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Effect of different calcium hydroxide formulations used during regenerative endodontic procedures on the viability, mineralization, and dentino/cemento/osteogenic differentiation potential of human periodontal ligament stem cells. An in-vitro study

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Aim: To evaluate the effect of CH and NCH on the viability, attachment, osteogenic, cementogenic and dentinogenic differentiation potential of hPDLSCs in-vitro.

Materials and Methods: Calcium Hydroxide (CH) and nano calcium hydroxide (NCH) powders were initially characterized using transmission electron microscope (TEM), X-ray Diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, and a zeta sizer instrument. Standard dentin discs were prepared, irrigated according to the Regenerative Endodontic Procedures (REP) protocol, then treated with 1 mg/ml of CH or NCH pastes, and incubated for 1 week. Untreated discs served as control. Then, they were washed off, and all discs were treated with 17% EDTA before seeding of human periodontal ligament stem cells (hPDLSCs) . Cell viability was assessed using MTT assay. Mineralized nodules and alkaline phosphatase (ALP) activity were determined to assess cell differentiation. Cell dentino/cementogenic and osteogenic potential were assessed through monitoring gene expression levels Dentin sialophosphoprotein (DSSP), Cementum protein 1 (CEMP1), Runt-related transcription factor 2 (RUNX2), Osteoprotegrin (OG), and Osteocalcin (OC) using RT-qPCR.

Results: TEM examination of NCH powder particles revealed a regular pattern and hexagonal shape with side dimension up to 60 ±10 nm. XRD analysis of NCH powder demonstrated its highly crystalline nature. FTIR spectroscopy confirmed that CH and NCH consisted mainly of pure calcium hydroxide. The zeta sizer instrument showed that NCH is positively charged with an average zeta potential of +27.8 mV. hPDLSCs seeded on NCH-treated discs showed significantly higher cell survival , more mineralization, higher alkaline phosphatase activity and increased expression of DSPP, CEMP1, RUNX2, OPG and OC markers.

Conclusion: NCH can be an alternative ICM during REP.

Keywords: nano-calcium hydroxide, intra-canal medication, stem cell viability, stem cell mineralization, stem cell differentiation.

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Introduction

Regenerative endodontic procedures (REPs) implemented in immature non-vital teeth are biologically based methodologies based on the principles of tissue engineering and the regenerative potential of resident mesenchymal stem cells. [1] Clinically, REP comprise minimal instrumentation, active irrigation, intra-canal medicament (ICM) application for 1-4 weeks, induction of bleeding, dressing by a bioactive material followed by an appropriate coronal seal. Clinical and radiographic outcomes are monitored up to 24 months. [2]

It is well acknowledged that eliminating all microorganisms from root canals is technically difficult due to the complex anatomy of teeth and the infiltration of bacterial products into dentin. [3] Because REPs involve stem cells, it is important to comprehend the interaction between the stem cells and the applied ICM. [4] ICM must have low cytotoxicity and high biocompatibility for their clinical application because they are likely to affect stem cells resident in the periapical tissues either by direct contact, or indirectly by altering dentin [5], such effect is time and dose-dependent.

The ICM frequently used in REPs is Calcium hydroxide (CH). [6] Its antimicrobial activity is via dissociation into calcium ions and hydroxyl ions creating an alkaline microenvironment that inhibits microbial growth. [7] On the other hand, CH may not be effective clinically; due to its inability to penetrate dentinal tubules, dentine buffering capacity, or the presence of resistant strains linked to refractory apical periodontitis. [8] CH is manufactured in different formulations including dry powder, paste in syringe and gels. Nanosized calcium hydroxide (NCH) has also been developed to benefit from the chemical and biological reactivity of nanomaterials. The higher charge density and higher surface area of nanomaterials allow better interaction with the microenvironment and the negatively-charged bacterial populations. [9] Also, they are expected to have deeper penetration into the dentinal tubules, hence can prevent bacterial repopulation.

Periodontal ligament stem cells (PDLSCs) represent a mesenchymal stem cell population vulnerable to be affected by ICM used during REPs. They can differentiate into various cell types. The strong association of PDLSCs with REPs is highlighted by hard tissue histologic analyses of case reports showing bone-like and/or cementum-like tissues after REP. [10]

Therefore, this study sought to evaluate the effect of CH and NCH on the viability, attachment, osteogenic, cementogenic and dentinogenic differentiation potential of hPDLSCs in-vitro. The null hypothesis tested was that there will be no difference between either material on the biological properties of stem cells.

