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Evaluating Iron Oxide Nanoparticles As An Alternative To Calcium Hydroxide Against E. Faecalis: An In Vitro Study

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Abstract
Background: *Persistent root canal infections, especially those caused by Enterococcus faecalis, pose a significant challenge in endodontic therapy. Traditional medicaments like calcium hydroxide (CH) have shown limited efficacy against resistant strains. Recently, iron oxide nanoparticles (IONPs) have emerged as promising antimicrobial agents.*
Aim: *This study aimed to compare the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of IONPs and CH, both alone and in combination, against E. faecalis.*
Methods: *Medications were divided into three groups: IONPs alone, CH alone, a combination of IONPs and CH. MIC and MBC were determined using standard dilution and culture methods. The zone of inhibition was evaluated using agar well diffusion. Statistical analysis was performed using ANOVA and Tukey's post hoc test.*
Results: *The combination of IONPs and CH demonstrated the highest antibacterial efficacy, with significant inhibition of E. faecalis growth at a lower concentration (2.5 mg/mL). CH showed MIC at 5 mg/mL, while IONPs required 10 mg/mL. The combination group also produced the largest inhibition zones across all concentrations tested.*
Conclusion: *IONPs in combination with CH offer enhanced antibacterial efficacy against E. faecalis and may be a superior alternative to CH alone in endodontic disinfection protocols.*
Keywords: *Iron oxide nanoparticles, calcium hydroxide, Enterococcus faecalis, intracanal medicament, paediatric dentistry.*

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I. Introduction

Dental caries is a widespread condition affecting populations worldwide and a primary contributor to endodontic pathologies.[1] When carious lesions progress deeply into the dentin, they allow bacterial infiltration into the pulp, potentially resulting in pulpitis, necrosis, and apical periodontitis.[2] One of the key bacterial agents identified in persistent endodontic infections is *Enterococcus faecalis*, known for its resistance to conventional treatment methods and its ability to thrive in nutrient-deprived environments.[3]

Successful endodontic therapy hinges on the thorough elimination of microbial flora from the root canal system.[4] This is primarily achieved through mechanical debridement and chemical irrigation, often supplemented with intracanal medicaments that serve to neutralize residual bacteria.[5] An ideal medicament should provide long-lasting antimicrobial action. At the same time, it should minimise irritation to the apical periodontium. This property is particularly important during multi-visit treatments, where the medicament must penetrate the dentinal tubules and eliminate any persistent microorganisms. The most commonly used intracanal medicament is calcium hydroxide. However, calcium hydroxide shows limited effectiveness against resistant species like *Enterococcus faecalis* and *Candida albicans*. Its high pH can cause dentin brittleness and weaken the tooth structure with prolonged use. Residues left in the canal may interfere with sealing and cause crown discolouration, especially in anterior teeth.[6]

Nanotechnology offers new avenues in the fight against persistent endodontic pathogens. Due to their small size and large surface area-to-volume ratio, nanoparticles possess unique chemical and physical properties that enhance their antimicrobial capabilities.[7] Among these, iron oxide nanoparticles (IONPs) have gained attention due to their ability to generate reactive oxygen species (ROS), penetrate biofilms, and maintain a favorable safety profile.[8]

Only a limited number of studies have explored the application of iron oxide nanoparticles in the medical and dental fields. This is particularly relevant in paediatric dentistry, where persistent infections in immature permanent teeth compromise root development. This study was designed to assess and compare the antibacterial efficacy of IONPs, CH, and their combination against *E. faecalis*, focusing on determining MIC, MBC, and zones of inhibition to establish their potential for clinical use.

II. Materials And Methods

Study Design

This was an *in vitro* experimental study conducted to evaluate the antimicrobial activity of different intracanal medicaments such as iron oxide nanoparticles, calcium hydroxide, and their combination against *E. faecalis*.

Preparation of Suspensions

IONPs were prepared by mixing 400 mg in 10 mL of deionized water and homogenized with an ultrasonic homogenizer. A calcium hydroxide suspension was prepared in a similar manner. For combination groups, equal volumes of IONP and CH suspensions were mixed.

Preparation of Bacterial Suspension

Bacterial cultures were incubated in BHI broth at 37°C for 24 hours. A 0.5 McFarland standard (equivalent to 1-1.5 × 10^8 CFU/mL) was used.

Minimum inhibitory concentration and Minimum bactericidal concentration Testing

Serial dilutions (40 mg/mL to 2.5 mg/mL) were prepared in test tubes. Three main concentrations for this study were 2.5 mg/mL, 5 mg/mL, and 10 mg/mL. Each tube received 0.1mL of bacterial suspension and was incubated at 37°C for 24 hours. MIC was determined based on turbidity. The MIC was determined by observing turbidity in bacterial suspensions after 24 hours of incubation. To determine MBC, samples from the MIC assay that exhibited no turbidity were sub-cultured onto Enterococcus-selective agar and incubated. Bactericidal activity was confirmed by the absence of colony growth. To avoid any false results, five tests were done for each concentration of each group of medication.

