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# Comparative Evaluation of Calcium Hydroxide Nanoparticles and Calcium Hydroxide–Loaded Poly(lactic-co-glycolic acid) Nanoparticles on Cytocompatibility, Osteoblastic Differentiation, and Mineralization under Inflammatory Conditions

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## Abstract

# Objective

This study aimed to compare the cytocompatibility, osteogenic differentiation, and mineralization potential of conventional calcium hydroxide ($Ca(OH)_2$), calcium hydroxide nanoparticles ($Ca(OH)_2$ NPs), and calcium hydroxide-loaded poly(lactic-co-glycolic acid) nanoparticles (CH-PLGA NPs) in primary human osteoblasts under inflammatory conditions.

# Material and Methods

Primary human osteoblasts from eight donors (n = 8) were used. Inflammation was induced using lipopolysaccharide 20 µg/mL for 72 hours. Cells were treated with ($Ca(OH)_2$), $Ca(OH)_2$ NPs, and CH-PLGA NPs at equivalent concentrations (0.5 mg/mL). Cytotoxicity was evaluated using MTT assay at 48 hours. Osteogenic differentiation was assessed by RT-qPCR analysis of *RUNX2*, *ALP*, and *COL1A1* mRNA expression at day 14. Mineralization was evaluated using Alizarin Red S staining at day 21.

# Results

All groups maintained metabolic activity above 80%, indicating no cytotoxicity. Both nanoparticle formulations enhanced osteogenic gene expression compared with the LPS group. $Ca(OH)_2$ NPs demonstrated the highest upregulation of *ALP* (~ 4.9-fold) ($p < 0.05$) and significantly increased *COL1A1* expression found in CH-PLGA NPs (~ 4.2-fold) ($p < 0.05$). *RUNX2* expression showed no significant differences. Mineralization was observed in all groups without significant differences.

# Conclusions

$Ca(OH)_2$ NPs and CH-PLGA NPs exhibited favorable cytocompatibility and enhanced osteogenic gene expression under inflammatory conditions, with more pronounced effects observed for CH-PLGA NPs. However, neither formulation significantly enhanced mineralization. Further studies are required to confirm their effects on functional bone regeneration and clinical outcomes.

## 1. Introduction

In root canal infections, periapical periodontitis arises as a consequence of the inflammatory response, leading to periapical bone destruction and associated clinical symptoms [1]. The primary goals of endodontic treatment are to eliminate infection and achieve resolution of periapical pathology while maintaining a symptom-free state [2]. To accomplish these objectives, endodontic therapy involves mechanical instrumentation and chemical irrigation to reduce microbial load and remove toxins from the

root canal system. However, mechanical instrumentation and chemical irrigation alone may not achieve complete eradication of bacteria within the root canal system [3].

Calcium hydroxide $(\mathrm{Ca(OH)_2})$ has long been considered the gold standard intracanal medicament [4]. However, conventional $Ca(OH)_2$ presents several limitations, including reduced efficacy due to buffering by dentin and tissue fluids, lack of sustained ion release, potential weakening of dentin structure, and limited penetration into dentinal tubules and complex canal spaces [5–7]. To overcome these limitations, nanotechnology has been introduced in endodontics. Nanoparticles possess unique properties such as a high surface-to-volume ratio, enhanced antimicrobial activity, and improved physicochemical characteristics, which may enhance their clinical performance [7,8].

Calcium hydroxide nanoparticles ($Ca(OH)_2$ NPs) and calcium hydroxide-loaded poly(lactic-co-glycolic acid) nanoparticles (CH-PLGA NPs) have been developed to improve delivery efficiency and penetration into anatomically complex regions. Previous studies have demonstrated that $Ca(OH)_2$ in nanoparticle form exhibits superior antimicrobial properties compared with its conventional counterpart [7,9]. In addition, CH-PLGA NPs provide a controlled release system that may further enhance biological performance. Despite these advancements, their effects on bone remodeling remain largely unexplored.

Therefore, this study aimed to compare the in vitro cytocompatibility and osteogenic potential of $Ca(OH)_2$ NPs and CH-PLGA NPs with those of conventional $Ca(OH)_2$ in primary human osteoblasts, focusing on cell viability, osteoblastic differentiation, and mineralization rather than physicochemical and structural characterization.

## 2. Materials & Methods

This in vitro experimental study was conducted from October 2025 to April 2026. The study protocol was approved by the Research Ethics Committee of the Faculty of Dentistry, Chiang Mai University (approval no. 54/2025). All methods were performed in accordance with the relevant guidelines and regulations and with the Declaration of Helsinki. Written informed consent was obtained from all participants before the collection of bone specimens. All personal information was kept strictly confidential. This study compared the osteoblastic differentiation and mineralization potential of $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs under inflammatory conditions. $Ca(OH)_2$ NPs and CH-PLGA NPs were synthesized by the National Nanotechnology Center (Pathum Thani, Thailand), whereas conventional $Ca(OH)_2$ was supplied by Chulalongkorn University (Bangkok, Thailand). CH-PLGA NPs were prepared using the emulsification–solvent evaporation method. Details of the preparation methods, formulations, and nanoparticle properties have been reported previously[5,8]

## 2.1 Cell Harvest and Cell culture

Primary human osteoblasts were isolated from trabecular bone fragments obtained during mandibular third molar extractions. Bone samples were collected under sterile conditions and immediately immersed in phosphate-buffered saline. Cells were isolated by enzymatic digestion using 1 mg/mL collagenase type II (Sigma-Aldrich, USA) at 37°C for 90 minutes with gentle agitation. The resulting cell suspensions were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator at 37°C with 5% $CO_2$.

