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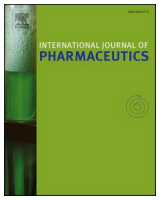

Novel strategies enhancing endodontic disinfection: Antibacterial biodegradable calcium hydroxide nanoparticles in an ex vivo model

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ABSTRACT

Due to the high failure rates associated to endodontic disinfection, this study aimed to investigate the antibacterial properties of poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with $Ca(OH)_2$ for endodontic disinfection procedures. $Ca(OH)_2$ NPs production and physicochemical characterization were carried out as well as multiple antibacterial tests using three bacterial strains and an ex vivo model of endodontic infection with extracted human teeth. Agar diffusion test and broth dilution determined the inhibition growth zones ($n = 5$) and the minimal inhibitory concentration (MIC, $n = 5$), respectively. Cell viability was assessed using Live/Dead staining with confocal microscopy ($n = 5$). Data was analysed using ANOVA followed by post-hoc analysis. After 24 h of incubation, $Ca(OH)_2$ NPs demonstrated a MIC of 10 μg/mL for *Porphyromonas gingivalis* ($p < 0.001$) and *Enterococcus faecalis* and 5 μg/mL for *Fusobacterium nucleatum* ($p < 0.001$). Although the agar diffusion test did not exhibit any inhibition area for $Ca(OH)_2$ nor for $Ca(OH)_2$ NPs, this was probably due to the buffering effect of the agar medium. However, the antibacterial capacity was confirmed in an ex vivo model, where instrumentalized teeth were infected with *Enterococcus Faecalis* and treated after 28 days of culture. A significant reduction in bacterial metabolic activity was confirmed for $Ca(OH)_2$ NPs (40 % reduction with a single dose) and confirmed by Live/Dead staining. In conclusion, $Ca(OH)_2$-loaded PLGA NPs present promising antibacterial efficacy for endodontic disinfection procedures.

1. Introduction

The American Association of Endodontics (AAE) reports that over 15.1 million root canal therapy procedures are performed annually in the United States (Kojima et al., 2004). While success rates for this procedure can be as high as 95 %, these rates decrease in cases diagnosed with necrotic non-vital pulp tissue, which is often caused by pathogenic microorganisms (Gulabivala and Ng, 2023; Burns et al., 2022). The root canal system possesses a complex architecture and the location of microorganisms in isthmuses, accessory and lateral canals, and dentinal tubules can make complete eradication of bacteria highly challenging (Trope and Bergenholtz, 2002; Ricucci and Siqueira, 2010; Nair et al., 2005; Vera et al., 2012; Narayanan and Vaishnavi, 2010). Even with mechanical debridement and chemical cleaning, completely eliminating bacteria from the root canal system is difficult to achieve (Siqueira and Rôças, 2022; Hulsmann et al., 2005). Intracanal medications are used between visits to decrease bacterial load, but their efficacy is limited, especially for bacteria residing in anatomically complex areas

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such as the dentinal tubules (Heling et al., 1992). Despite advances in endodontics, success rates have remained similar over the decades, indicating that current intracanal medications have limitations and cannot achieve the desired effect (Burns et al., 2022; Goldberg et al., 2020).

Calcium hydroxide ($Ca(OH)_2$) is the most commonly used to supplement chemomechanical preparation to enhance disinfection within the root canal system (Roig-soriano et al., 2022). Its antibacterial activity is due to its alkalinity, which produces highly oxidant hydroxyl ions that act on the bacterial cell wall, resulting in damage to the cytoplasmic membrane through protein denaturation as well as DNA damage (Prathita et al., 2019). In addition, a high basic pH must be maintained to sustain the hydroxyl ions antibacterial activity, which alters the pH gradient of the cytoplasmic membrane, leading to protein denaturation (Mohammadi and Dummer, 2011). $Ca(OH)_2$ elevated pH also damages the organic components of the cytoplasmic membrane, inhibits nutrient delivery, and causes DNA strand splitting, ultimately leading to DNA replication inhibition and harmful mutations, disrupting cellular activity (Imlay and Linn, 1988). However, evidence suggests that all three mechanisms of damage to the bacterial cytoplasmic membrane, protein denaturation and DNA damage, may occur simultaneously, and it is challenging to establish a chronological order in which these events take place (Imlay and Linn, 1988; Tamara et al., (2018), 2022 (2022).). $Ca(OH)_2$ uncoupling into calcium and hydroxyl ions is highly dependent on the vehicle used for the application, affecting the pH value and the degree of penetration inside the tubules (Pacios et al., 2004). Therefore, a suitable vehicle should allow high penetration through the tubules and a slow and steady release of calcium and hydroxyl ions with no undesirable effects on the initiation of hard tissue formation. In terms of intracanal medications there is still research and patent work to be undertaken, specially regarding the precise dose, treatment duration, dispensing method and delivery vehicle (Shabbir et al., 2022). Although different vehicles have been used to administer $Ca(OH)_2$, such as water-soluble, viscous, and oil-based vehicles (Mohammadi and Dummer, 2011), all of them carry a certain degree of adverse effects, ultimately affecting $Ca(OH)_2$ clinical performance (Mohammadi and Dummer, 2011). Therefore, there is an urgent medical need for a suitable and safe vehicle able to maintain $Ca(OH)_2$ properties during a prolonged time.

Additionally, in endodontics, $Ca(OH)_2$ paste has also been utilized in the majority of regenerative endodontic procedures ahead of the triple antibiotic paste, according to a study conducted in 13 countries (Hatipoğlu et al., 2023). This may be because the triple antibiotic has unfavorable side effects like staining teeth. Therefore, in cases of regenerative endodontic procedure, the American Association of Endodontics and the European Endodontic Society currently advise using $Ca(OH)_2$ paste.

Over the years, mixed results have been obtained regarding the antibacterial effectiveness of $Ca(OH)_2$ in eliminating bacteria from the root canal system (Han et al., 2001; Shuping et al., 2000; Estrela et al., 2001; Liewehr, 2001). While many studies reported the high efficacy of $Ca(OH)_2$ as an antibacterial agent, others documented its inefficacy in eradicating bacteria and their by-products, especially in conditions similar to the clinical environment (Weiger et al., 2002; Gluskin et al., 2020; Roig-soriano et al., 2022). Furthermore, $Ca(OH)_2$ has been shown to be ineffective in removing microorganisms that settle inside the dentinal tubules (Heling et al., 1992) because it cannot directly contact the bacteria inside the tubules, which is essential for exerting its antibacterial effects (Jr and Lopes, 1999; Shaaban et al., 2023). This is likely due to the limited penetration of $Ca(OH)_2$, which can only reach up to 28 and 126 μm inside the dentinal tubules, whereas bacteria can penetrate up to 400 μm in some circumstances (Info, 2017; Taschieri et al., 2014).

