over the entire agar surface. This protocol was applied identically to each of the plates included in the study.

Preparation of Wells in the Culture Medium

With a Bunsen burner lit to maintain aseptic conditions, the Petri dishes were placed near the flame and carefully uncovered. Using the wide end of a sterile disposable micropipette tip, three or four previously marked wells (≈ 6 mm in diameter) were made, according to the experimental distribution for each plate. The wells were created equidistant from each other and at a uniform distance from the plate edge to standardize material diffusion.

Subsequently, using a metal loop previously heated to red hot and allowed to cool, the agar residues from the perforations were removed, ensuring a clean and sterile well formation.

Once the wells were prepared, 10 µL of previously melted bile esculin agar (BEA), cooled to approximately 45 °C, were dispensed into the bottom of each well. This thin layer of semi-liquid agar served as a base for the treatments, preventing dispersion of the experimental material across the medium surface and ensuring precise delimitation of the application area without altering the integrity of the agar or compromising the sample.

Preparation and Dosing of Experimental Materials

All powders used in the formulations were previously weighed using an analytical precision balance (Galaxy HR-250AZ, SHS). For the procedure, each substance was placed in a disposable plastic laboratory weighing tray and, with the aid of a sterile spatula, the powders were carefully transferred to the balance to obtain the exact amount required for each mixture, thus ensuring precise measurement.

A working proportion of 0.6 g of powder and 0.8 mL of glycerin was established. For formulations combining two powders and one liquid, a proportion of 0.6:0.6:1.6 (g:g:mL) of glycerin was used. This ratio was defined to obtain homogeneous mixtures with intermediate consistency, facilitating their application onto the culture medium without causing dispersion or excessive density.

Glycerin (Laturi, 100 mL bottle) was used as a humectant agent and vehicle for the preparation of the experimental mixtures.

Each mixture was prepared according to the previously described proportions, ensuring a uniform texture suitable for dispensing. The resulting mixture was then loaded into a sterile 3 mL syringe, from which 0.15 mL (1.5 syringe scale lines) of the material was dispensed into each well of the culture medium, ensuring a consistent volume per application.

The formulations were prepared as many times as necessary, maintaining constant proportions and application volume in each well. In this way, homogeneity, precision, and reproducibility in treatment application were ensured for each experimental assay.

Calcium hydroxide was considered the reference group (positive control) due to its known antimicrobial activity and was compared with NAC and $Ca(OH)_2$ + NAC. Glycerin was used as a negative control in independent wells to confirm that the vehicle did not exhibit antibacterial activity; the presence of an inhibition zone would have indicated vehicle interference.

Data Collection Forms

Data collection was performed manually and visually by recording the inhibition zone diameters (mm) of *Enterococcus faecalis* for each experimental compound on a standardized observation sheet. The procedure was carried out on a clean, aseptic illuminated work surface, with a Bunsen burner lit nearby to help maintain sterile conditions throughout the process.

The first plate reading was performed at 24 h, verifying the absence of contaminants or alterations in the plates included in the study. Using a Truper digital Vernier caliper, an initial measurement was obtained, followed by two additional measurements per well to improve precision and reduce measurement error. The inhibition zone diameter was recorded in millimeters (mm), measured from edge to edge including the well diameter. The mean of the three measurements per well was used for statistical analysis.

Subsequent inhibition zone diameter measurements were performed at 48 and 72 h, allowing appropriate monitoring of the effects of the evaluated compounds over time. Between each measurement point, the plates were stored at 37.5 °C and removed only for inhibition zone measurement, then immediately returned to the incubator to maintain constant conditions.

Sample Disposal

Because the plates used were made of plastic material, they were disinfected by immersion. A plastic container was filled to approximately two-thirds of its volume with 5.25% sodium hypochlorite solution.

The plates were kept immersed in the solution for 24 h to ensure elimination of the studied microorganism.

Afterward, the plates were discarded in a properly labeled red bag, in compliance with established biohazard waste disposal regulations.

Results

Antibacterial activity was assessed by measuring inhibition zone diameters (mm) at 24 h, 48 h, and 72 h.