# 2.2 Cytotoxicity test

For this assay, 1×10³ cells were seeded into each well of a 96-well plate and incubated with 200 µL of DMEM with 10% FBS for durations 48 hours. Cells were assigned to the following seven experimental groups: (1) Basal group; control (Basal medium alone), (2) LPS group (Basal medium + LPS 20 µg/mL), (3) Cal Con group (Basal medium + $Ca(OH)_2$ 0.5 mg/mL), (4) Cal NP group (Basal medium + $Ca(OH)_2$ NPs 0.5 mg/mL), (5) Cal PLGA group (Basal medium + CH-PLGA NPs 0.5 mg/mL, $Ca(OH)_2$ equivalent), (6) PLGA group; vehicle control (Basal medium + PLGA), (7) PG group; vehicle control (Basal medium + Propylene Glycol). All plates were incubated at 37°C in a 5% $CO_2$ environment. All experimental groups were performed in triplicate, and absorbance was measured at 570 nm with a reference wavelength of 630–650 nm using a TECAN microplate reader. A concentration of 0.5 mg/mL was selected for subsequent osteoblastic differentiation and mineralization assays because it was the highest concentration within the tested range (0.25–0.5 mg/mL) at which all three formulations—conventional $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs—maintained cell viability ≥ 70% relative to the untreated control after 48 hours, consistent with the cytotoxicity interpretation criterion of ISO 10993-5:2009[10]. Cell viability results across the tested concentrations are presented in **Supplementary Figure S1**.

# 2.3 Evaluation of osteoblast differentiation via Real-time quantitative PCR

For osteogenic differentiation, 2.5 × $10^4$ cells were seeded into each well of a 6-well plate and cultured under standard conditions. After initial attachment, osteogenic differentiation was induced by supplementing the culture medium with 50 µg/mL L-ascorbic acid and 10 mM β-glycerophosphate, based on established protocols [11]. Cells were then treated under the eight experimental conditions: (1) Basal group; control (Basal medium), (2) Osteogenic group (Osteogenic medium alone), (3) LPS group (Osteogenic medium + LPS 20 µg/mL), (4) Cal Con group (Osteogenic medium + $Ca(OH)_2$ 0.5 mg/mL), (5) Cal NP group (Osteogenic medium + $Ca(OH)_2$ NPs 0.5 mg/mL), (6) Cal PLGA group (Osteogenic medium + CH-PLGA NPs 0.5 mg/mL, $Ca(OH)_2$ equivalent), (7) PLGA group; vehicle control (Osteogenic medium + PLGA). (8) PG group; vehicle control (Osteogenic medium + Propylene Glycol). Lipopolysaccharide (LPS; 20 µg/mL) was applied for 72 hours to simulate a pathological microenvironment in group 3–8. All cultures were maintained at 37°C in a humidified atmosphere with 5% $CO_2$. Total RNA was extracted at day 14 using the RNAspin Mini RNA Isolation Kit (Cytiva, USA), and RNA purity was assessed by A260/A280 ratio. Real-time quantitative PCR (RT-qPCR) was performed using SYBR Green chemistry (SensiFAST SYBR Lo-ROX kit, Bioline, USA) with gene-specific primers

(Table 1). Gene expression levels were normalized to the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1
Primer sequences used in this study Primer Sequence (5′-3′) References

| | | |
|---|---|---|
| $GAPDH^{\dagger}$ | **F: 5′-GTCAACGGATTTGGTCGTATTG-3′**<br>**R: 5′-TGTAGTTGAGGTCAATGAAGGG-3′** | **28** |
| $RUNX2^{\S}$ | F: 5′-AACCCACGAATGCACTATCCA-3′<br>R: 5′-CGGACATACCGAGGGACATG-3′ | 22 |
| $ALP^{\backslash P}$ | F: 5′-ACGTGGCTAAGAATGTCATC-3′<br>R: 5′-CTGGTAGGCGATGTCCTA-3′ | 29. |
| $COL1A1^{\ddagger}$ | F: 5′-CTGGTCTTCCTGGCCCCTCTGGTG-3′<br>R: 5′-CCTCTGTCGCCCTGTTCGCCTGTC-3′ | 30 |

† *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase; § *RUNX2*, Runt-related transcription factor 2;

$\backslash P$ *ALP*, Alkaline phosphatase; ‡ $COL1A1$, Collagen type I alpha 1 chain.

## 2.4 Evaluation of osteoblast mineralization with Staining

For mineralization assessment, 8 × 10³ cells were seeded into each well of a 6-well plate and cultured under the same experimental conditions as described for the RT-qPCR assay. Mineralization was evaluated at day 21 using Alizarin Red S (ARS) staining to detect calcium deposition. Cells were fixed and stained with 2% ARS solution (pH 4.3). After staining, excess dye was removed by gentle washing, and the bound stain was subsequently eluted using 10% (w/v) hexadecylpyridinium chloride monohydrate. The extracted dye was quantified by measuring absorbance at 540–550 nm with a reference wavelength of 650 nm using a TECAN microplate reader.