To overcome these challenges without using new molecules that would require an extended follow-up at clinical level and improve the efficacy of $Ca(OH)_2$, recent drug delivery procedures have focused on nanotechnological approaches able to load active compounds and deliver them in a prolonged manner retaining the pharmaceutical properties (Bhatia, 2016; Kishen, 2012; Esteruelas et al., 2021; Fernandes et al., 2022; Diogo et al., 2023). Among several nanoscopic systems, biodegradable polymeric nanoparticles (NPs) have shown to possess suitable properties and, especially poly-(lactic-co-glycolic) acid (PLGA), is accepted by the main regulatory agencies (Sánchez-López et al., 2020; Galindo et al., 2022; Esteruelas et al., 2022). PLGA NPs may be able to decrease potential adverse effects, and prevent or reduce the buffering effect produced by dentin and hydroxyapatite, as well as maintain the high alkaline pH value in which $Ca(OH)_2$ can retain its antibacterial capability (Mohammadi and Dummer, 2011; Diogo et al., 2023). In a previous study (Elmsmari et al., 2021), our research team successfully optimized $Ca(OH)_2$-loaded PLGA NPs ($Ca(OH)_2$ NPs that displayed an extended drug release profile compared to free $Ca(OH)_2$ and significantly greater infiltration inside dentinal tubules of extracted teeth in contrast to free $Ca(OH)_2$. The current study aims to examine the antibacterial capacity of this optimized $Ca(OH)_2$ NPs against three bacterial strains in order to elucidate the suitability of $Ca(OH)_2$ NPs for endodontic disinfection. The null hypothesis was that there was no difference in the antibacterial activity between $Ca(OH)_2$, $Ca(OH)_2$ nanoparticles, and control group against endodontic bacteria.

2. Materials and methods

This study was conducted with the approval of the ethical committee with code (END-ELB-2020–01) to assess the antibacterial effect of $Ca(OH)_2$ NPs for endodontics disinfection procedures. The antibacterial efficacy of the NPs was evaluated through several antibacterial tests using three bacterial strains: *Porphyromonas gingivalis* (Pg) (ATCC 33277), *Fusobacterium nucleatum* (Fn) (ATCC 25586), and *Enterococcus faecalis* (Ef) (ATCC 19433) (Alghamdi, 2020; Tomazinho et al., 2007; Eduardo and De, 2002), in accordance with the guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI) (M07-A10, 2015; M02-A12, 2015).

2.1. Preparation of calcium hydroxide nanoparticles

The preparation and characterization of $Ca(OH)_2$ NPs has been carried out as described elsewhere (Elmsmari et al., 2021). Briefly, $Ca(OH)_2$ NPs were prepared using the solvent displacement method, and they were optimized using a central composite design. The optimized $Ca(OH)_2$ NPs were measured using photon correlation spectroscopy (PCS) to determine the average size and polydispersity index (PI) after 1:10 dilution at 25 °C, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). In addition, transmission electron microscope observation was carried out after negative staining using uranyl acetate (2 %) and measurement of the diameter of the $Ca(OH)_2$ NPs was carried out using ImageJ software (Sánchez-López et al., 2017; Cano et al., 2018; Sánchez-López et al., 2018). Subsequently, the antibacterial capacity was assessed using several methods (Fig. 1).

2.2. Minimal inhibitory concentration (MIC)

To determine the minimal inhibitory concentration (MIC), a broth microdilution method was used according to the guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI) (M07-A10, 2015; M02-A12, 2015) (Fig. 2A). Serial dilutions were carried out (1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200; 1:500) for both the $Ca(OH)_2$ nanoparticles (NPs) and Calcium hydroxide 98 % extra pure ACROS Organics™ (Fisher Scientific, USA) mixed with Milli-Q water at the same concentrations, starting at a concentration of 1 mg/mL. Subsequently, 100 μL from each dilution was added to a 96-Well Microplate (Fisher Scientific, USA).

The bacterial suspension turbidity for the three bacterial strains *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Enterococcus faecalis* was adjusted to an optical density of 0.1 (equivalent to 0.5

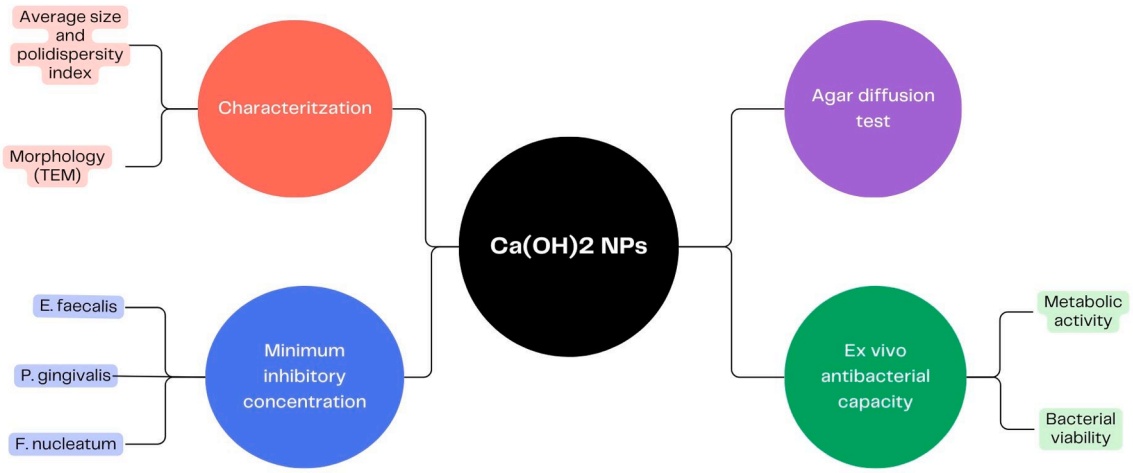

Fig. 1. Flowchart of the experiments carried out in the present study.