Ethical clearance:

This study was approved by the Institutional Ethics Committee [XXX], and all procedures were conducted in accordance with the Declaration of Helsinki.

Statistical Analysis

Data were analysed using SPSS version 28.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA and Tukey's post hoc test were used for group comparisons, with significance set at $p \leq 0.05$.

III. Results

Minimum Inhibitory Concentration (MIC)

The combination group (IONPs + CH) demonstrated the greatest antimicrobial activity, achieved complete inhibition of *E. faecalis* at the lowest tested concentration of 2.5 mg/mL. Calcium hydroxide alone showed inhibitory activity starting at 5 mg/mL, while IONPs alone required a concentration of 10 mg/mL to achieve complete inhibition. These findings suggest a synergistic antibacterial effect when IONPs and CH are combined. One-way ANOVA revealed statistically significant differences in MIC among the three groups ($p \leq 0.05$). (Figure 1-3)

FIGURE 1: Test-tubes with IONPs showing no turbidity only at 10 mg/mL concentration

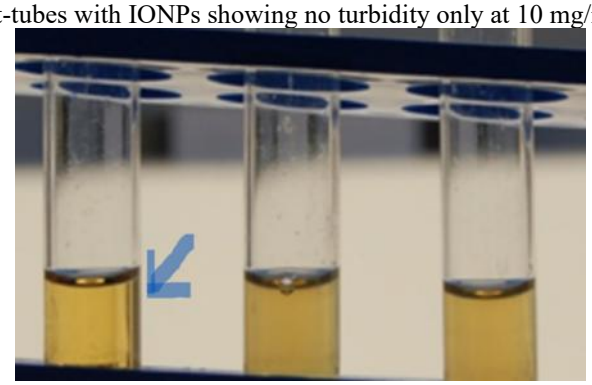


FIGURE 2: Test-tubes with combination of IONPs and CH showing no turbidity at all three concentrations

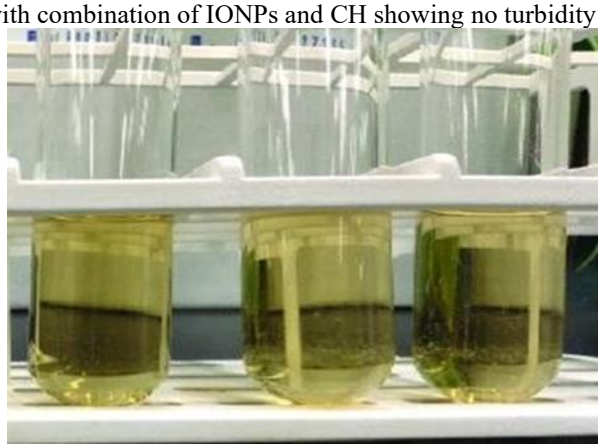
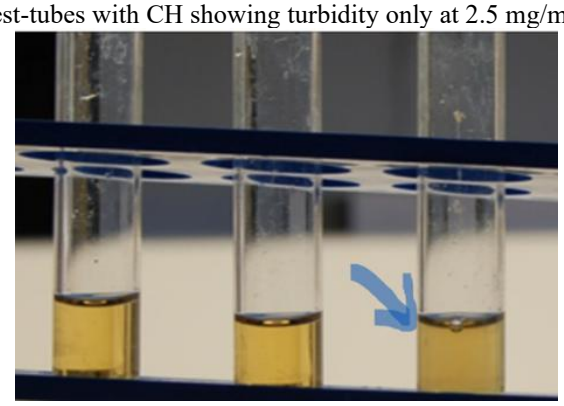


FIGURE 3: Test-tubes with CH showing turbidity only at 2.5 mg/mL concentration



Minimum Bactericidal Concentration (MBC)

IONPs + CH demonstrated bactericidal effects at all three concentrations (2.5, 5, and 10 mg/mL), whereas CH alone demonstrated this effect at 5 and 10 mg/mL. IONPs alone showed bactericidal action at 10 mg/mL, indicating reduced bactericidal efficiency compared to the combination.

Zone of Inhibition

At 2.5 mg/mL, the combination group exhibited the highest inhibition zone, followed by CH and IONPs. At 5 mg/mL, zones were IONPs + CH > IONPs > CH. At the highest concentration of 10 mg/mL, the combination group again showed the largest zone of inhibition, followed by CH and IONPs. Statistically significant differences were observed among all groups at each concentration ($p \leq 0.05$).