## 2.5 Data analysis

Data were analyzed using GraphPad Prism version 10.2.3 (GraphPad Software, Inc., San Diego, CA, USA). The sample size (n = 8 donors) was selected based on a previous study[12]. Normality was assessed using the Shapiro–Wilk test. Comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference (LSD) post hoc test. Statistical significance was set at $p < 0.05$.

# 3. Results

## 3.1 Cytocompatibility

All experimental groups maintained metabolic activity above 80% at 48 hours, indicating no cytotoxic effects. Both $\mathrm{Ca(OH)_2}$ NPs and CH-PLGA NPs demonstrated cytocompatibility comparable to conventional $\mathrm{Ca(OH)_2}$ and control groups. Exposure to lipopolysaccharide (LPS; 20 µg/mL) did not significantly reduce metabolic activity, confirming its suitability for inducing an inflammatory environment without compromising cell viability (Fig. 1).

## 3.2 Effects of $Ca(OH)_2$ NPs and CH-PLGA nanoparticles on osteogenic genes expression

Treatment with $Ca(OH)_2$ NPs and CH-PLGA NPs modulated osteogenic gene expression in primary human osteoblasts under inflammatory conditions. For alkaline phosphatase (*ALP*) mRNA expression (Fig. 2A), $Ca(OH)_2$ NPs demonstrated the highest upregulation (4.90 ± 6.05-fold), which was significantly higher than the LPS group. CH-PLGA NPs also showed increased *ALP* expression (4.08 ± 5.38-fold), although this did not reach statistical significance compared with LPS. Conventional $Ca(OH)_2$ exhibited moderate upregulation (3.02 ± 3.17-fold), while the osteogenic medium showed 2.28 ± 1.62-fold. For collagen type I alpha 1 chain (*COL1A1*) mRNA expression (Fig. 2B), CH-PLGA NPs exhibited the greatest upregulation (4.26 ± 3.79-fold), which was significantly higher than basal (1.24 ± 0.77-fold), LPS (1.05 ± 0.59-fold), conventional $Ca(OH)_2$ (0.64 ± 0.73-fold), and $Ca(OH)_2$ NPs (0.10 ± 0.08-fold, n = 7) ($p < 0.05$–0.001). In contrast, $Ca(OH)_2$ NPs and conventional $Ca(OH)_2$ demonstrated minimal or suppressed *COL1A1* expression relative to basal conditions. In contrast, runt-related transcription factor 2 (*RUNX2*) mRNA expression (Fig. 2C) showed no statistically significant differences among all experimental groups ($p > 0.05$). Overall, $Ca(OH)_2$ NPs predominantly enhanced early osteogenic differentiation as reflected by increased *ALP* mRNA, whereas CH-PLGA NPs exerted a more pronounced effect on extracellular matrix–related gene expression, as indicated by *COL1A1* upregulation.

## 3.3 Effects of $Ca(OH)_2$ NPs and CH-PLGA nanoparticles on mineralization

All experimental groups demonstrated increased mineral deposition (Fig. 3A). Treatment with $Ca(OH)_2$ resulted in moderate mineralization (2.05 ± 0.74 OD), comparable to the osteogenic medium. $Ca(OH)_2$ NPs (2.18 ± 0.81 OD) and CH-PLGA NPs (2.12 ± 0.76 OD) (Fig. 3B) also demonstrated increased mineral deposition compared with the LPS group. However, one-way ANOVA revealed no statistically significant differences among all experimental groups ($p > 0.05$) (Fig. 3B). Overall, although $Ca(OH)_2$ NPs and CH-PLGA NPs showed a trend toward increased mineralization compared with LPS, none of the nanoparticle formulations significantly enhanced mineral deposition under the present experimental conditions (Fig. 3B).

## 4. Discussion

The present study evaluated the effects of $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs on osteoblastic differentiation and function under inflammatory conditions. Overall, both nanoparticle formulations modulated osteogenic gene expression; however, their effects differed depending on the stage of osteogenic differentiation. A previous study demonstrated that CH-PLGA NPs inhibit osteoclast activity at a concentration of 5 mg/mL [13]. In the present study, the same formulation and preparation method were employed to further investigate their effects on bone remodeling, specifically focusing on cytocompatibility, osteoblastic differentiation, and mineralization.

Both conventional $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs were able to modulate osteogenic differentiation in primary human osteoblasts; however, their effects differed depending on the stage of differentiation. Notably, CH-PLGA NPs exhibited the highest *COL1A1* expression, suggesting enhanced extracellular matrix formation. This effect may be attributed to the controlled and sustained release of calcium ($Ca^{2+}$) and hydroxyl ($OH^-$) ions from the PLGA carrier [5,8], which may provide a more stable microenvironment for collagen synthesis. This is consistent with the findings of Han et al., who demonstrated that sustained release of $Ca(OH)_2$ loaded in microcapsules prolonged the upregulation of *COL1* [14]. In contrast, both conventional $Ca(OH)_2$ and $Ca(OH)_2$ NPs demonstrated relatively lower *COL1A1* expression. A possible explanation is that rapid ion release may induce cellular stress [15], which could negatively affect collagen production despite preserved cell viability [16]. These findings are consistent with Dvorak et al., who reported that extracellular calcium levels regulate *COL1A1* expression in a time- and concentration-dependent manner [17].