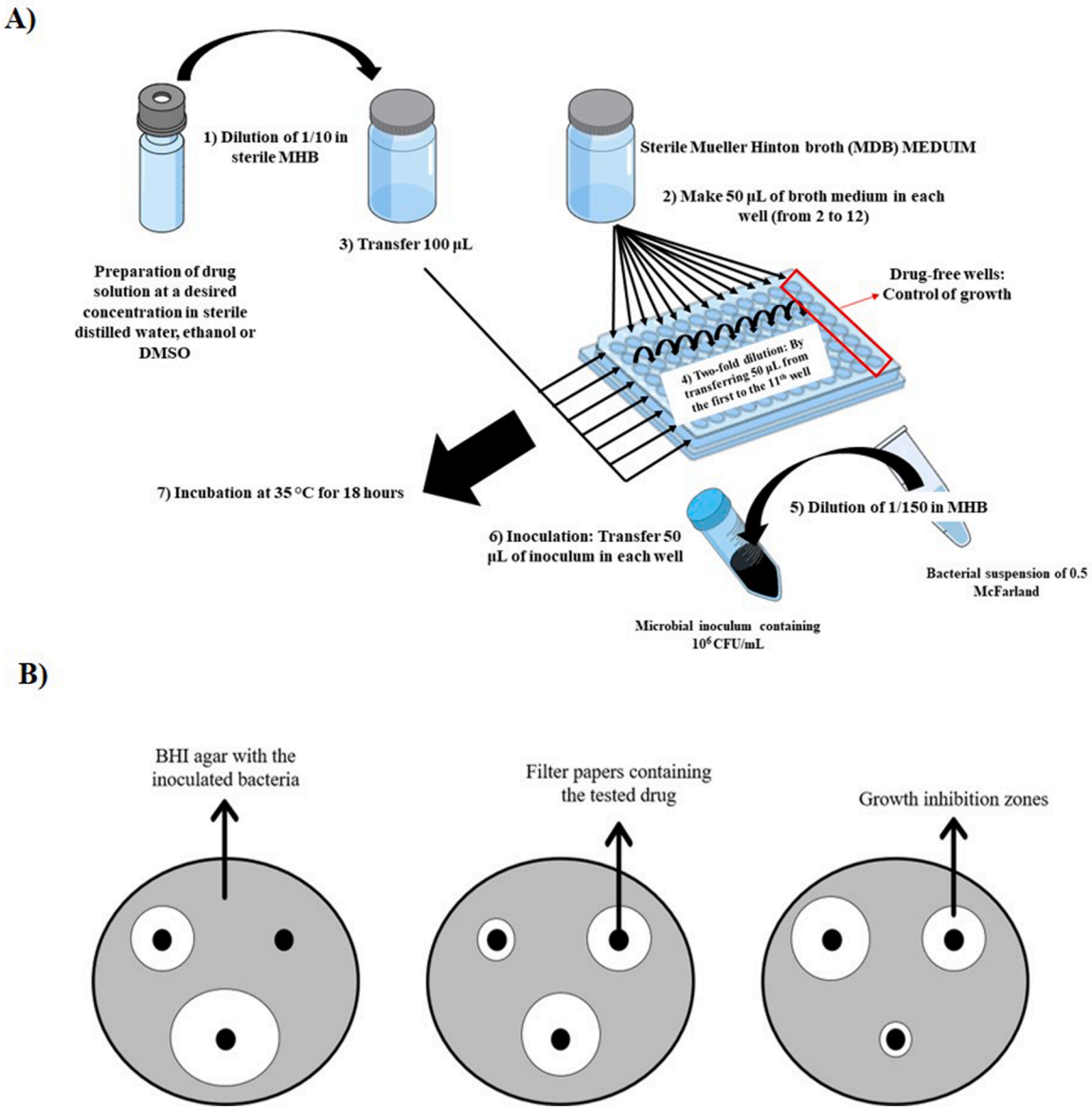

Fig. 2. Scheme of the bacterial assays developed. A) Microdilutions used for antibacterial assesments as recommended by CLSI protocol and B) Agar diffusion test protocol.

McFarland) by adding brain heart infusion (BHI, Condalab, Spain) media and measuring absorbance in a spectrophotometer (Cary 60 UV–Vis Spectrophotometer, Agilent, USA) at a wavelength of 600 nm. Then, each diluted well was inoculated with 100 µL of the adjusted bacterial suspensions (Jarkhi et al., 2022; Afdilla et al., 2022). All experiments were performed by triplicate.

After 24 h, bacterial growth was assessed in terms of turbidity, which was measured at 600 nm using a plate spectrophotometer (Infinite M Nano, TECAN, Switzerland). Wells containing the broth medium only were used as a negative control to examine the equipment and medium sterility. Additionally, some wells containing the broth growing medium and bacteria were used as a positive control to test the growing ability of the medium. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited bacterial growth (M07-A10, 2015), and the significance level was determined.

2.3. In vitro antibacterial therapeutic efficacy

To measure the growth inhibition zones, the agar diffusion test was used (n = 3) (Jarkhi et al., 2022; Afdilla et al., 2022). Brain heart infusion agar (BHI Agar) (Condalab, Spain) plates were prepared and inoculated with *Enterococcus faecalis*, which has a high prevalence in cases of necrotic endodontic infections (Jr et al., 2008; Lacevic et al., 2004; Gomes et al., 2005). The antibacterial properties of $Ca(OH)_2$ NPs, free $Ca(OH)_2$, and a control (1x PBS) were tested as described elsewhere (M02-A12, 2015). The plates were incubated under anaerobic conditions at 37 °C for 24 h, and the diameters of the inhibition growth were measured for each compound. All experiments were performed by triplicate (Fig. 2B).

2.4. Ex vivo antibacterial therapeutic efficacy

For the *ex vivo* model, extracted single-root teeth with straight canals were used, with the patient informed consent. No data associated with the patient was recorded concerning the extracted teeth. The extracted teeth were preserved in individual containers in a saline solution with 0.5 % sodium hypochlorite (NaOCl, Proclinic, Spain) (Elmsmari et al., 2021).

Root canal opening access was performed with a round diamond bur at high speed, and then the teeth were crowned, standardizing their length to 13 mm. Subsequently, root canals were instrumented with Reciproc Blue R25® (VDW, Germany) according to manufacturer instructions. 4.2 % NaOCl irrigation was used during the instrumentation process to allow the correct progression of the instrument inside the root canal and to simulate clinical conditions. Then, teeth were cut horizontally with a diamond blade and a clinical handpiece at 3 and 6 mm from the apex, dividing the teeth into coronal, medial, and apical blocks. Finally, teeth were randomly divided into three experimental groups (control, $Ca(OH)_2$ NPs, and $Ca(OH)_2$) and were sterilized by gas plasma (Sterrad, ASP, USA).

Sterile samples were incubated in BHI media for two days to ensure complete rehydration and sterility. Then, they were inoculated with *Enterococcus faecalis* at an optical density of 0.1 at a wavelength of 600 nm. Samples were incubated under anaerobic conditions for 28 days with media renewal every two days to develop a mature biofilm (Azim et al., 2016). Finally, treatments with $Ca(OH)_2$ NPs or $Ca(OH)_2$ at 1 mg/mL were applied.

2.5. In vitro metabolic activity

Resazurin assay was used to quantify the metabolic activity, as it is proportional to the number of bacteria and their viability (n = 5). The samples were washed twice with PBS and incubated with 300 µL of resazurin sodium salt at 30 µg/mL (Sigma-Aldrich, Spain) for 30 min at 37 °C. The absorbance was measured at 570 and 600 nm using 100 µL of each sample (Infinite M Nano, TECAN, Switzerland). The metabolic activity was normalized against the control, consisting of teeth incubated for 28 days without antibacterial treatment, and considered 100 % metabolic activity.

2.6. Visual observation of bacterial viability

In order to visually observe bacterial viability, it was assessed using Live/Dead staining with confocal microscopy observation. The samples were stained with LIVE/DEAD® BackLight™ Bacterial Viability Kit (Invitrogen™, Spain) according to the manufacturer's instructions. Adherent bacteria were stained with 300 µL of the dye-solution reagent for 15 min at 37 °C, and then washed with 1x PBS. Images were acquired at three random coronal, medial, and apical regions using a confocal laser microscope at 10x magnification (DMI8, Leica, Germany) using FITC and Texas Red excitation/emission filters for live and dead cells, respectively.

2.7. Statistical analysis

All experiments were performed in duplicate by independent operators and the same supervisor. The data were analyzed using GraphPad Prism v6 (Graphpad software, Inc). The mean and standard deviation (±) were used to present the data. Significant differences were assessed by applying either one or two-way ANOVA followed by post-hoc analysis, with a significance threshold of 0.05.

3. Results and discussion

3.1. Average size of calcium hydroxide nanoparticles

The optimized formulation of $Ca(OH)_2$ NPs was characterized by means of PCS obtaining a PI of 0.077 and an average size around 170 nm (Elmsmari et al., 2021). Moreover, surface charge was highly negative and NPs demonstrated the ability to encapsulate $Ca(OH)_2$. These results are in accordance with other formulations based on PLGA nanoparticles encapsulating different compounds (Esteruelas et al., 2022; Sánchez-López et al., 2017; Sánchez-López et al., 2018). Moreover, these data was supported by TEM nanoparticles observation where $Ca(OH)_2$ NPs were found to be spherical and non-aggregated (Fig. 3A). Moreover, the average size of the obtained images was analyzed and frequency distribution was calculated (Fig. 3B). An average size of 129 nm was calculated with all diameters being less than 200 nm, lower than the obtained using photon correlation spectroscopy. Moreover, frequency distribution obtained by both techniques show similar results with smaller nanoparticles in the case of TEM measurements (Fig. 3C, 3D).

Since a single parameter can not be used to adequately describe sample distribution, less than 200 nm particle size and spherical shape was confirmed using PCS and TEM investigations (López-Machado et al., 2021). Moreover, due to the measurement of the hydrodynamic ratio by PCS, TEM results provide slightly small nanoparticles since it constitutes a direct measurement (Sánchez-López et al., 2018). Moreover, pH was also measured obtaining an alkaline pH (9.65) that favours calcium hydroxide therapeutic efficacy.