Materials and Methods

All the experiments were conducted after approval of the Research Ethics Committee of the Faculty of Dentistry at The British University in Egypt (FD BUE REC 22-010).

NCH preparation

NCH was synthesized using ball milling from CH bulk powder (Nanogate, Cairo, Egypt). The powder was milled in a Planetary Ball Mill PM 400 milling machine (Retsch GmbH, Haan, Germany) for 10 hours at 3 minutes interval at speed 350 rpm.

NCH characterization

Transmission Electron Microscope (TEM)

NCH powder particles were observed under the JEOL JEM-2100 TEM to determine their morphology, size, and shape.

X-ray Diffraction (XRD)
An XRD analysis was performed on CH and NCH powders to determine the phases and degree of crystallinity using XPERT-PRO Powder Diffractometer system.

Fourier transform infrared (FTIR) spectroscopy
Molecular composition of CH and NCH powders were assessed by JASCO FTIR-6200 Spectrometer.

Particle size (PS) and Zeta potential (ZP) determination
Particle size and surface charge of NCH was determined by measuring its Zeta potential using Malvern Zeta sizer Instruments over 12 zeta runs for 120 s, with time interval 10 s at room temperature of 25°C.

CH and NCH pastes preparation
1 mg/ml of CH and NCH pastes were prepared by mixing 500 mg of CH and NCH powders with 0.5 ml of sterile water, respectively.

Dentin discs preparation
Dentin discs of 2 mm thickness were prepared from shaped root canals of mandibular molars using an IsoMet saw with a diamond disc under sterile phosphate-buffered saline (PBS) irrigation, and finally sterilized by ethylene oxide gas sterilization.

CH and NCH pastes application
REP protocol was followed to treat all the dentin discs. Discs were divided into 3 groups; discs treated with 2 ml of CH paste (CH group), discs treated with 2 ml of NCH paste (NCH group), and untreated discs that served as control (Control group). All samples were incubated for 7 days at 37°C. Then, ICM was washed off the treated discs with saline until complete removal was achieved. All discs were finally immersed in 17% EDTA for 1 min, then washed by saline solution before seeding of the hPDLSCs.

Isolation and characterization of hPDLSCs
Stem cell isolation, characterization, and confirmation of their multilineage differentiation was performed as described previously. [11-13]

Cell Viability Assay
The effect of the ICM on hPDLSCs viability was determined by the MTT assay as described previously. [14]

Osteogenic differentiation of hPDLSCs
Dentin discs were irrigated following the REP protocol, then incubated with CH and NCH for 7 days. At day 7, medicaments were removed and PDLSCs were seeded and cultured in osteogenic medium for 14 days. [15]

Alizarin red assay
The degree of mineralization was measured using Alizarin Red S stain as described previously. [16]

Alkaline phosphatase (ALP) enzyme activity determination
The activity of ALP enzyme was determined by following the kinetics of colorless para-nitrophenyl phosphate (p-NPP) conversion into yellow colored para-nitrophenolate (p-NP) as described previously. [13]

Osteo/odonto/cementogenic markers measurement
The expression of osteo/odontogenic and cementogenic markers was determined after osteogenic differentiation using qRT-PCR as described previously.[16] The primer sequences used for amplification are provided in Table 1.

Table 1: Primers used in RT-qPCR

	Forward sequence	Reverse sequence
OPG	CTAATTCAGAAAGGAAATGC	GCTGAGTGTTCTGGTGGACA
RUNX2	GTTATGAAAAACCAAGTAGCCAGGT	GTAATCTGACTCTGTCCTTGTGGAT
OC	CGCCTGGGTCTCTTCACTAC	CTCACACTCCTCGCCCTATT
DSPP	TCACAAGGGAGAAGGGAATG	TGCCATTTGCTGTGATGTTT
CEMP1	CCACACCTCAAAATCATCCTGCAGC	ATGGTTGGTCCACAGGGCTAGC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Statistical Analysis

Data were analyzed using SPSS version 22.0 statistical software (SPSS, Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± standard deviation (SD). Cell viability data was analyzed statistically by t-test and all other experiments were assessed using ANOVA test and the Tukey post-hoc analysis. *p*-value < 0.05 was considered significant.

Results

NCH characterization

Transmission Electron Microscope (TEM)

TEM images of NCH powder particles revealed that the particles are overlapped, had a regular pattern with a random orientation (Fig.1a). Higher magnification images (Fig.1b) confirmed that the particles are hexagonal in shape with dimension up to 60 ±10 nm.