In contrast, $Ca(OH)_2$ NPs significantly increased expression of *ALP* mRNA compared with the LPS group, indicating enhanced early osteoblastic differentiation. Although CH-PLGA NPs also showed increased *ALP* expression, this effect was not statistically significant. These findings suggest that $Ca(OH)_2$ NPs may preferentially promote early-stage osteogenic activity, whereas CH-PLGA NPs may be more effective during later stages of differentiation [18]. Furthermore, both nanoparticle formulations appeared to attenuate the suppressive effects of LPS on osteoblastic activity. LPS is known to impair osteoblast differentiation through Toll-like receptor 4 (TLR4)-mediated inflammatory signaling and activation of MAPK pathways, leading to reduced *ALP* expression and increased apoptosis [19,20]. Therefore, the observed partial recovery of *ALP* expression may indicate a protective effect of these materials under inflammatory conditions.

*RUNX2* expression showed no statistically significant differences among groups, which may be explained by the timing of gene expression analysis. *RUNX2* is an early transcription factor that peaks during the initial stages of osteoblast differentiation and decreases as cells progress toward maturation [21–23].

Despite differences in gene expression, no statistically significant differences in mineralization were observed among groups. Although $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs showed a trend toward increased mineral deposition compared with the LPS group, these differences were not significant. This

suggests that enhanced osteogenic gene expression does not necessarily translate into increased functional mineralization under the present experimental conditions. Several factors may explain this discrepancy. Mineralization by osteoblasts requires an adequate supply of $Ca^{2+}$, and previous studies have shown that $Ca(OH)_2$ at concentrations as low as 0.25 mg/mL can initiate mineral deposition [24]. However, ARS staining has inherent limitations, particularly in systems containing calcium-based materials. Both $Ca(OH)_2$ and $Ca(OH)_2$ NPs exist in colloidal form and may directly bind to ARS, potentially leading to overestimation of mineralization. ARS is a non-specific calcium chelating dye and cannot distinguish between biologically deposited mineral and residual calcium-containing compounds [25]. Similar limitations have been reported in previous studies, where mineralization was assessed qualitatively without quantitative validation [26,27]. Taken together, these findings indicate that $Ca(OH)_2$ NPs and CH-PLGA NPs exert differential effects on osteoblastic differentiation. $Ca(OH)_2$ NPs appear to enhance early-stage osteogenic activity, whereas CH-PLGA NPs more effectively support extracellular matrix formation. However, neither formulation significantly improved mineralization under the present experimental conditions.

# 5. Limitation

Several limitations should be considered in the present study. First, the use of calcium-containing materials introduces inherent constraints in mineralization assays. Both $Ca(OH)_2$ and $Ca(OH)_2$ NPs exist in colloidal form and may directly interact with ARS, a non-specific calcium-binding dye. This interaction may lead to overestimation of mineral deposition, as ARS cannot differentiate between biologically formed mineral and residual calcium-containing particles. Second, gene expression was assessed at a single time point (day 14), which may not fully capture the dynamic progression of osteoblastic differentiation. To address these limitations, mineralization should be evaluated using complementary techniques, including calcium quantification assays, von Kossa staining, or advanced imaging modalities such as scanning electron microscopy to improve specificity and accuracy. Furthermore, time-course analyses at multiple intervals (e.g., days 3, 7, 14, and 21) are recommended to better characterize the temporal dynamics of osteogenic differentiation.

# 6. Conclusions

This study aimed to compare cytocompatibility, osteoblastic differentiation, and mineralization in primary human osteoblasts, with osteogenic responses evaluated under inflammatory conditions. The materials investigated were conventional calcium hydroxide, calcium hydroxide nanoparticles ($Ca(OH)_2$ NPs), and calcium hydroxide-loaded poly(lactic-co-glycolic acid) nanoparticles (CH-PLGA NPs). All three formulations showed favorable cytocompatibility at 0.5 mg/mL. Both nanoparticle formulations enhanced selected osteogenic gene expression relative to the LPS control, but neither significantly increased mineralization. These findings highlight the importance of controlled calcium ion delivery in modulating stage-specific osteogenic responses. From a translational perspective, $Ca(OH)_2$ NPs and CH-PLGA NPs may have potential applications in regenerative endodontics. However, further in vivo and

clinical studies are essential to confirm their safety, efficacy, and long-term outcomes in periapical tissue regeneration.

## Declarations

# Ethic approval

ethical approval obtained from the research ethics committee of the Faculty of Dentistry, Chiang Mai University (54/2025).

# Conflict of interest

The authors deny any conflicts of interest related to this study.

# Author Contribution

K.K.: Investigation, manuscript writing – Original Draft; P.R. and P.L.: investigation; W.L.: elaboration of concept, acquisition of funds; N.I.: elaboration of concept, manuscript writing - review and editing, supervision, acquisition of funds

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# Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## References

1. Siqueira, J. F. Jr. & Rôças, I. N. Present status and future directions: Microbiology of endodontic infections. *Int. Endod J.* **55** (Suppl 3), 512–530. 10.1111/iej.13677 (2022).
2. Chugal, N., Mallya, S. M., Kahler, B. & Lin, L. M. Endodontic Treatment Outcomes. *Dent. Clin. North. Am.* **61**, 59–80. 10.1016/j.cden.2016.08.009 (2017).
3. Usta, S. N. et al. Effectiveness of conservative instrumentation in root canal disinfection. *Clin. Oral Investig*. **27**, 3181–3188. 10.1007/s00784-023-04929-z (2023).