3.2. Minimal inhibitory concentration (MIC)

After 24 h of incubation, the antibacterial activity of $Ca(OH)_2$ NPs and free $Ca(OH)_2$ was assessed against three bacterial strains, *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Enterococcus faecalis,* and was evaluated after several dilutions (Fig. 3). As can be observed on Fig. 4, both $Ca(OH)_2$ NPs and free $Ca(OH)_2$ showed statistical differences ($p < 0.0001$) against the control thus highlighting their antibacterial activity. Furthermore, at low concentrations (2 µg/mL for E. faecalis *and F. nucleatum* and 2 and 5 µg/mL *for P. gingivalis)*, due to $Ca(OH)_2$ NPs prolonged release, statistical differences were obtained.

As can be observed in Table 1, $Ca(OH)_2$ NPs had a MIC of 10 µg/mL

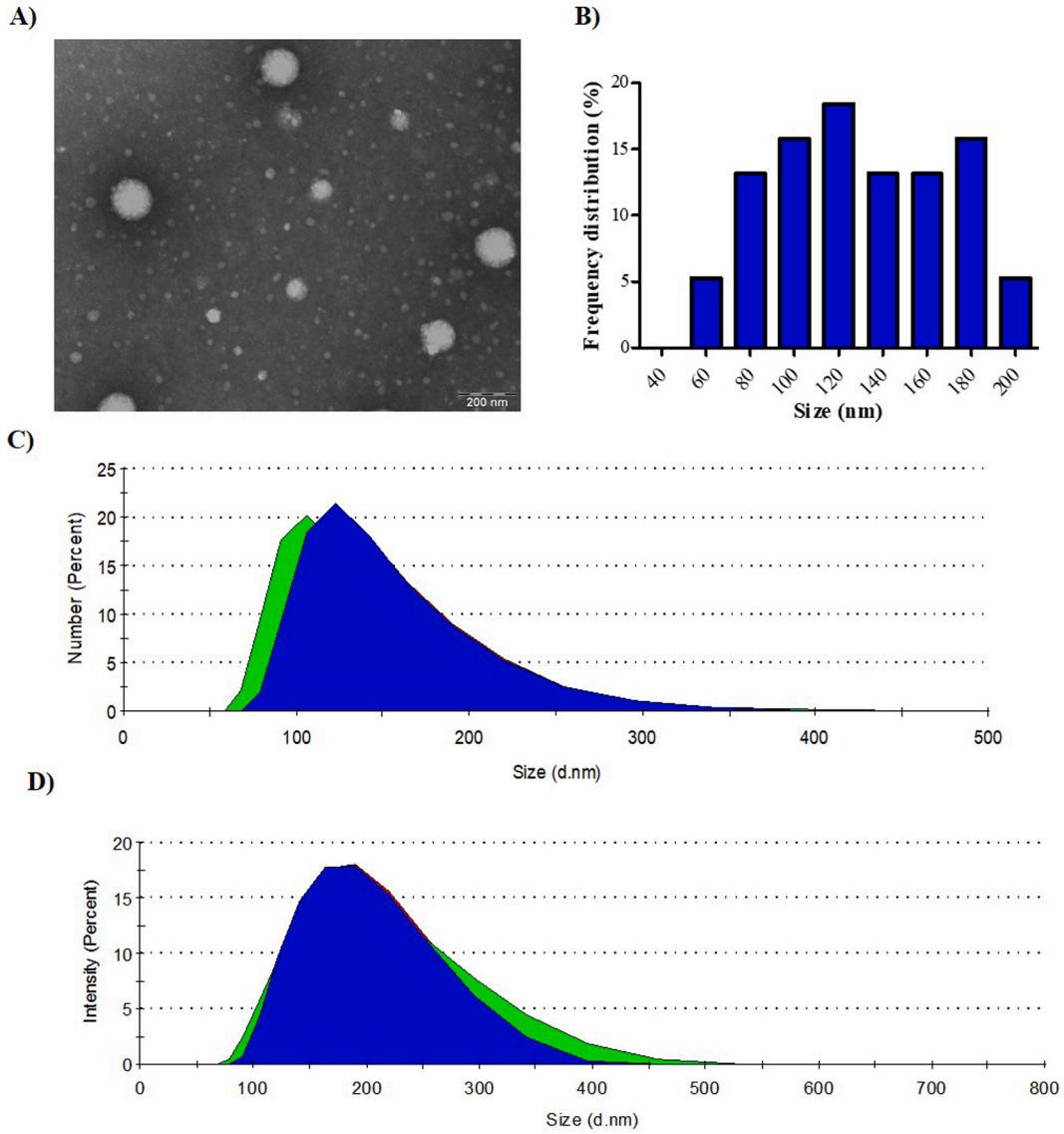

Fig. 3. Morphological and physicochemical characterization of calcium hydroxide nanoparticles. A) Transmission electron microscope results, B) Frequency distribution results of the average size of transmission electron images obtained, C) Frequency distribution in number of particles obtained using dynamic light scattering, D) Frequency distribution in intensity obtained using dynamic light scattering.

for *Porphyromonas gingivalis* and *Enterococcus faecalis* and 5 μg/mL for *Fusobacterium nucleatum*. $Ca(OH)_2$ had a MIC of 5 μg/mL for *Enterococcus faecalis* and was below 1 μg/mL for *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Table 1). Therefore, $Ca(OH)_2$ NPs showed the ability to retain $Ca(OH)_2$ antibacterial activity against different bacterial strains. Moreover, as previously demonstrated, $Ca(OH)_2$ NPs released the active compound in a prolonged manner showing higher MIC. The proposed $Ca(OH)_2$ NPs reduced the MIC concentration reported by previous studies against *Enterococcus faecalis* and *Porphyromonas gingivalis* (Sabrah et al., 2013) and, in addition, a previous attempt to produced $Ca(OH)_2$ NPs obtained a similar MIC reduction (6 μg/ml approximately). However, these NPs were not biodegradable (Bhardwaj et al., 2015).

In addition to the agar diffusion test, the minimum inhibitory concentration (MIC) of $Ca(OH)_2$ NPs was compared to commercial $Ca(OH)_2$ in terms of inhibiting bacterial growth for three bacterial species. It was noted that after 24 h of incubation, both $Ca(OH)_2$ NPs and the commercial $Ca(OH)_2$ could inhibit bacterial growth at all tested concentrations (Shrestha et al., 2010; Carpio-perochena et al., 2017; Fan et al., 2015; Wu et al., 2014; Kishen et al., 2008). Moreover, $Ca(OH)_2$ NPs MIC was higher than the commercial free $Ca(OH)_2$ thus confirming $Ca(OH)_2$ prolonged release from $Ca(OH)_2$ NPs.

3.3. Agar diffusion test

The antibacterial performance of $Ca(OH)_2$ and $Ca(OH)_2$ NPs was also evaluated using the agar diffusion test against *Enterococcus faecalis* strain, which measures the growth inhibition zones in an agar plate. PBS was used as a control. However, using this assessment, neither $Ca(OH)_2$ nor $Ca(OH)_2$ NPs induced an observable inhibition area (Fig. 5). Therefore, these results indicate that the active compound is not able to produce inhibition of the bacterial growth under the study conditions. This may be due to the fact that the agar diffusion test uses BHI agar media which possesses a neutral pH (pH 7.4 ± 0.2). Therefore, this assessment confirmed that either free $Ca(OH)_2$ or $Ca(OH)_2$ released from NPs both need a high pH environment in order to exert its effect due to the fact that the dissociation of calcium and hydroxyl radicals is necessary in order to obtain bacterial inhibition (Athanassiadis et al., 2007).