Normality tests of inhibition zone diameters (mm)

Table 1: Normality tests of inhibition zone diameters (mm): Kolmogorov–Smirnov and Shapiro–Wilk

Time	Substance	Kolmogorov-Smirnov			Shapiro-Wilk		
		Statistic	df	p-value	Statistic	df	p-value
24 h	$Ca(OH)_2$	0.185	24	0.032	0.889	24	0.013
	NAC	0.165	24	0.090	0.948	24	0.242
	$Ca(OH)_2$ + NAC	0.126	24	0.200	0.921	24	0.063

48 h	$Ca(OH)_2$	0.221	24	0.004	0.811	24	0.001
	NAC	0.214	24	0.006	0.760	24	0.001
	$Ca(OH)_2$ + NAC	0.138	24	0.200	0.935	24	0.123
72 h	$Ca(OH)_2$	0.399	24	0.001	0.503	24	0.001
	NAC	0.197	24	0.016	0.938	24	0.144
	$Ca(OH)_2$ + NAC	0.081	24	0.200	0.990	24	0.997

Note: *n* = 24 per group and time point. Normality assessed with Kolmogorov–Smirnov and Shapiro–Wilk tests. $p < 0.05$ indicates a departure from normality. $Ca(OH)_2$ = calcium hydroxide; NAC = N-acetylcysteine.

Data distribution was evaluated using normality tests (Kolmogorov–Smirnov and Shapiro–Wilk). Since several distributions yielded $p < 0.05$ in at least one normality test, deviations from normality were assumed. Accordingly, paired comparisons were performed using the Wilcoxon signed-rank test when normality assumptions were not met and the paired Student's t-test when distributions were approximately normal. Statistical significance was set at $p < 0.05$. All analyses were conducted with n = 24 plates per group and time point.

Comparison of inhibition zones between groups at 24 h

Table 2: Comparison of substances at 24 h using the Wilcoxon signed-rank test and the paired Student's t-test

Pair	Substances Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)	Student's t-test (p)
Pair 1	$Ca(OH)_2$	24	20.71	0.42	0.001	0.001
	NAC	24	16.57	0.21		
Pair 2	$Ca(OH)_2$	24	20.71	0.42	0.001	0.001
	$Ca(OH)_2$ + NAC	24	16.61	0.30		
Pair 3	NAC	24	16.57	0.21	0.304	0.532
	$Ca(OH)_2$ + NAC	24	16.61	0.30		

Note: Values are mean ± SD (mm); n = 24 plates. Wilcoxon signed-rank and paired Student's t-tests were applied. Statistical significance set at $p < 0.05$.

At 24 h of evaluation, statistically significant differences were observed among the evaluated compounds. Calcium hydroxide showed a higher mean inhibition zone diameter (20.71 mm) compared with NAC (16.57 mm) and $Ca(OH)_2$ + NAC (16.61 mm). Calcium hydroxide presented the largest inhibition zone against *Enterococcus faecalis* compared with N-acetylcysteine and with the $Ca(OH)_2$ + NAC combination ($p < 0.05$). These findings were consistent when related-sample tests (Wilcoxon and Student's t-test) were applied, as reported in the table.

In contrast, N-acetylcysteine and the $Ca(OH)_2$ + NAC combination showed similar inhibition zone diameters, with no statistically significant differences between them ($p > 0.05$). This suggests that, at 24 h, the $Ca(OH)_2$ + NAC combination did not increase the inhibition zone compared with NAC used individually.

The negative control (glycerin) was used to verify the absence of antibacterial effect of the vehicle; its results were not included in the comparative analysis because it was not part of the statistically evaluated experimental groups.

Comparison between samples at 48 h

Table 3: Comparison of substances at 48 h using the Wilcoxon signed-rank test and the paired Student's t-test

Pair	Substances Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)	Student's t-test (p)
Pair 1	$Ca(OH)_2$	24	20.61	0.47	0.001	0.001
	NAC	24	15.65	0.34		
Pair 2	$Ca(OH)_2$	24	20.61	0.47	0.001	0.001
	$Ca(OH)_2$ + NAC	24	15.41	0.19		
Pair 3	NAC	24	15.65	0.34	0.007	0.013
	$Ca(OH)_2$ + NAC	24	15.41	0.19		

Note: Values are mean ± SD (mm); n = 24 plates. Wilcoxon signed-rank and paired Student's t-tests were applied. Statistical significance set at $p < 0.05$.

At 48 h of evaluation, statistically significant differences were observed among the tested compounds. Calcium hydroxide showed the highest mean inhibition zone diameters (20.61 mm), compared with N-acetylcysteine (15.65 mm) and the $Ca(OH)_2$ + NAC combination (15.41 mm). Pairwise comparisons demonstrated significant differences between calcium hydroxide and NAC, as well as between calcium hydroxide and $Ca(OH)_2$ + NAC ($p < 0.05$). Statistically significant differences were also observed between N-acetylcysteine and the $Ca(OH)_2$ + NAC combination, with NAC showing a larger inhibition zone ($p < 0.05$), according to both Wilcoxon and paired Student's t-tests.