4. Naeem, M. M. et al. Effect of Propolis on Root Dentine Microhardness When Used as an Intracanal Medicament: An In Vitro Study. *J. Funct. Biomater.* **14** 10.3390/jfb14030144 (2023).
5. Chaiyosang, P., Mahatnirunkul, T. & Leelapornpisid, W. The Effects of Calcium Hydroxide-loaded Poly(Lactic-co-glycolic Acid) Biodegradable Nanoparticles in the ex vivo External Inflammatory Root Resorption Model. *J. Contemp. Dent. Pract.* **24**, 351–356. 10.5005/jp-journals-10024-3522 (2023).
6. Al-Hiyasat, A. S., El-Farraj, H. S. & Alebrahim, M. A. The effect of calcium hydroxide on dentine composition and root fracture resistance of human teeth: An in vitro study. *Eur. J. Oral Sci.* **129**, e12798. 10.1111/eos.12798 (2021).
7. Louwakul, P., Saelo, A. & Khemaleelakul, S. Efficacy of calcium oxide and calcium hydroxide nanoparticles on the elimination of Enterococcus faecalis in human root dentin. *Clin. Oral Invest.* **21**, 865–871. 10.1007/s00784-016-1836-x (2017).
8. Leelapornpisid, W., Wanwatanakul, P. & Mahatnirunkul, T. Efficacy of calcium hydroxide-loaded poly(lactic-co-glycolic acid) biodegradable nanoparticles as an intracanal medicament against endodontopathogenic microorganisms in a multi-species biofilm model. *Aust Endod J.* **50**, 89–96. 10.1111/aej.12812 (2024).
9. Dianat, O., Saedi, S., Kazem, M. & Alam, M. Antimicrobial Activity of Nanoparticle Calcium Hydroxide against Enterococcus Faecalis: An In Vitro Study. *Iran. Endod J.* **10**, 39–43 (2015).
10. Ramakrishnan, R., Daly, A. C. & Revisiting ISO 10993-5 In Vitro Cytotoxicity Standard Tests for Evaluation of Extracellular Matrix-Based Biomaterials. *International Journal of Biomaterials* 7395612, (2026). https://doi.org/10.1155/ijbm/7395612 (2026).
11. Chevalier, C. et al. Primary mouse osteoblast and osteoclast culturing and analysis. *STAR. Protocols*. **2**, 100452. https://doi.org/10.1016/j.xpro.2021.100452 (2021).
12. Kostina, D. et al. Isolation of Human Osteoblast Cells Capable for Mineralization and Synthetizing Bone-Related Proteins In Vitro from Adult Bone. *Cells* **11**, 3356 (2022).
13. Promta, P. et al. The Evaluation of Anti-Osteoclastic Activity of the Novel Calcium Hydroxide Biodegradable Nanoparticles as an Intracanal Medicament. *J. Endod.* **50**, 667–673. https://doi.org/10.1016/j.joen.2024.02.023 (2024).
14. Han, B. et al. The biological performance of calcium hydroxide-loaded microcapsules. *J. Endod*. **39**, 1030–1034. 10.1016/j.joen.2013.04.014 (2013).
15. Hao, Y., Yang, N., Sun, M., Yang, S. & Chen, X. The role of calcium channels in osteoporosis and their therapeutic potential. *Front. Endocrinol. (Lausanne)*. **15**, 1450328. 10.3389/fendo.2024.1450328 (2024).
16. Han, Y. High concentrations of calcium suppress osteogenic differentiation of human periodontal ligament stem cells in vitro. *J. Dent. Sci.* **16**, 817–824. 10.1016/j.jds.2021.02.011 (2021).
17. Dvorak, M. M. et al. Physiological changes in extracellular calcium concentration directly control osteoblast function in the absence of calciotropic hormones. *Proc. Natl. Acad. Sci. U S A*. **101**, 5140–5145. 10.1073/pnas.0306141101 (2004).