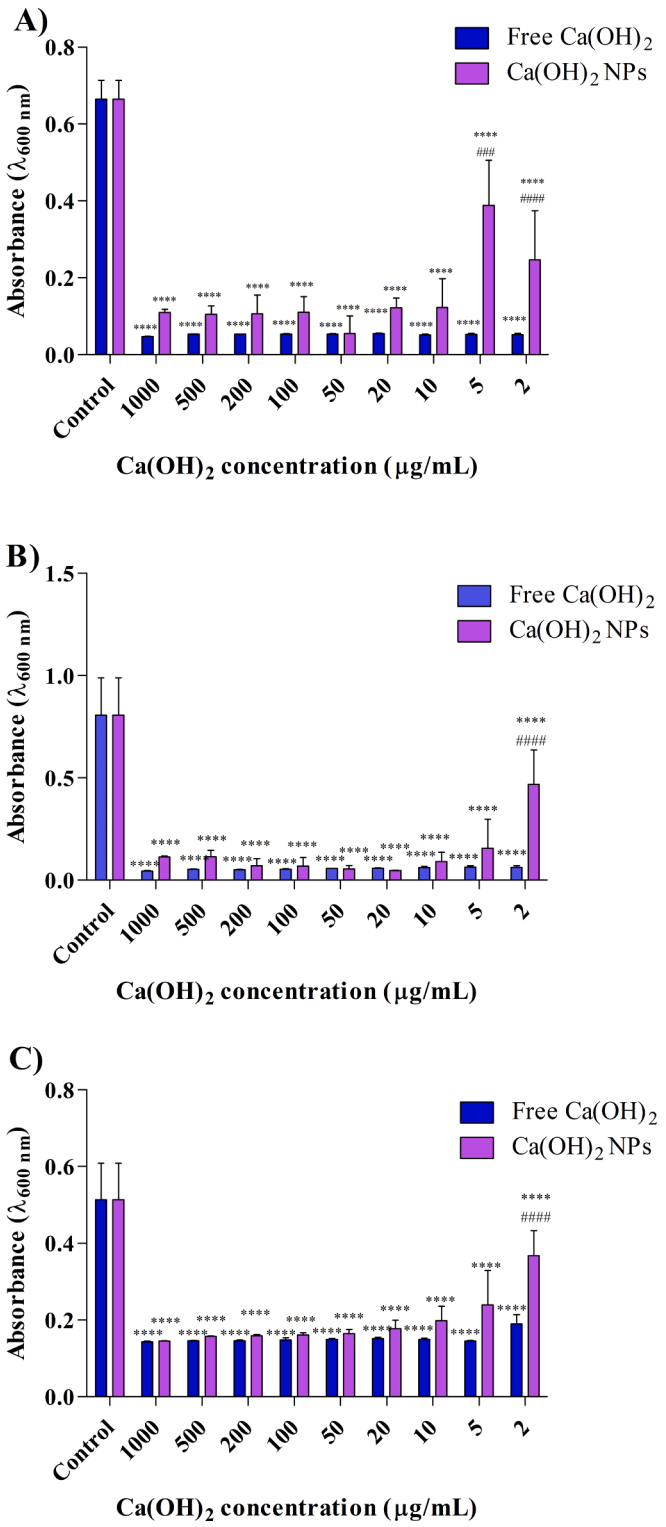

Fig. 4. Absorbance obtained assessing the minimal inhibitory concentration (MIC) of three bacterial strains **A)** *Porphyromonas gingivalis*, **B)** *Fusobacterium nucleatum* and **C)** *Enterococcus faecalis* with different concentrations after 24 h. Statistical significance was calculated using two-way ANOVA followed by Bonferroni post-hoc test. Significant differences against the control are represented as **** $p < 0.0001$ and differences between the same concentration between free $Ca(OH)_2$ and $Ca(OH)_2$ NPs are represented as ### $p < 0.001$ and ##### $p < 0.0001$.

Table 1
Minimum inhibitory concentration values of Free $Ca(OH)_2$ and $Ca(OH)_2$NPs.

	Free $Ca(OH)_2$	$Ca(OH)_2$NPs
Porphyromonas gingivalis	< 1 µg/mL	10 µg/mL
Fusobacterium nucleatum	< 1 µg/mL	5 µg/mL
Enterococcus faecalis	5 µg/mL	10 µg/mL

3.4. Ex vivo metabolic activity and bacterial viability

The metabolic activity of bacteria infecting teeth and treated with either $Ca(OH)_2$ NPs or free $Ca(OH)_2$ was analysed using the resazurin reduction assay. The results demonstrated that both $Ca(OH)_2$ NPs and $Ca(OH)_2$ significantly reduced ($p < 0.05$) the metabolic activity compared to the control (Fig. 6). Furthermore, although no significant differences between free $Ca(OH)_2$ and $Ca(OH)_2$ NPs were observed ($p > 0.05$), $Ca(OH)_2$ NPs showed a trend towards a more marketed reduction of the metabolic bacterial activity. This may be due to the fact that the NPs are able to interact with the bacterial membranes in a more effective manner and also due to NPs prolonged release, $Ca(OH)_2$ protection against degradation and higher internalization in the dentinal tubules. Similarly, Podbielski et al. (Podbielski et al., 2003) showed that $Ca(OH)_2$ reduced bacteria viability, but none of the conditions they sued led to the complete biofilm eradication (Podbielski et al., 2003).

Moreover, fluorescent images with *ex vivo* extracted human teeth were obtained staining viable bacteria using green fluorescence and dead ones using red label. These results confirm that both $Ca(OH)_2$ and $Ca(OH)_2$ NPs were able to decrease metabolic bacterial activity due to the release of hydroxyl ions at the therapeutic site of action. Despite the fact that no statistical differences were obtained on the metabolic activity against free $Ca(OH)_2$, $Ca(OH)_2$ NPs are able to decrease bacterial survival in a more effective manner than free $Ca(OH)_2$ and, at the same time, guarantee an increased internalization on the dentinal tubules as well as prolonged calcium hydroxide release (Elmsmari et al., 2021). In the apical region, a greater amount of dead bacteria was observed after the use of NPs (Fig. 7). Clinically this is the most important and critical area because it is the area where the bacteria have the greatest capacity to obtain nutrients, and the most difficult to disinfect because it is also the area furthest away from access to the canal system. Therefore, the higher penetration of $Ca(OH)_2$ NPs (Elmsmari et al., 2021) guarantee a more effective disinfection specifically in this complex area.

In addition, previous research has examined the efficacy of nanoparticles against *E. Faecalis* by confocal microscopy. In this area, Keskin et al. (Keskin et al., 2021) found that when Chitosan NPs were applied against E. Faecalis biofilms, there were no appreciable differences when compared to NaOCL (6 %) (Keskin et al., 2021). Other nanoparticles types, like silver, have also demonstrated to decrease *E. Faecalis* bacterial biofilms in LIVE/DEAD ® testing conducted under Confocal Laser Scanning Microscopy (Rodrigues et al., 2018; Arias-Moliz et al., 2020).