These results indicate that, at 48 h, the NAC combination did not potentiate the inhibitory effect of calcium hydroxide and showed lower inhibition zone diameters even compared with NAC used alone.

Comparison between samples at 72 h

Table 4: Comparison of substances at 72 h using the Wilcoxon signed-rank test and the paired Student's t-test

air	Substances Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)	Student's t-test (p)
Pair 1	$Ca(OH)_2$	24	20.29	1.46	0.001	0.001
	NAC	24	15.55	0.21		
Pair 2	$Ca(OH)_2$	24	20.29	1.46	0.001	0.001

	$Ca(OH)_2$ + NAC	24	15.44	0.15		
Pair 3	NAC	24	15.55	0.21	0.076	0.061
	$Ca(OH)_2$ + NAC	24	15.44	0.15		

Note: Values are mean ± SD (mm); n = 24 plates. Wilcoxon signed-rank and paired Student's t-tests were applied. Statistical significance set at $p < 0.05$.

At 72 h of evaluation, statistically significant differences were observed among the tested compounds. Calcium hydroxide showed the highest mean inhibition zone diameters (20.29 mm), compared with N-acetylcysteine (15.55 mm) and the $Ca(OH)_2$ + NAC combination (15.44 mm). Pairwise comparisons demonstrated significant differences between calcium hydroxide and NAC, as well as between calcium hydroxide and $Ca(OH)_2$ + NAC ($p < 0.05$), according to both Wilcoxon and paired Student's t-tests.

In contrast, no statistically significant differences were found between N-acetylcysteine and the $Ca(OH)_2$ + NAC combination ($p > 0.05$), indicating similar inhibition zone diameters between these two treatments.

These findings suggest that at 72 h, the incorporation of NAC did not enhance the inhibitory effect of calcium hydroxide and maintained an antibacterial activity comparable to NAC used alone.

Temporal comparison within each substance

Calcium Hydroxide ($Ca(OH)_2$).

Table 5: Temporal comparison of calcium hydroxide ($Ca(OH)_2$) at 24 h, 48 h, and 72 h using the Wilcoxon signed-rank test (mean values ± standard deviation and between-time differences)

Pair	Time Points Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)
Pair 1	24 h vs 48 h	24	20.71 vs 20.61	0.42 vs 0.47	0.021
Pair 2	24 h vs 72 h	24	20.71 vs 20.29	0.42 vs 1.46	0.019
Pair 3	48 h vs 72 h	24	20.61 vs 20.29	0.47 vs 1.46	0.157

Note: Values are mean ± SD (mm); n = 24 plates. Within-group time comparisons were performed using the Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$.

Calcium hydroxide showed a slight progressive decrease in inhibition zone diameter over the evaluation period. Statistically significant differences were observed between 24 and 48 h and between 24 and 72 h ($p < 0.05$), with the highest inhibition values recorded at 24 h. In contrast, no statistically significant differences were found between 48 and 72 h ($p > 0.05$), indicating that the antibacterial effect remained relatively stable after 48 h. These results were obtained using the Wilcoxon signed-rank test (Table 5).

N-acetylcysteine (NAC)

Table 6: Temporal comparison of N-acetylcysteine (NAC) at 24 h, 48 h, and 72 h using the Wilcoxon signed-rank test (mean values ± standard deviation and between-time differences)

Pair	Time Points Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)
Pair 1	24 h vs 48 h	24	16.57 vs 15.65	0.21 vs 0.34	0.001
Pair 2	24 h vs 72 h	24	16.57 vs 15.55	0.21 vs 0.21	0.001
Pair 3	48 h vs 72 h	24	15.65 vs 15.55	0.34 vs 0.21	0.259

Note: Values are mean ± SD (mm); n = 24 plates. Within-group time comparisons were performed using the Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$.

N-acetylcysteine showed a gradual decrease in the mean inhibition zone diameters over the evaluation period. When the temporal measurements were compared, statistically significant differences were observed between 24 and 48 h and between 24 and 72 h ($p < 0.05$), with the highest inhibition values recorded at 24 h, as shown in Table 6. In contrast, no statistically significant differences were found between the measurements obtained at 48 and 72 h ($p > 0.05$), indicating that the antibacterial activity of N-acetylcysteine remained relatively stable after 48 h. These results were obtained using the Wilcoxon signed-rank test (Table 6).