18. Zhu, S., Chen, W., Masson, A. & Li, Y. P. Cell signaling and transcriptional regulation of osteoblast lineage commitment, differentiation, bone formation, and homeostasis. *Cell. Discovery*. **10**, 71. 10.1038/s41421-024-00689-6 (2024).
19. Alonso-Pérez, A. et al. Role of Toll-Like Receptor 4 on Osteoblast Metabolism and Function. *Front. Physiol.* **9**, 504. 10.3389/fphys.2018.00504 (2018).
20. Guo, C. et al. Lipopolysaccharide (LPS) induces the apoptosis and inhibits osteoblast differentiation through JNK pathway in MC3T3-E1 cells. *Inflammation* **37**, 621–631. 10.1007/s10753-013-9778-9 (2014).
21. Komori, T. Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. *Int. J. Mol. Sci.* **20** 10.3390/ijms20071694 (2019).
22. Huang, W., Yang, S., Shao, J. & Li, Y. P. Signaling and transcriptional regulation in osteoblast commitment and differentiation. *FBL* 12, 3068–3092, (2007). 10.2741/2296
23. Komori, T. Regulation of bone development and extracellular matrix protein genes by RUNX2. *Cell Tissue Res.* **339**, 189–195. 10.1007/s00441-009-0832-8 (2010).
24. Narita, H., Itoh, S., Imazato, S., Yoshitake, F. & Ebisu, S. An explanation of the mineralization mechanism in osteoblasts induced by calcium hydroxide. *Acta Biomater.* **6**, 586–590. 10.1016/j.actbio.2009.08.005 (2010).
25. Addison, W. N. et al. Extracellular matrix mineralization in murine MC3T3-E1 osteoblast cultures: an ultrastructural, compositional and comparative analysis with mouse bone. *Bone* **71**, 244–256. 10.1016/j.bone.2014.11.003 (2015).
26. Khosropanah, H., Lashkarizadeh, N., Ayatollahi, M., Kaviani, M. & Mostafavipour, Z. The Impact of Calcium Hydroxide on the Osteoinductive Capacity of Demineralized Freeze-Dried Bone Allograft: an In-Vitro Study. *J. Dent. (Shiraz)*. **19**, 19–27 (2018).
27. Shalaby, R. A., Abdel-Aziz, A. M., Rashed, L. A. & Radwan, M. Z. The Effect of Calcium hydroxide, Glass Ionomer and light cured resin modified calcium silicate on viability, proliferation and differentiation of stem cells from human exfoliated deciduous teeth. *BMC Oral Health*. **23**, 721. 10.1186/s12903-023-03429-6 (2023).
28. Gavali, S. et al. Estrogen enhances human osteoblast survival and function via promotion of autophagy. *Biochim. Biophys. Acta Mol. Cell. Res.* **1866**, 1498–1507. 10.1016/j.bbamcr.2019.06.014 (2019).
29. Rickard, D. J. et al. Isolation and characterization of osteoblast precursor cells from human bone marrow. *J. Bone Miner. Res.* **11**, 312–324. 10.1002/jbmr.5650110305 (1996).
30. Bosetti, M., Zanardi, L., Hench, L. & Cannas, M. Type I collagen production by osteoblast-like cells cultured in contact with different bioactive glasses. *J. Biomed. Mater. Res. A*. **64**, 189–195. 10.1002/jbm.a.10415 (2003).