Although this results show the capacity of $Ca(OH)_2$ NPs to achieve suitable antibacterial capacity, the design of $Ca(OH)_2$ NPs may be further improved to increase its antibacterial properties either by increasing the $Ca(OH)_2$ concentration within the NPs or by combining it with other antibacterial agents such as antibiotics or ions (Godoy-Gallardo et al., 2021). Moreover, the use of $Ca(OH)_2$ has been suggested to denature the collagen matrix or breakdown of the inorganic matrix of dentine (Mohammadi and Dummer, 2011). This potential detrimental effect should be quantified in future experiments.

The null hypothesis has been rejected, and we found significant differences in antibacterial efficacy between the nanoparticle group and the control group. The minimum inhibitory concentration (MIC) in the nanoparticle group was higher than that in the commercial $Ca(OH)_2$ group. Regarding metabolic activity, there were no significant differences between the commercial $Ca(OH)_2$ group and the nanoparticle group, but there were differences compared to the control group.

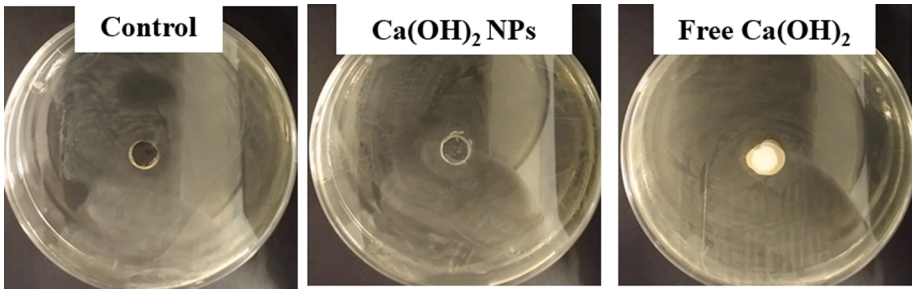

Fig. 5. Agar diffusion test of $Ca(OH)_2$ NPs and calcium hydroxide at 1 mg/mL using *Enterococcus faecalis* strain at 24 h. 1x PBS as control.

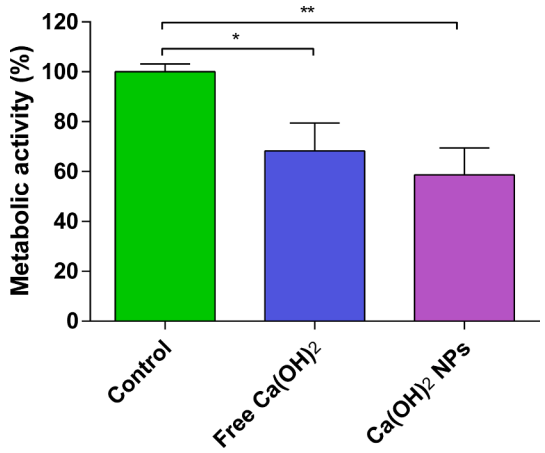

Fig. 6. Metabolic activity of $Ca(OH)_2$ NPs and $Ca(OH)_2$ at 1 mg/mL using *Enterococcus faecalis* strain at 24 h. 1x PBS was used as control. Statistical differences were calculated using one-way ANOVA and represented as *$p < 0.05$ and **$p < 0.01$.

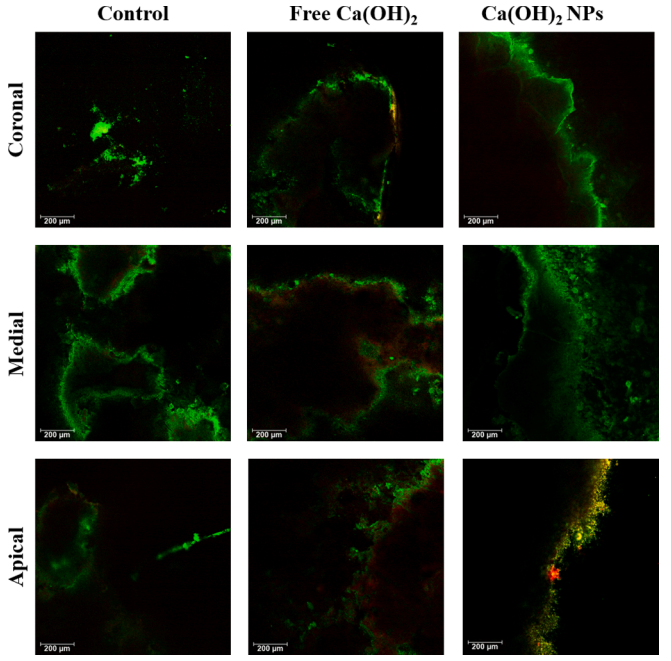

Fig. 7. Metabolic activity of *Enterococcus faecalis* treated with $Ca(OH)_2$ NPs and calcium hydroxide at 1 mg/mL for 24 h. Green bacteria are alive, red bacteria are dead and yellow bacteria, due to coalescence of live and dead staining, are cells that are metabolic actively but their membranes are compromised. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

Calcium hydroxide ($Ca(OH)_2$) has been considered the gold standard antibacterial agent in endodontics. However, innovative approaches have been explored to address limitations such as decreased antibacterial activity due to the buffering effect of dentin and difficulties accessing intricate root canal networks. In this area, nanotechnology-based medications offer potential solutions for these challenges.

This study shows that $Ca(OH)_2$ NPs possess an average size below 200 nm and exhibited a minimum inhibitory concentration (MIC) of 10 μg/mL for *Porphyromonas gingivalis* and *Enterococcus faecalis*, and 5 μg/mL for *Fusobacterium nucleatum*. Although no inhibition area was observed in the agar diffusion test, probably due to the agar buffering effect, $Ca(OH)_2$ NPs significantly reduced bacterial metabolic activity, thus preserving the active compound effectiveness.

To conclude, our findings highlight the potential of $Ca(OH)_2$ NPs as an antibacterial agent against several bacterial strains involved in endodontic infections being able to attain anatomically complicated infected areas such as the apical region. However, further testing, particularly against more resilient bacterial biofilms, is required before their clinical application can be carried out.

5. Data availability statement

Data described in this paper are available on request.

Declaration of competing interest

Dr. Firas Elmsmari, Dr. Fernando Duran-Sindreu, Dr. Marisa García López Dr. Jose Antonio González Sánchez and Dr Elena Sánchez López, report a licensed patent with the Universitat Internacional De Catalunya and University of Barcelona—Composition comprising nanoparticles, method for the preparation of a composition comprising nanoparticles and uses of the composition for dental treatment. European patent with the file number: EP20382504. The other authors have stated explicitly that there are no conflicts of interest in connection with this article.

All authors have reviewed the contents of the manuscript being submitted, approved its contents, and validated the accuracy of the data.

Author contributions

F.M and MT.T were involved in performing all the antibacterial tests and experiments. All the antibacterial tests were done with the direct help and assistance of LM.D and RA.P. Moreover, both JA.G and E.S.L were directly responsible for developing and designing the methodology of the study. Finally, L.M.D, K.I.A, ML.G, E.S.L and FD.S were involved in supervision of the writing process plus, article correction and editing.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Elena Sanchez-Lopez has patent #Composition comprising nanoparticles, method for the preparation of a composition comprising nanoparticles and uses of the composition for dental treatment. EP20382504.7 issued to Explotation rights: Universitat Internacional de Catalunya and Universitat de Barcelona. Regarding the patent mentioned other inventors are also included in this manuscript: García López, M. L.; Sánchez López, E.; Durán-Sindreu, F.; González Sánchez, J. A. and Elmasmari, F.].

Data availability

No data was used for the research described in the article.