$Ca(OH)_2$ + NAC

Table 7: Temporal comparison of the $Ca(OH)_2$ + NAC combination at 24 h, 48 h, and 72 h using the Wilcoxon signed-rank test

Pair	Time Points Compared	N	Mean (mm)	SD (mm)	Wilcoxon (p)
Pair 1	24 h vs 48 h	24	16.61 vs 15.41	0.30 vs 0.19	0.001
Pair 2	24 h vs 72 h	24	16.61 vs 15.44	0.30 vs 0.15	0.001
Pair 3	48 h vs 72 h	24	15.41 vs 15.44	0.19 vs 0.15	0.568

Note: Values are mean ± SD (mm); n = 24 plates. Within-group time comparisons were performed using the Wilcoxon signed-rank test. Statistical significance was set at $p < 0.05$.

The $Ca(OH)_2$ + NAC combination showed a gradual decrease in the inhibition zone diameters over the evaluation period. When the temporal measurements were compared, statistically significant differences were observed between 24 and 48 h and between 24 and 72 h ($p < 0.05$), with the largest inhibition zone recorded at 24 h, as shown in Table **7**. In contrast, no statistically significant differences were found between the measurements obtained at 48 and 72 h ($p > 0.05$), indicating that the antibacterial activity of the combination remained relatively stable after 48 h. These results were obtained using the Wilcoxon signed-rank test (Table 7).

Temporal Comparison of Antibacterial Activity by Substance

Table 8: Summary of inhibition zone diameters (mean values in mm) at 24 h, 48 h, and 72 h

Substances	24 h (mm)	48 h (mm)	72 h (mm)
$Ca(OH)_2$	20.71	20.61	20.29
NAC	16.57	15.65	15.55
$Ca(OH)_2$ + NAC	16.61	15.41	15.44

Note: Mean inhibition zone diameter (mm); *n* = 24 plates per time point. $Ca(OH)_2$ = calcium hydroxide; NAC = N-acetylcysteine.

In the overall comparative analysis of the temporal behavior of the evaluated substances, calcium hydroxide consistently showed the largest inhibition zone diameter against *Enterococcus faecalis* at all evaluation time points, although with a slight progressive decrease in mean values over time.

N-acetylcysteine and the $Ca(OH)_2$ + NAC combination showed similar inhibition zone diameters to each other and consistently lower values than calcium hydroxide, with a mild decrease and a relatively stable pattern after 48 h.

Overall, none of the tested substances exceeded the antibacterial activity of calcium hydroxide at any of the three evaluated time points, as summarized in Table 8. Statistical comparisons were based on related-sample analyses according to data distribution criteria.

These findings describe the comparative and temporal antibacterial behavior of the tested substances under the defined experimental conditions.

Discussion

Calcium hydroxide showed the largest inhibition zone diameters at all three evaluation time points, which is consistent with its well-recognized antimicrobial activity in agar diffusion *in vitro* models. This behavior is mainly attributed to its high pH and the release of hydroxyl ions, which create an unfavorable environment for bacterial survival by disrupting cell membranes and denaturing bacterial proteins (Gomes *et al*., 2002) [7]. However, although its antimicrobial efficacy has been widely documented, a systematic review reported that calcium hydroxide remains one of the most commonly used intracanal medicaments, yet its ability to completely eliminate bacteria from root canals is limited when evaluated using culture techniques (Sathorn *et al*., 2007) [17].

It has also been documented that *Enterococcus faecalis* can survive under highly alkaline conditions, particularly when in the stationary phase or under nutritional stress, which restricts the prolonged antimicrobial action of this material (Portenier *et al*., 2003) [15]. This behavior is consistent with the statistical findings obtained through related-sample tests (Wilcoxon signed-rank test and paired Student's t-test), in which significant differences between groups were observed ($p < 0.05$).

Similarly, previous reports have shown that calcium hydroxide demonstrates greater antibacterial activity against *Enterococcus faecalis* compared with other intracanal medicaments evaluated through *in vitro* assays, as evidenced by larger inhibition zones in agar, which supports the findings of the present study (Govindaraju *et al*., 2021, S158) [8].