# Figures

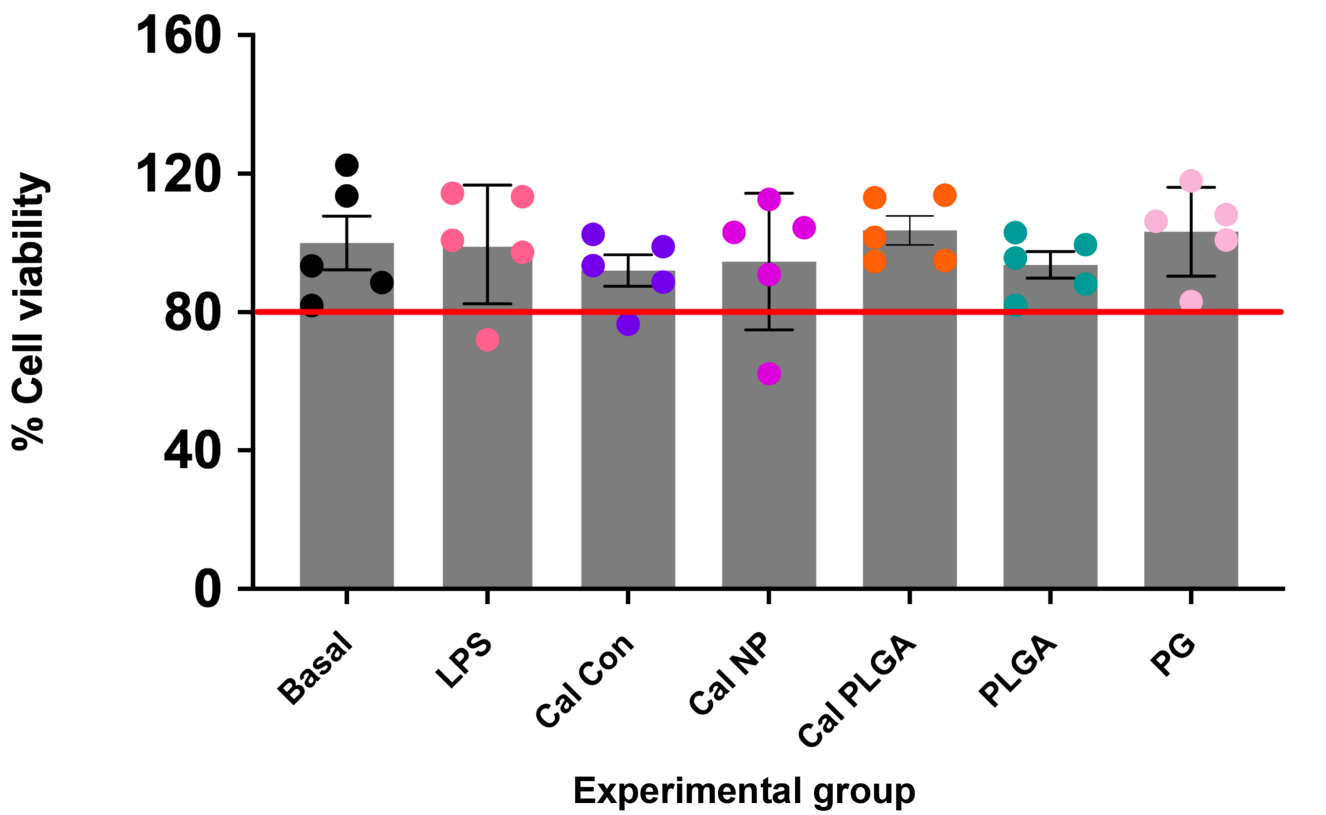
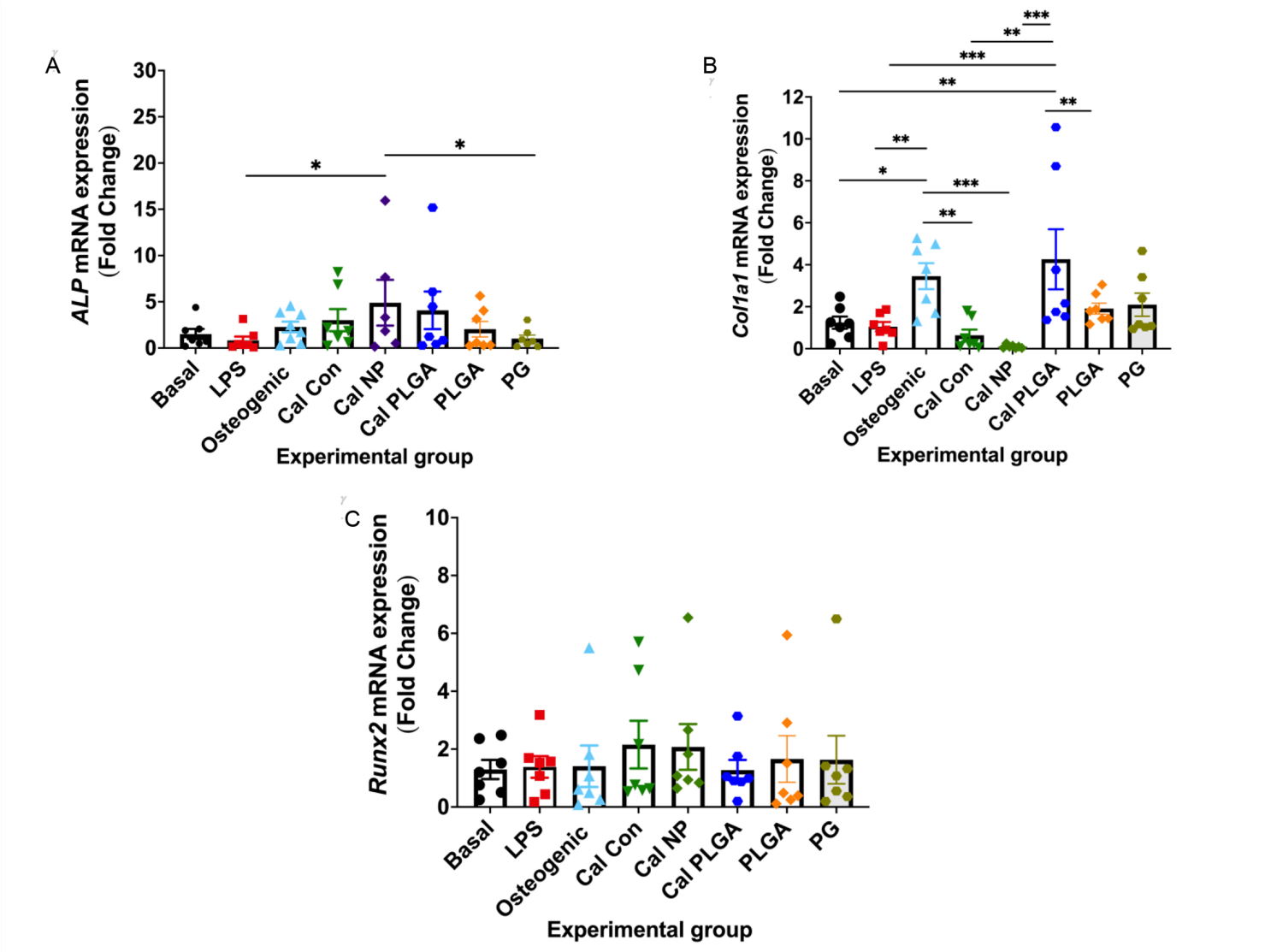



**Figure 1**

**Cell viability of primary human osteoblasts following treatment with $Ca(OH)_2$, $Ca(OH)_2$ NPs, and CH-PLGA NPs under inflammatory conditions.** Data are presented as mean ± standard deviation (SD) with individual data points representing cells derived from each donor. Cal Con, calcium hydroxide; Cal NP, calcium hydroxide nanoparticles; Cal PLGA, calcium hydroxide–loaded poly(lactic-co-glycolic acid) nanoparticles; Osteogenic, Osteogenic medium alone; PLGA, poly(lactic-co-glycolic acid); LPS, lipopolysaccharide; PG, Propylene Glycol.




## Figure 2

**Effects of $Ca(OH)_2$, $Ca(OH)_2$ NPs and CH-PLGA NPs on osteogenic gene expression in primary human osteoblasts.** Relative mRNA expression levels of osteogenic markers following treatment under different experimental conditions. **(A)**ALP, **(B)** COL1A1, and **(C)** RUNX2 mRNA expression were evaluated by real-time quantitative PCR at day 14. Data are presented as mean ± standard deviation (SD), with individual data points representing cells derived from each donor. Statistical significance was determined using one-way ANOVA followed by post hoc multiple comparisons ($p < 0.05$). ALP, alkaline phosphatase; COL1A1, collagen type I alpha 1 chain; Cal Con, calcium hydroxide; Cal NP, calcium hydroxide nanoparticles; Cal PLGA, calcium hydroxide–loaded poly(lactic-co-glycolic acid) nanoparticles; Osteogenic, Osteogenic medium alone; PLGA, poly(lactic-co-glycolic acid); LPS, lipopolysaccharide; PG, Propylene Glycol; RUNX2, runt-related transcription factor 2.