Intergroup Comparisons

Tukey's post hoc test revealed significant differences between 10 mg/mL and 2.5 mg/mL in all three groups. The IONPs + CH group showed a statistically significant improvement in zone of inhibition compared to CH or IONPs alone, particularly at lower concentrations. These results reinforce the enhanced efficacy of the combined formulation. (Table 1-2)

Table 1: Intergroup comparison of inhibition zones of test materials against *E. faecalis* at different concentrations

Concentrations mg/mL	IONPs Mean (mm)	IONPs + CH Mean (mm)	CH Mean (mm)	*p* value
2.5	4.86 ± 0.00	5.96 ± 0.00	5.06 ± 0.00	0.001
5	7.6 ± 0.58	9.6 ± 0.60	7.36 ± 0.36	0.002
10	9.96 ± 1.12	13.76 ± 1.00	11.06 ± 1.10	0.010

One-way ANOVA test; indicates significant difference at $p \leq 0.05$

Table 2: Pairwise intergroup comparison of inhibition zones of test materials against *E. faecalis* at different concentrations

Concentrations mg/mL	IONPs *p* value	IONPs + CH *p* value	CH *p* value
2.5 vs 5	0.078 (NS)	0.028	0.07 (NS)
2.5 vs 10	0.03	0.04	0.016
5 vs 10	0.067 (NS)	0.060 (NS)	0.020

Tukey's post hoc test; indicates significant difference at $p \leq 0.05$; NS - Nonsignificant

IV. Discussion

Untreated dental caries provides a niche for microbial invasion, leading to pulpal damage and the need for endodontic treatment. The predominant microorganisms responsible for such infections are anaerobic bacteria, with *Enterococcus faecalis* recognized as the most persistent species. Other commonly implicated pathogens include *Streptococcus mitis*, *Streptococcus sanguinis*, various *Actinomyces* species, *Fusobacterium*, *Spirochetes*, and *Prevotella* species.[9]

In paediatric patients, these infections present an additional challenge. Necrotic pulps in immature permanent teeth often compromise root development, leaving thin dentinal walls that are prone to fracture. Effective intracanal medicaments are therefore critical in children, not only to eradicate resistant microorganisms such as *E. faecalis* but also to promote an environment conducive to continued root maturation (apexogenesis and apexification).

Calcium hydroxide ($Ca(OH)_2$) is commonly employed as an intracanal medicament in endodontics due to its ability to release hydroxyl ions and raise the pH of the root canal environment. This disrupts bacterial membranes, enzymes, and DNA, thereby exerting antimicrobial effects. However, its effectiveness against resistant organisms such as *E. faecalis* and *Candida albicans* remains debated. Mattigatti *et al*.[10] reported lower antimicrobial efficacy compared with chlorhexidine, and Chen *et al*.[11] also found $Ca(OH)_2$ ineffective against *E. faecalis* biofilms.

Nanoparticulate compounds are increasingly explored for their antibacterial potential.[12] They act mainly by disrupting bacterial membranes, altering electrochemical gradients, and interfering with essential cellular functions, ultimately leading to cell death.[13] Iron oxide nanoparticles, in particular, have shown potential in disrupting biofilms and modulating bacterial activity.[12]

Al-Mallah *et al*.[14] in 2021 found that IONPs alone enhanced bacterial growth at high concentrations and showed bacteriostatic effects at lower concentrations. Similarly, Torres-Gómez *et al*.[15] reported low antibacterial activity of IONPs at concentrations above 0.07 mg/mL. In contrast, the present study demonstrated that combining IONPs with calcium hydroxide enhanced antibacterial activity against *E. faecalis*, indicating a synergistic effect. This may be explained by the high pH created by calcium hydroxide, which facilitates ROS-mediated bacterial killing by IONPs.

Previous studies have shown *E. faecalis* to be resistant to high pH environments and capable of surviving calcium hydroxide exposure.[16],[17] In contrast, the addition of nanoparticles enhances penetration into dentinal tubules and disrupts biofilms, mechanisms that are critical for eliminating persistent infections.[13]

Additionally, the zone of inhibition data reinforces the superior efficacy of the combination, particularly at lower concentrations. The results align with Bukhari *et al*.[18], who observed enhanced efficacy of IONPs when combined with oxidizing agents like hydrogen peroxide.

An ideal endodontic medicament should combine strong antimicrobial properties with selective toxicity, ensuring minimal harmful effects on periradicular tissues.[19] The concentration of nanoparticles is a key determinant of their cytotoxic effects on cells.[20] At lower concentrations, cells can efficiently eliminate nanoparticles through the normal phagocytosis process. However, at higher concentrations, this clearance mechanism becomes impaired, leading to potential adverse health effects over time.[21] IONPs may have relatively weak antimicrobial activity against certain strains and limited biocompatibility with eukaryotic cells.[22] Although concerns exist regarding the cytotoxicity of nanoparticles, studies have shown IONPs to have acceptable biocompatibility at lower concentrations.[14] Nonetheless, further *in vivo* research is necessary before clinical translation.

V. Conclusion

The combination of iron oxide nanoparticles and calcium hydroxide demonstrated superior antibacterial efficacy against *Enterococcus faecalis* compared with either agent alone. This synergistic effect supports its potential as an intracanal medicament, warranting further *in vivo* and clinical evaluation.

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