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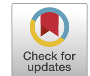

ORIGINAL ARTICLE

The viability of human dental pulp cells and apical papilla cells after treatment with conventional calcium hydroxide and nanoparticulate calcium hydroxide at various concentrations

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KEYWORDS

Nanoparticulate $Ca(OH)_2$;
Dental pulp cells;
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Abstract *Introduction:* Nanoparticulate $Ca(OH)_2$ had greater antibacterial effect than conventional $Ca(OH)_2$. Conversely, a study reported that nanoparticulate $Ca(OH)_2$ had toxicity against murine fibroblast. However, the study of nanoparticulate $Ca(OH)_2$, involving human dental pulp cells (DPCs) and apical papilla cells (APCs) is lacking. The aim of this study is to compare the effects of conventional $Ca(OH)_2$ and nanoparticulate $Ca(OH)_2$ on the viability of DPCs and APCs.

Methods: Primary human DPCs/APCs from the 3rd to 5th passage were divided into control and experimental groups. In the control group, cells were cultured in complete media. In the experimental group, cells were cultured in complete media containing 10, 100, or 1000 μg/mL of either conventional $Ca(OH)_2$ or nanoparticulate $Ca(OH)_2$ for 1, 3, 5, and 7 days. After the treatment period, the cells were tested for viability using MTT assay.

Results: DPCs treated with conventional $Ca(OH)_2$ in all concentrations at day 5 revealed significantly higher proliferation compared to nanoparticulate $Ca(OH)_2$ treated groups. In additions, DPCs treated with 1000 μg/ml nanoparticulate $Ca(OH)_2$ at day7 were significantly lower proliferation compared to DPCs treated with conventional $Ca(OH)_2$. In contrast, APCs treated with 1000 μg/ml nanoparticulated $Ca(OH)_2$ were significantly higher proliferation than APCs treated with 1000 μg/ml conventional $Ca(OH)_2$ at day7.

Conclusions: Nanoparticulate $Ca(OH)_2$ increased the viability of APCs and can be an alternative choice of intracanal medication for regenerative endodontic procedures. However, Nanoparticulate $Ca(OH)_2$ exerted some effects on DPCs. The use of nanoparticulate $Ca(OH)_2$ has no advantages over the conventional $Ca(OH)_2$ for vital pulp therapy.
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1. Introduction

Bacteria are the main causative agents of dental diseases. When bacteria are involved in dental pulp, it leads to pulpal infection and apical periodontitis (García et al., 2007; Rafatjou et al., 2019). Without treatment, it subsequently causes periapical rarefaction, indicating the need for endodontic treatment, such as vital pulp therapy (VPT), root canal treatment, and regenerative endodontic procedures (REPs).

VPT, including indirect and direct pulp capping and partial or complete pulpotomy, is the method of preserving the vitality and function of the dental pulp and enhancing the formation of reparative dentin after injuries (Aguilar and Linsuwanont, 2011). VPT involves the removal of local irritants and placement of a dressing material over the pulp stump (Hargreaves et al., 2015). The VPT procedures have traditionally used calcium hydroxide ($Ca(OH)_2$) as a gold standard (Patel et al., 2020). Pulp dressing with $Ca(OH)_2$ is suitable when the exposed pulp is in normal condition or when pulpitis is reversible (Alex, 2018). It is a relatively simple procedure, less invasive and affordable treatment (Asl Aminabadi et al., 2016).

REPs are biologically based procedures that desire to replace damaged structures, including dentin, root structures, and cells in the pulp-dentin complex (Murray et al., 2007). Intracanal medication should stimulate the activities of dental pulp cells (DPCs) and stem cells from apical papilla (SCAPs). It is crucial to conserve the most suitable cells with potential competence for tissue regeneration. Several reports have affirmed that SCAPs have high cellular proliferation, multilineage differentiation, mineralization, self-renewal ability, and low immunogenicity, making them a suitable choice for pulp regeneration (Kang et al., 2019; Kitikuson and Srisuwan, 2016; Langer and Vacanti, 1993). Apart from SCAPs, dental pulp stem cells (DPSCs) are also suitable for tissue regeneration because they have a high proliferation and capability to differentiate into various cell types (Kawashima et al., 2017).