Temporal analysis revealed a progressive reduction in the antibacterial effect of calcium hydroxide between 24 and 72 h, reflected by decreasing inhibition zone diameters. This pattern may be explained by the fact that the antimicrobial activity of calcium hydroxide depends on hydroxyl ion dissociation and sustained alkaline pH levels. Experimental studies have demonstrated that ion release and pH maintenance may vary over time depending on the physicochemical characteristics of the system, directly influencing material activity (Carvalho *et al*., 2016) [4]. Accordingly, the greater inhibitory effect observed at 24 h may be related to higher initial hydroxyl ion availability, whereas the reductions observed at 48 and 72 h likely reflect a gradual decrease in antibacterial activity.

In the present study, N-acetylcysteine showed smaller inhibition zones than calcium hydroxide at all evaluated time points, although its activity remained relatively stable throughout the observation period. These findings indicate that NAC exhibits moderate antimicrobial activity under *in vitro* conditions, without reaching the antibacterial effectiveness observed for calcium hydroxide. This behavior may be explained by evidence that N-acetylcysteine is more effective in inhibiting and disrupting *Enterococcus faecalis* biofilms than in eliminating planktonic bacterial cells, which may limit its apparent performance in agar diffusion models based on inhibition zone (Quah *et al*., 2012) [16].

In agar diffusion assays, inhibition zone size depends not only on antimicrobial potency but also on diffusion capacity in the medium and physicochemical properties such as compound solubility and viscosity in agar (Balouiri *et al*., 2016) [3]. Reported outcomes may also vary according to the technique used (disc versus well diffusion), due to differences in agent release and diffusion dynamics within the medium, which can modify the observed inhibition zones (Valgas *et al*., 2007) [23]. In addition, for medicaments evaluated using these assays, the vehicle plays an important role in active compound release and diffusion; substances such as glycerin, propylene glycol, and polyethylene glycol may alter antimicrobial behavior in agar diffusion tests (Nalawade *et al*., 2016) [12].

Regarding the $Ca(OH)_2$ + N-acetylcysteine combination, the results did not demonstrate a statistically significant synergistic antibacterial effect against *Enterococcus faecalis* based on inhibition zone measurements. Observed values were comparable to those obtained with NAC alone, with no significant increase across the three evaluated time points. This finding may be explained by the fact that N-acetylcysteine has been described as having limited direct

antimicrobial activity compared with calcium hydroxide, with its primary action directed toward interference with biofilm structure and bacterial adhesion rather than a direct bactericidal effect against *E. faecalis* (Ulusoy *et al*., 2016) [22].

Conclusions

Under the evaluated *in vitro* conditions, calcium hydroxide demonstrated the highest antibacterial activity against *Enterococcus faecalis* at all analyzed time points, as evidenced by the largest inhibition zone diameters. Its antibacterial activity showed a progressive decrease between 24 and 72 h, indicating that its inhibitory effect is more pronounced during the initial phases of contact with the microorganism.

N-acetylcysteine exhibited moderate antibacterial activity against *Enterococcus faecalis*, with smaller inhibition zones compared with calcium hydroxide, but with a relatively stable behavior over time. The combination of calcium hydroxide with N-acetylcysteine did not demonstrate a synergistic antibacterial effect, as the values obtained were comparable to those of N-acetylcysteine used alone at all three evaluated time points.

Overall, the findings of this study indicate that calcium hydroxide remains the most effective material within the experimental model used, while NAC did not provide an additional advantage when used in combination with this compound.

Limitations

The present study has several limitations that should be considered when interpreting the results. As an *in vitro* study, the experimental conditions do not fully reproduce the clinical environment of the root canal system, where multiple biological and physicochemical factors interact. Additionally, antibacterial activity was evaluated using the agar diffusion test, which depends on the diffusion capacity of the materials in the medium and does not allow differentiation between bactericidal and bacteriostatic effects. Furthermore, the evaluation was conducted exclusively against *Enterococcus faecalis* in its planktonic form, without considering mature biofilms, which exhibit greater resistance and clinical relevance. Finally, potential cytotoxic effects and interactions with periapical tissues were not analyzed; therefore, the results should not be directly extrapolated to clinical practice.

Future directions

It is recommended that future studies employ experimental models other than agar diffusion in order to evaluate the antibacterial activity of materials under conditions that more closely resemble the clinical environment. Additionally, the incorporation of specific methodologies for biofilm assessment is suggested, along with expanding the analysis through the use of different concentrations and exposure times in alternative experimental models.

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