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References

Afdilla, N., Fatmasari, D., Djamil, M., Hadisaputro, S., Daniati, N., 2022. J. Appl. Heal Manag. Technol. 4 (2), 36–41.
Alghamdi, F., Shakir, M., 2020. The Influence of Enterococcus Faecalis as a Dental Root Canal Pathogen on Endodontic Treatment : A Systematic Review Material and Methods. 12 (3), 1–10.
Arias-Moliz, M.T., Baca, P., Solana, C., et al., 2020. Doxycycline-functionalized polymeric nanoparticles inhibit Enterococcus faecalis biofilm formation on dentine. Int. Endod. J. 54 (3), 413–426.
Athanassiadis, B., Abbott, P.V., Walsh, L.J., 2007. The use of calcium hydroxide, antibiotics and biocides as antimicrobial medicaments in endodontics. Aust. Dent. J. 52 (1 SUPPL.).
Azim, A.A., Aksel, H., Zhuang, T., Mashtare, T., Babu, J.P., Huang, G.-T.-J., 2016. Efficacy of 4 Irrigation Protocols in Killing Bacteria Colonized in Dentinal Tubules Examined by a Novel Confocal Laser Scanning Microscope Analysis. J. Endod. 42 (6), 928–934.
Bhardwaj, A., Ballal, S., Velmurugan, N., 2015. Comparative evaluation of the antimicrobial activity of natural extracts of Morinda calcium hydroxide, against Enterococcus faecalis : An in vitro study. Iran. Endod. J. 15 (3), 293–297.
Bhatia S. Nanotechnology and Its Drug Delivery Applications. In: Natural Polymer Drug Delivery Systems. 1–24 (2016).
Burns, L.E., Kim, J., Wu, Y., Alzwaideh, R., McGowan, R., Sigurdsson, A., 2022. Outcomes of primary root canal therapy: An updated systematic review of longitudinal clinical studies published between 2003 and 2020. Int. Endod. J. 55 (7), 714–731.
Cano, A., Ettcheto, M., Espina, M., et al., 2018. Epigallocatechin-3-gallate loaded PEGylated-PLGA nanoparticles: A new anti-seizure strategy for temporal lobe epilepsy. Nanomedicine Nanotechnology. Biol. Med. 14 (4), 1073–1085.
Carpio-perochena, A., Kishen, A., Felitti, R., et al., 2017. Antibacterial Properties of Chitosan Nanoparticles and Propolis Associated with Calcium Hydroxide against Single- and Multispecies Biofilms : An In Vitro and In Situ Study. J. Endod. 43 (8), 1332–1336.
Diogo P, Amparo M, Palma PJ, Rai A, Graça P. M. S. Neves M, Santos JM. May carriers at nanoscale improve the Endodontic's future? Adv. Drug Deliv. Rev. 195, 114731 (2023).
Eduardo, L., De, P.C., 2002. Fusobacterium Nucleatum in Endodontic Flare-Ups. 179–183.
Elmsmari, F., González Sánchez, J.A., Duran-Sindreu, F., et al., 2021. Calcium hydroxide-loaded PLGA biodegradable nanoparticles as an intracanal medicament. Int. Endod. J. 54 (11), 2086–2098.
Esteruelas, G., Halbaut, L., García-Torra, V., et al., 2021. Development and optimization of Riluzole-loaded biodegradable nanoparticles incorporated in a mucoadhesive in situ gel for the posterior eye segment. Int. J. Pharm. 612, 121379.
Esteruelas, G., Souto, E.B., Espina, M., et al., 2022. Diclofenac Loaded Biodegradable Nanoparticles as Antitumoral and Antiangiogenic Therapy. Pharmaceutics 15 (1), 102.
Estrela, C., Rodrigues, C., Estrela, D.A., 2001. Two Methods to Evaluate the Antimicrobial Action of Calcium Hydroxide Paste. J. Endod. 27 (12), 12–15.
Fan, W., Wu, D., Ma, T., Fan, B., 2015. Ag-Loaded Mesoporous Bioactive Glasses against Enterococcus Faecalis Biofilm in Root Canal of Human Teeth. 34 (1), 54–60.
Fernandes, A.R., dos Santos, T., Granja, P.L., et al., 2022. Permeability, anti-inflammatory and anti-VEGF profiles of steroidal-loaded cationic nanoemulsions in retinal pigment epithelial cells under oxidative stress. Int. J. Pharm. 617 (February), 121615.
Galindo, R., Elena, S., Espina, M., et al., 2022. Development of Peptide Targeted PLGA-PEGylated Nanoparticles Loading Licochalcone-A for Ocular Inflammation. Pharmaceutics 14, 285.
Gluskin, A.H., Lai, G., Peters, C.I., Peters, O.A., 2020. The double-edged sword of calcium hydroxide in endodontics: Precautions and preventive strategies for extrusion injuries into neurovascular anatomy. J. Am. Dent. Assoc. 151 (5), 317–326.
Godoy-Gallardo, M., Eckhard, U., Delgado, L.M., et al., 2021. Antibacterial approaches in tissue engineering using metal ions and nanoparticles: From mechanisms to applications. Bioact. Mater. 6 (12), 4470–4490.
Goldberg, F., Cantarini, C., Alfie, D., Macchi, R.L., Arias, A., 2020. Relationship between unintentional canal overfilling and the long-term outcome of primary root canal treatments and nonsurgical retreatments: a retrospective radiographic assessment. Int. Endod. J. 53 (1), 19–26.
Gomes, B.P.F.A., Jacinto, R.C., Pinheiro, E.T., Sousa, E.L.R., Zaia, A.A., Ferraz, C.C.R., 2005. Porphyromonas gingivalis. Porphyromonas Endodontalis, Prevotella Intermedia and Prevotella Nigrescens in Endodontic Lesions Detected by Culture and by PCR. 5, 211–215.
Gulabivala, K., Ng, Y.L., 2023. Factors that affect the outcomes of root canal treatment and retreatment—A reframing of the principles. Int. Endod. J. 56 (S2), 82–115.
Han, G.Y., Park, S., Yoon, T.C., 2001. Antimicrobial Activity of Ca (OH). 2 Containing Pastes with Enterococcus Faecalis in Vitro.
Hatipoğlu, F.P., Ömer Hatipoğlu, N., Taha, A.P., et al., 2023. Attitude and practice of regenerative endodontic procedures among endodontists and paediatric dentists: A multinational survey from 13 countries. Int. J. Paediatr. Dentristry 33 (5), 521–534.
Heling, I., Steinberg, D., Kenig, S., Gavrilovich, I., Sela, M.N., Friedman, M., 1992. Efficacy of a sustained-release device containing chlorhexidine and Ca(OH)2 in preventing secondary infection of dentinal tubules. Int. Endod. J. 25 (1), 20–24.
Hulsmann, M., Peters, O.A., Dummer, P.M.H., 2005. Mechanical preparation of root canals: shaping goals, techniques and means. Endod. Top. 10 (1), 30–76.
Imlay, J.A., Linn, S., 1988. DNA Damage and Oxygen Radical Toxicity. Science (80-) 240, 1302–1309.
Info, A., 2017. Comparison of the Penetration Depth of Conventional and Nano-Particle Calcium Hydroxide into. Dentinal Tubules. 12 (3), 366–370.
Jarkhi, A., Hui, A., Lee, C., 2022. Antimicrobial Effects of L -Chg 10 -Teixobactin against Enterococcus faecalis In Vitro. Microorganisms 10, 1099.
Jr, J.F.S., Lopes, H.P., 1999. Mechanisms of antimicrobial activity of calcium hydroxide : a critical review. Int. Endod. J. 32, 361–369.
Jr, J.F.S., Rôças, I.N., Silva, M.G., 2008. Prevalence and Clonal Analysis of Porphyromonas Gingivalis in Primary Endodontic Infections. 34 (11), 1332–1336.
Keskin, N.B., Aydın, Z.U., Uslu, G., Özyürek, T., Erdönmez, D., Gündoğar, M., 2021. Antibacterial efficacy of copper-added chitosan nanoparticles: a confocal laser scanning microscopy analysis. Odontology 109 (4), 868–873.
Kishen, A., 2012. Advanced Therapeutic Options for Endodontic Biofilms. 99–123.
Kishen, A., Shi, Z., Shrestha, A., Neoh, K.G., 2008. An Investigation on the Antibacterial and Antibiofilm Efficacy of Cationic Nanoparticulates for Root Canal Disinfection. J. Endod. 34 (12), 1515–1520.
Kojima, K., Inamoto, K., Nagamatsu, K., et al., 2004. Success rate of endodontic treatment of teeth with vital and nonvital pulps. a meta-analysis. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 97 (1), 95–99.
Lacevic, A., Vranic, E., Zulic, I., 2004. Etiological findings in endodontic-periodontal infections. Bosn. J. Basic Med. Sci. IV 57–61.
Liewehr, F., 2001. Antimicrobial Activity of Several Calcium Hydroxide Preparations in Root Canal Dentin. J. Endod. 27, 765–767.
López-Machado, A., Díaz, N., Cano, A., et al., 2021. Development of topical eye-drops of lactoferrin-loaded biodegradable nanoparticles for the treatment of anterior segment inflammatory processes. Int. J. Pharm. 609, 121188.
M02-A12: Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard—Twelfth Edition. (January) (2015).
M07-A10: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition. (January) (2015).
Mohammadi, Z., Dummer, P.M.H., 2011. Properties and applications of calcium hydroxide in endodontics and dental traumatology. Int. Endod. J. 44 (8), 697–730.
Nair, P.N.R., Henry, S., Cano, V., Vera, J., 2005. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after 'one-visit' endodontic treatment. Oral Surgery, Oral Med. Oral Pathol. Oral Radiol Endodontology 99 (2), 231–252.
Narayanan, L., Vaishnavi, C., 2010. Endodontic Microbiology. J. Conserv. Dent. 13, 233.
Pacios, M.G., Luisa, M., Casa, D., Bulacio, M.D.L.Á., López, M.E., 2004. Influence of different vehicles on the pH of calcium hydroxide pastes. J. Oral Sci. 46 (2), 107–111.
Podbielski, A., Spahr, A., Haller, B., 2003. Additive Antimicrobial Activity of Calcium Hydroxide and Chlorhexidine on Common Endodontic Bacterial Pathogens. J. Endod. 29 (5), 340–345.
Prathita, T., Djauharie, N.K., Meidyawati, R., 2019. Antimicrobial activity of mineral trioxide aggregate and calcium hydroxide sealer on enterococcus faecalis strain ATCC29212. Int. J. Appl. Pharm. 11 (1), 123–125.
Ricucci, D., Siqueira, J.F., 2010. Biofilms and apical periodontitis: Study of prevalence and association with clinical and histopathologic findings. J. Endod. 36 (8), 1277–1288.
Rodrigues, C., de Andrade, F., de Vasconcelos, L.R.S.M., Midena, R., et al., 2018. Antibacterial properties of silver nanoparticles as a root canal irrigant against Enterococcus faecalis biofilm and infected dentinal tubules. Int. Endod. J. 51 (8), 901–911.