$Ca(OH)_2$ is the standard medication currently used in VPT and REPs (Poggio et al., 2014; American Academy of Endodontists, 2021). The particle size of conventional $Ca(OH)_2$ ranges from 1 to 10 μm, while the diameter of dentinal tubules near the pulp is 2.0–2.5 μm. Hence, conventional $Ca(OH)_2$ could not reach the deeper parts of dentin, and it was unable to kill *Enterococcus faecalis* (*E. faecalis*), which is the predominant resistant species, in the dentinal tubule (Dianat et al., 2015a; Sirén et al., 2004). Bacteria can penetrate 300–1500 μm deep into the dentinal tubules (Tasanarong et al., 2016), which are often inaccessible by medicaments (Dianat et al., 2015b). In addition to having antimicrobial efficacy, the ability to promote the viability of DPCs and apical papilla cells (APCs) is also critical for success in VPT and REPs. To improve the efficacy of $Ca(OH)_2$, size reduction into a nanoparticulate form may be an alternative to enhance its properties (Dianat et al., 2015a).

Nanotechnology aims to improve therapeutic effects and physicochemical characteristics (Patel et al., 2020; Balto et al., 2020). Nanotechnology has been adopted to produce $Ca(OH)_2$ nanoparticles to achieve greater antimicrobial effects (Dianat et al., 2015b). It was reported that $Ca(OH)_2$ nanoparticles not only kill *E. faecalis* up to 200–400 μm deep into dentinal tubules, displaying better penetration than conventional $Ca(OH)_2$ (Dianat et al., 2015b; Zand et al., 2017; Shrestha and Kishen 2016) but they also have a lesser effect on dentin microhardness (Naseri et al., 2019). Conversely, it was reported that $Ca(OH)_2$ nanoparticles have higher toxicity against murine fibroblasts than conventional $Ca(OH)_2$ (Dianat et al., 2015a). However, the study of $Ca(OH)_2$ nanoparticles, especially in VPT and REPs involving human DPCs and APCs, is still unclear and should be elucidated. Therefore, the aim of this study was to compare the effects of conventional $Ca(OH)_2$ and $Ca(OH)_2$ nanoparticles at various concentrations on the viability of human DPCs and APCs.

2. Materials and methods

2.1. Patient recruitment

This study was approved by the Human Experimentation Committee of Thammasat University, Thailand (Ethics no. 033/2564). After verbal and written informed consent was obtained, nonpathologically impacted third molars with mature and immature roots from healthy patients (aged 18–25 years) were collected. Volunteers who had severe underlying diseases and teeth with pulpal or periapical disease were excluded.

2.2. Culture of primary human DPCs

The tissue was harvested, and cells were cultured as previously described (Phumpatrakom and Srisuwan, 2014). The extracted teeth with mature roots were rinsed using sterile normal saline solution and stored on ice in containers with serum-free α-minimum essential medium (α-MEM) (Sigma-Aldrich, St Louis, MO, USA). To obtain pulp tissue, teeth were soaked and inverted into 5.25% NaOCl for 1 min except 3 mm from the root apex. Periodontal ligaments were removed, and teeth were sectioned into buccal and lingual parts. Then, pulp tissue was obtained using a sterile endodontic spoon and transferred into serum-free α-MEM.

Pulp tissues were minced and digested in 3.00 mg/mL type I collagenase (Gibco/Invitrogen; Gaithersburg, MD, USA) and 4.00 mg/mL dispase (Sigma-Aldrich) for 45 min at 37 °C. After that, the cells were centrifuged at 1500 rpm for 5 min at 25 °C. The extracted cells were cultured in complete α-MEM containing 10% fetal bovine serum (Gibco/Invitrogen), 1% penicillin–streptomycin (Sigma-Aldrich) and 100 μmol/L L-ascorbic (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 and 95% air.

2.3. Culture of primary human APCs

For immature teeth, the entire tooth surface was thoroughly cleaned and irrigated with sterile phosphate-buffered saline solution (PBS) (Sigma-Aldrich). The apical papilla was gently separated from the root surface by tweezers, copiously irrigated with sterile normal saline, and transferred into serum-free α-MEM, and the cell harvesting protocol for APCs was similar to that for pulp tissue.

Both types of cells were monitored using an inverted-light microscope, and photographs were taken by a DP-12 Olympus camera (Olympus, Tokyo, Japan). The culture medium was regularly replaced every three days. The subculture process

at a 1:3 ratio was achieved when both types of cells reached 80% confluence. DPCs and APCs from the 3rd to 5th passage were used.