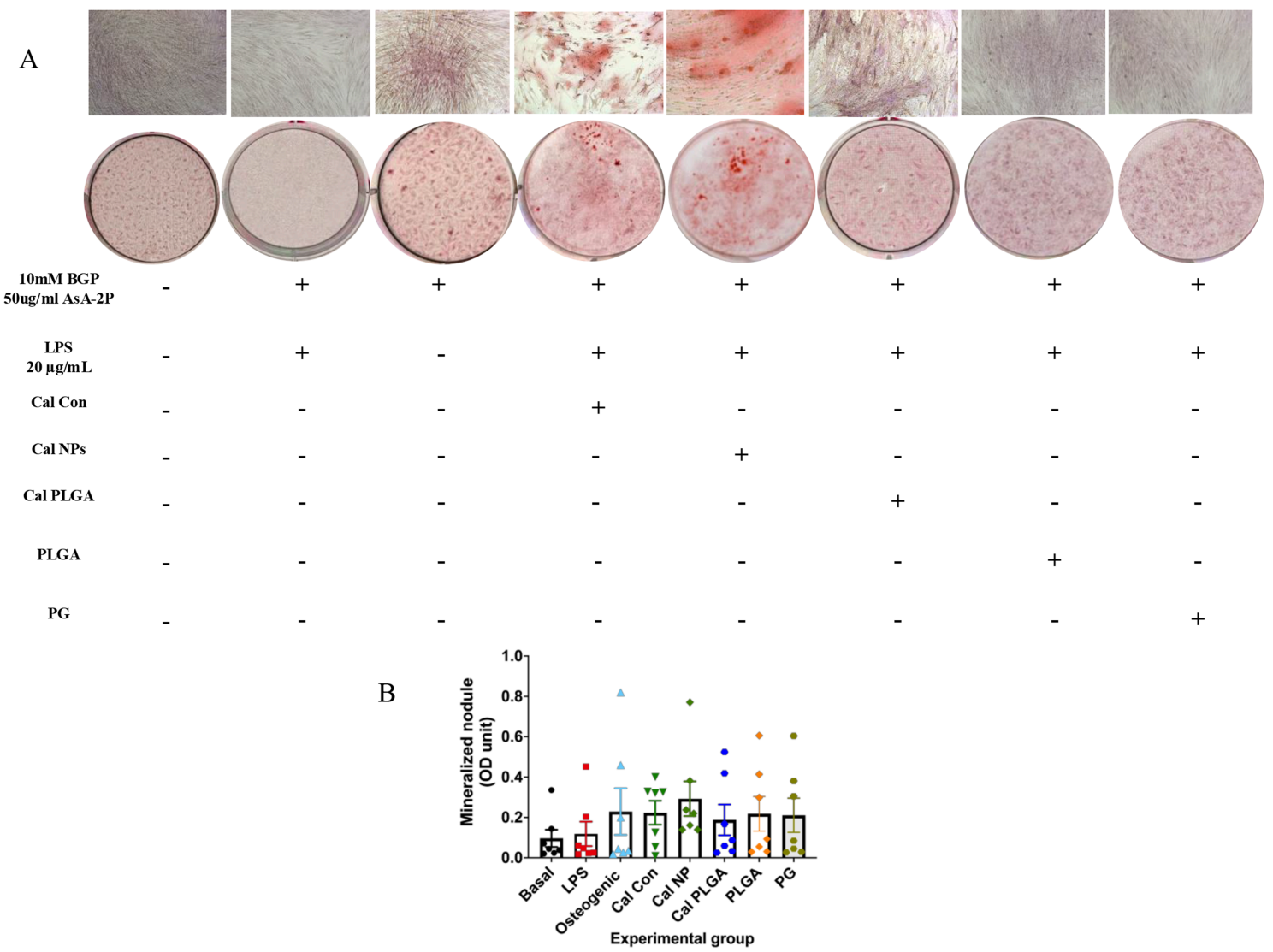



**Figure 3**

**Effects of $Ca(OH)_2$, $Ca(OH)_2$ NPs and CH-PLGA NPs on osteoblastic mineralization. (A)** Representative images mineralized nodule formation in primary human osteoblasts following Alizarin Red S (ARS) staining at day 21 under different experimental conditions**. (B)** Quantitative analysis of mineralization expressed as OD unit. BGP, β-glycerophosphate; AsA-2P, L-ascorbic acid; Cal Con, Conventional calcium hydroxide; Cal NP, calcium hydroxide nanoparticles; Cal PLGA, calcium hydroxide–loaded poly(lactic-co-glycolic acid) nanoparticles; Osteogenic, Osteogenic medium alone; PLGA, poly(lactic-co-glycolic acid); LPS, lipopolysaccharide; PG, Propylene Glycol; OD, optical density. Data are presented as mean ± standard deviation (SD), with individual data points representing cells derived from each donor.

# Supplementary Files

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- SupplementaryFigureS1.docx