Roig-soriano, X., Souto, E.B., Elmsmari, F., et al., 2022. Nanoparticles in Endodontics Disinfection : State of the Art. Pharmaceutics 14, 1519.
Sabrah, A.H.A., Yassen, G.H., Gregory, R.L., 2013. Effectiveness of Antibiotic Medicaments against Biofilm Formation of Enterococcus faecalis and Porphyromonas gingivalis. J. Endod. 39 (11), 1385–1389.
Sánchez-López, E., Ettcheto, M., Egea, M.A., et al., 2017. New potential strategies for Alzheimer's disease prevention: pegylated biodegradable dexibuprofen nanospheres administration to APPswe/PS1dE9. *Nanomedicine Nanotechnology*. Biol. Med. 13 (3), 1171–1182.
Sánchez-López, E., Ettcheto, M., Egea, M.A., et al., 2018. Memantine loaded PLGA PEGylated nanoparticles for Alzheimer's disease : in vitro and in vivo characterization. J. Nanobiotechnology 16 (32), 1–16.
Sánchez-López, E., Egea, M.A., Davis, B.M., et al., 2018. Memantine-Loaded PEGylated Biodegradable Nanoparticles for the Treatment of Glaucoma. Small 14 (2), 1–12.
Sánchez-López, E., Esteruelas, G., Ortiz, A., et al., 2020. Dexibuprofen biodegradable nanoparticles: One step closer towards a better ocular interaction study. Nanomaterials 10 (4), 1–24.
Shaaban, S., Genena, S., Elraggal, A., Hamad, G.M., Meheissen, M.A., Moussa, S., 2023. Antibacterial effectiveness of multi-strain probiotics supernatants intracanal medication on Enterococcus faecalis biofilm in a tooth model. BMC Oral Health 23 (1), 1–11.
Shabbir, J., Najmi, N., Zehra, T., et al., 2022. Intracanal Medicaments. in: Biomaterials in Endodontics. 5–81.
Shrestha, A., Zhilong, S., Gee, N.K., Kishen, A., 2010. Nanoparticulates for Antibiofilm Treatment and Effect of Aging on Its Antibacterial Activity. J. Endod. 36 (6), 1030–1035.
Shuping, G.B., Ørstavik, D., Sigurdsson, A., Trope, M., 2000. Reduction of Intracanal Bacteria Using Nickel- Titanium Rotary Instrumentation and Various. 13.
Siqueira, J.F., Rôças, I.N., 2022. Present status and future directions: Microbiology of endodontic infections. Int. Endod. J. 55 (S3), 512–530.
Tamara Yuanita T., Listiana ID., Prasetyo I *et al.* Comparison of the Effect of Calcium Hydroxide Combination with Cocoa Pod Husk Extract and Green Tea Extract On C-Fos and Dmp-1 Expression in Exposed Dental Pulp. J. Int. Dent. Med. Res. (2018), 2022 (2022).
Taschieri, S., Del, F.M., Samaranayake, L., Chang, J.W.W., Corbella, S., 2014. Microbial Invasion of Dentinal Tubules : a Literature Review and a New Perspective. 163–170.
Tomazinho LF, Avila-campos MJ, Paulo S. Detection of Porphyromonas gingivalis , Porphyromonas endodontalis , Prevotella intermedia , and Prevotella nigrescens in chronic endodontic infection. 285–288 (2007).
Trope, M., Bergenholtz, G., 2002. Microbiological basis for endodontic treatment: can a maximal outcome be achieved in one visit? Endod. Top. 1, 40–53.
Vera, J., Siqueira, J.F., Ricucci, D., et al., 2012. One- versus two-visit endodontic treatment of teeth with apical periodontitis: A histobacteriologic study. J. Endod. 38 (8), 1040–1052.
Weiger, R., De, L.J., Decker, H.E., Löst, C., 2002. Vitality Status of Microorganisms in Infected Human Root Dentine. 166–171.
Wu, D., Fan, W., Kishen, A., Gutmann, J.L., Fan, B., 2014. Evaluation of the Antibacterial Efficacy of Silver Nanoparticles against Enterococcus faecalis Biofilm. J. Endod. 40 (2), 285–290.