2.4. Experimental group

Both types of cells were divided into four groups:

(1) Negative (−) control group: DPCs and APCs were cultured in complete media.
(2) Positive (+) control group: DPCs and APCs were cultured in 6% NaOCl.
(3) Experimental group treated with conventional $Ca(OH)_2$: DPCs and APCs were separately cultured in complete media containing 10, 100, and 1000 µg/ml conventional $Ca(OH)_2$ (M Dent, Bangkok, Thailand) (Ruparel et al., 2012).
(4) Experimental group treated with nanoparticulate $Ca(OH)_2$: DPCs and APCs were separately cultured in complete media containing 10, 100, and 1000 µg/ml of nanoparticulate $Ca(OH)_2$ (NanoShel LLC, Wilmington, DE, USA).

All groups in this experiment were cultured for 1, 3, 5, and 7 days.

2.5. MTT cell viability assay

Conventional $Ca(OH)_2$-treated, nanoparticulate $Ca(OH)_2$-treated and untreated DPCs/APCs, which were plated and cultured in complete α-MEM, were used to determine their proliferative capacities. Briefly, DPCs and APCs were separately seeded into 24-well plates at 5000 cells/well in regular complete media. Cell proliferation was measured at 1, 3, 5, and 7 days using colorimetric qualification of 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich). A freshly mixed 5 mg/mL MTT in PBS solution was added to growing cells at 80 µL/well and incubated for 3 h at 37 °C. After that, the medium was removed and flushed with sterile PBS, and then 800 µL of dimethyl sulfoxide (Sigma-Aldrich) was added to each well. One hundred microliters of the mixed solution was transferred into a 96-well plate, and the absorbance at 550 nm was measured using a spectrophotometer (Sunrise; Tecan, Mannerdorf, Switzerland).

2.6. Statistical analysis

The experiments were carried out in 3 trials. Each trial comprised 3 replicates of control and different concentrations of conventional $Ca(OH)_2$ and $Ca(OH)_2$ nanoparticles.

The data were analyzed by 2-way analysis of variance (ANOVA), followed by Dunnett's and Sidak's tests using GraphPad Prism 9.4 software (Dotmatics, San Diego, CA, USA). The difference between the experimental groups was statistically significant at $p < 0.05$.

3. Results

3.1. DPC viability

There were significant differences ($p < 0.05$) in the proliferative capacity of DPCs treated with 10 and 100 µg/ml conventional $Ca(OH)_2$ on Days 1, 3 and 5 compared to the (+) and (−) control groups. In addition, DPCs treated with 10, 100 and 1000 µg/ml conventional $Ca(OH)_2$ on Day 7 had a significantly higher proliferative capacity than the controls. At this time point, the results revealed that the higher concentration led to greater proliferation of DPCs (Fig. 1).

Nanoparticulate $Ca(OH)_2$ showed a significantly higher ($p < 0.05$) proliferative capacity of DPCs at all concentrations on Day 3. However, DPCs treated with 1000 µg/ml $Ca(OH)_2$ nanoparticles on Days 5 and 7 had a significantly lower proliferative capacity than the controls (Fig. 1).

DPCs treated with conventional $Ca(OH)_2$ at all concentrations on Day 5 revealed a significantly higher proliferative capacity compared to that of the nanoparticulate $Ca(OH)_2$-treated groups. In addition, DPCs treated with 1000 µg/ml $Ca(OH)_2$ nanoparticles had a significantly lower proliferative capacity than DPCs treated with conventional $Ca(OH)_2$ on Day 7 ($p < 0.05$) (Fig. 2).

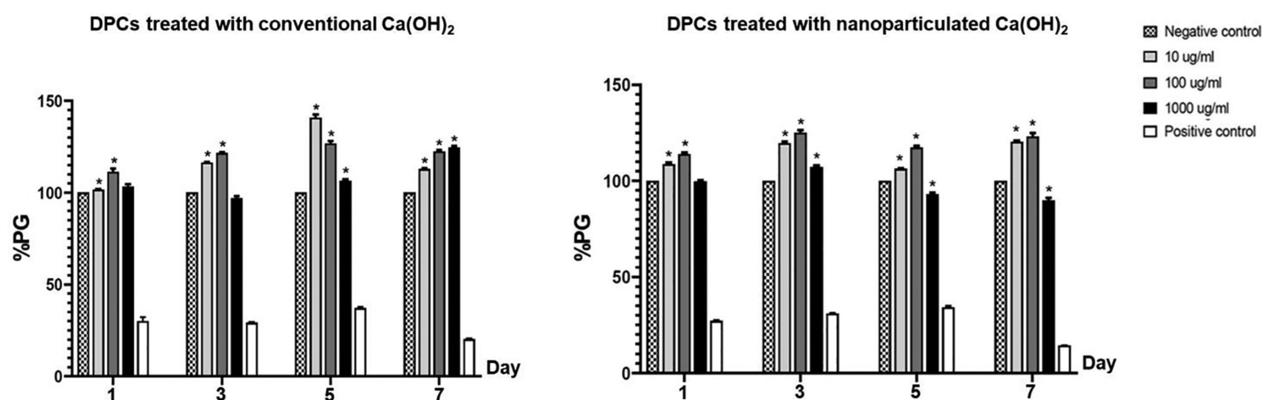

Fig. 1 The viability of DPCs after treatment with either conventional or nanoparticulate $Ca(OH)_2$ at 10, 100, and 1000 µg/ml. Differences between groups were determined by 2-way ANOVA followed by Dunnett's test compared to the controls. *Indicated a significant difference compared with the controls ($p < 0.05$).

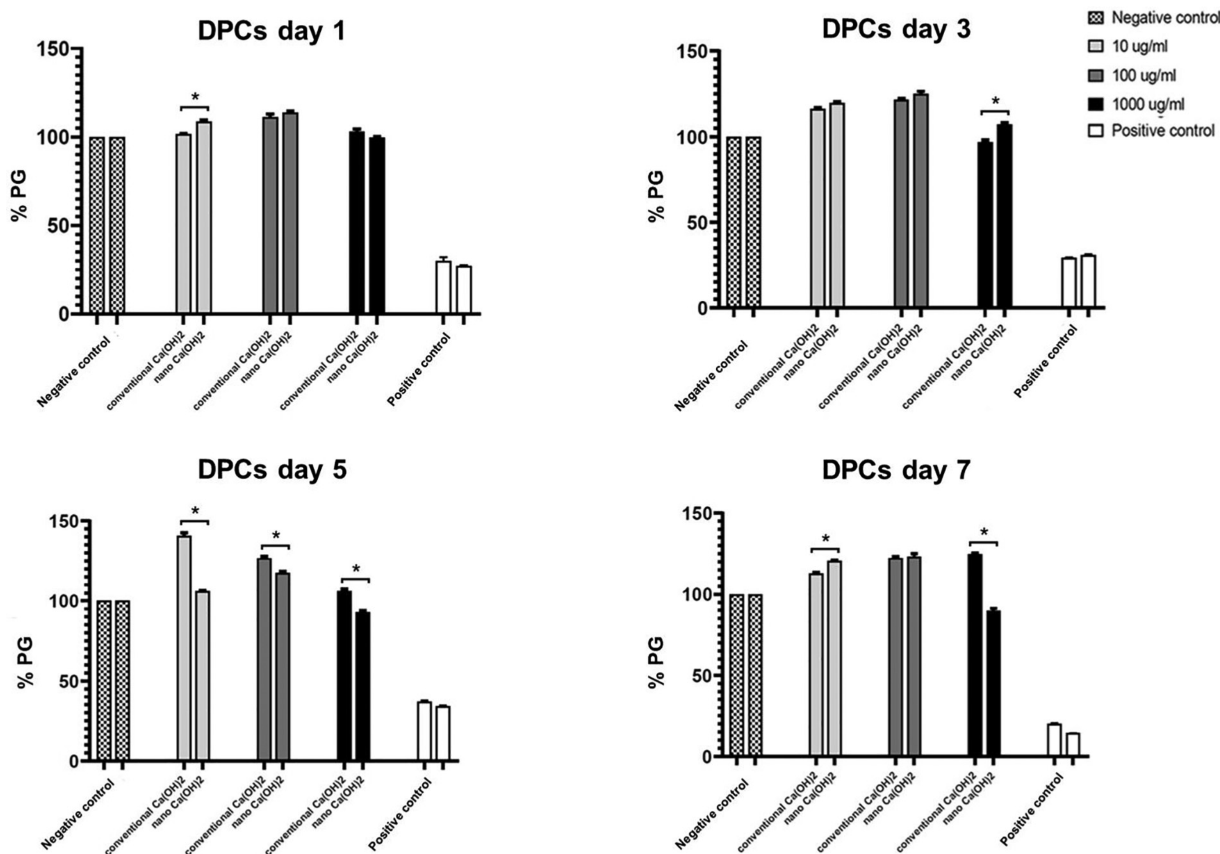

Fig. 2 Comparison of cell viability between DPCs treated with conventional and nanoparticulate $Ca(OH)_2$ on Days 1, 3, and 5. Differences between groups were determined by 2-way ANOVA followed by Sidak's test. *Indicated a significant difference ($p < 0.05$).

3.2. APC viability

There was a significantly higher proliferative capacity of APCs treated with 10, 100, and 1000 µg/ml conventional $Ca(OH)_2$ on Days 3, 5, and 7 compared to the control groups ($p < 0.05$). However, APCs treated with 1000 µg/ml $Ca(OH)_2$ nanoparticles had a significantly lower proliferative capacity on Day 1 than control groups. However, the proliferative capacity reversed on Days 3, 5, and 7 to be significantly higher than that in the control groups ($p < 0.05$) (Fig. 3).

In addition, APCs treated with 1000 µg/ml $Ca(OH)_2$ nanoparticles on Days 1, 3, and 5 had a significantly lower proliferative capacity than APCs treated with 1000 µg/ml conventional $Ca(OH)_2$. However, this study revealed that the number of APCs treated with 1000 µg/ml $Ca(OH)_2$ nanoparticles was significantly higher than that of APCs treated with 1000 µg/ml conventional $Ca(OH)_2$ on Day 7 ($p < 0.05$) (Fig. 4).

4. Discussion

The use of nanotechnology utilizes materials with diameters < 100 nm to improve therapeutic effects and physicochemical characteristics (Laurent et al., 2010). Previous studies showed that $Ca(OH)_2$ nanoparticles had greater antibacterial properties, a higher ability to penetrate into root canal biofilms, and better diffusion into the dentinal tubules (Dianat et al., 2015b; Louwakul et al., 2017). However, there is no evidence showing whether there would be more survival of DPCs and APCs, which are the key factors for VPT and REPs.

$Ca(OH)_2$ has a high pH (12.5–12.8) and low solubility in water of approximately 1.2 mg/ml at 25 °C (Fava and Saunders, 1999). We selected concentrations of 10, 100, and 1000 µg/ml because the higher concentration (1000 µg/ml) is comparable to the solubility of $Ca(OH)_2$ in clinical practice. For lower concentrations (10 and 100 µg/ml), the results were comparable to those of a previous study on SCAPs (Ruparel et al., 2012).

$Ca(OH)_2$ has been recommended as an intracanal medication for VPT and REPs (Hermann 1920; Galler et al., 2016; Almutairi et al., 2022) because of its various advantages. The main actions of $Ca(OH)_2$ come from the ionic dissociation of Ca^{2+} and OH^- ions. The action of these ions on vital tissue and bacteria generates the induction of hard tissue deposition and the antibacterial effect. However, when Ca^{2+} ions encounter CO_2 or CO_3^{2-} ions in biological tissue, $CaCO_3$ is formed, which limits mineralization by the consumption of Ca^{2+}. Moreover, $CaCO_3$ has neither biological nor antibacterial properties (Fava and Saunders, 1999). The release of OH^- ions from $Ca(OH)_2$ can increase the pH to 12 (Dianat et al., 2015b), establishing an alkaline environment (Louwakul et al., 2017). Their high alkalinity inflicts bacterial DNA (Del Carpio-Perochena et al., 2017), cellular membranes, and protein structures (Dianat et al., 2015b). Therefore, it can neutralize the virulence factors of gram-negative bacteria, promote pulp

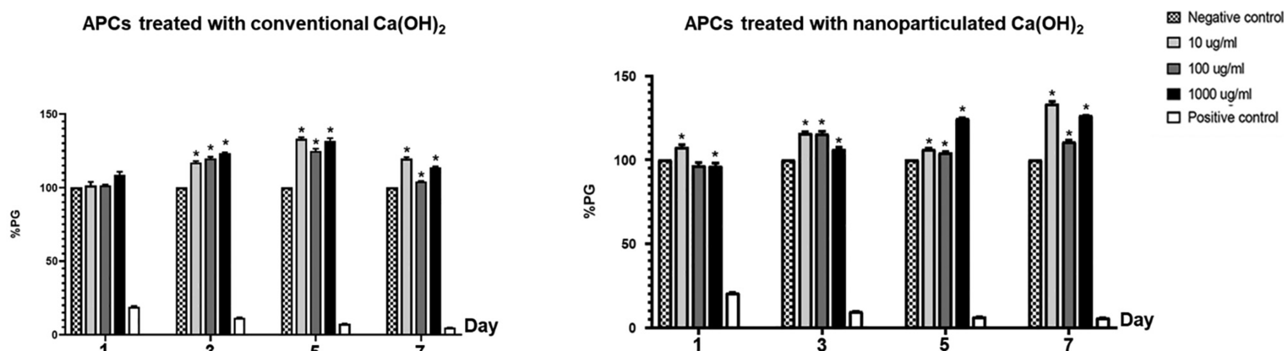

Fig. 3 The viability of APCs after treatment with either conventional or nanoparticulate $Ca(OH)_2$ at 10, 100, and 1000 µg/ml. Differences between groups were determined by 2-way ANOVA followed by Dunnett's test compared to the controls. *Indicated a significant difference compared with the control groups ($p < 0.05$).

Fig. 4 Comparison of cell viability between APCs treated with conventional and nanoparticulate $Ca(OH)_2$ on Days 1, 3, 5, and 7. Differences between groups were determined by 2-way ANOVA followed by Sidak's test. *Indicated a significant difference ($p < 0.05$).

healing, induce dentin bridge formation, dissolve debris, and inhibit root resorption (Aguiar et al., 2015; Dianat et al., 2015a; Naseri et al., 2019; Zand et al., 2017).

For DPCs, the results revealed that low concentrations of both conventional and nanoparticulate $Ca(OH)_2$ (10 and 100 µg/ml) could stimulate cell proliferation at all time points, whereas high concentrations (1000 µg/ml) of conventional $Ca(OH)_2$ could stimulate cell proliferation on Day 7. Interestingly, a high concentration of nanoparticulate $Ca(OH)_2$ inhibited cell proliferation on Days 5 and 7. A previous study reported that a high concentration of conventional $Ca(OH)_2$ could stimulate human DPC proliferation on Day 7, which is consistent with our study (Dou et al., 2020). Conversely, our study found that a high concentration of $Ca(OH)_2$

nanoparticles inhibited cell proliferation on Days 5 and 7. This is probably due to a greater number of reactive OH^- radicals being released from nanoparticles. It causes cell membrane and DNA damage and protein breakdown, which trigger cell death (Paramitta et al., 2011).

For APCs, previous studies reported that conventional $Ca(OH)_2$ promoted the survival and proliferation of SCAPs (Ruparel et al., 2012; Valverde et al., 2017). Additionally, it drastically increased the proliferation of SCAPs at a concentration of 1 mg/ml (Ruparel et al., 2012), which is consistent with our study that all concentrations of both conventional and nanoparticulate $Ca(OH)_2$ could stimulate APC proliferation on Days 3, 5, and 7 compared to the controls. Low concentrations of $Ca(OH)_2$ upregulated phosphorylated extracellular signal-related kinase (pERK), which is a marker of dental pulp and periodontal ligament stem cell proliferation (Ji et al., 2010). In addition, $Ca(OH)_2$-medicated dentine had no detrimental effect on SCAP attachment. It seems that the rest of the $Ca(OH)_2$ remaining on dentin after rinsing positively affected the SCAPs (Althumairy et al., 2014). Additionally, $Ca(OH)_2$ induces the release of TGF-β1 from dentin (Graham et al., 2006), which has a proliferative effect on mesenchymal stem cells and stimulates secondary dentin genesis, especially in REPs (Patel et al., 2020; Shimabukuro et al., 2009). This implied that $Ca(OH)_2$ nanoparticles enhance the positive effects on APCs.

In summary, our study has shown the beneficial effects of nanoparticulate $Ca(OH)_2$ on APC proliferation. The limitation of this study is that the cellular mechanism of each cell type that revealed a different response to nanoparticulate $Ca(OH)_2$ is not clearly understood. Further exploration should be focused especially on concentrations for appropriate clinical use, odontogenic capacity, and the molecular mechanisms of nanoparticulate $Ca(OH)_2$ to shed light on the clinical usage of intracanal medicaments in REPs.

5. Conclusion

This study revealed that nanoparticulate $Ca(OH)_2$ has no advantages over conventional $Ca(OH)_2$ for VPT, but the use of nanoparticulate $Ca(OH)_2$ can be an alternative choice of intracanal medication for REPs.

CRediT authorship contribution statement

Kamolparn Pugdee: Methodology, Software, Validation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration. **Awiruth Klaisiri:** Software, Writing – original draft, Supervision. **Panupat Phumpatrakom:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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