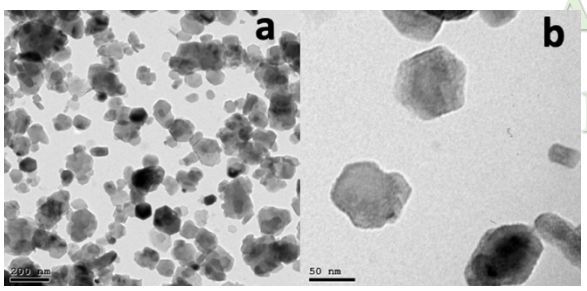

Fig. 1. TEM images of NCH powder.

X-ray Diffraction (XRD)

CH and NCH demonstrated a highly crystalline nature of their powders. Absence of a hump in the 2θ region 20° to 80° confirms the absence of amorphous structures in the synthesized powder. This confirms that there was no change to CH powder in the nanoform.

Fourier transform infrared (FTIR) spectroscopy

CH and NCH powders exhibited a sharp absorption band at 3641 cm^{-1} which, corresponds to the OH stretching and bending indicating that the powder consists mainly of pure CH. The broad band centered at 1400 cm^{-1} and 1447 cm^{-1} corresponds to the asymmetric stretching of the carbonate group. The sharp peak at 874 cm^{-1} and 876 cm^{-1} correspond to symmetric deformation of the carbonate group.

Zeta potential (ZP) determination

Result showed that NCH is positively charged with an average zeta potential of +27.8 mV.

Stem cell characterization

Cells displayed an adherent and fibroblast-like spindle shape, as well as growth in the form of colonies (Figures 2a & 2b). After culturing the hPDLSCs in special media for adipogenic, chondrogenic, and osteogenic differentiation, hPDLSCs showed trilineage differentiation potential. The multilineage differentiation was confirmed by morphological changes and special stains (Figure 2c).

Flow cytometry

hPDLSCs possessed high expression (>95%) of the MSCs-specific markers CD73, CD90, and CD105. The cell population also showed negative expression (<5%) for the hematopoietic stem cell markers CD34, CD45, and HLA-DR. Immunophenotyping results confirmed that these cells have the features of MSCs.

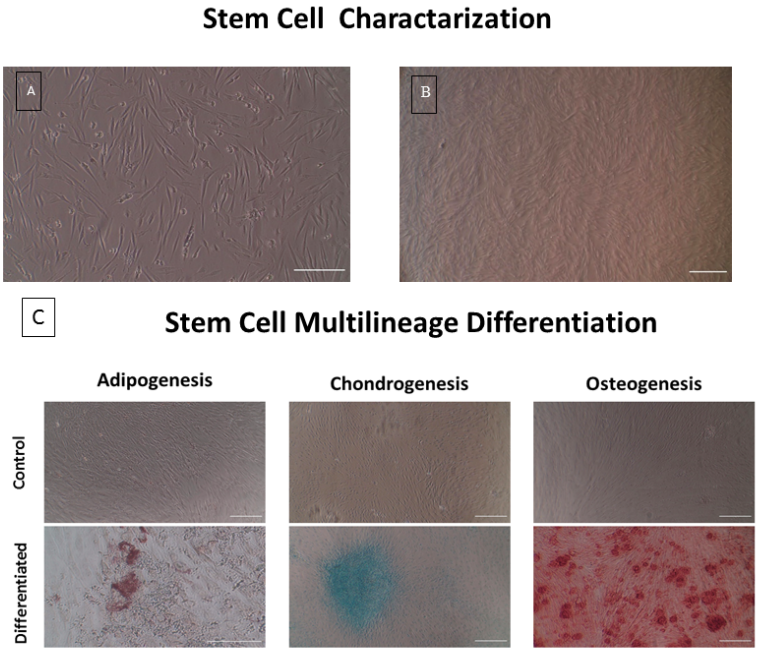

Fig. 2. a. Photomicrograph showing PDLSC cell morphology at passage 0 (P0), b. confluent PDLSC at passage 4 (P4), c. Multilineage differentiation of hPDLSCs. (Magnification 400x).

Cell viability assay

Results showed that CH and NCH had excellent cytocompatibility throughout all observation points (Figure 3). Inter-group comparison showed that NCH was associated with a significantly higher % of hPDLSCs viability at Day 3 and Day 7 ($p< 0.05$) compared to the control group.

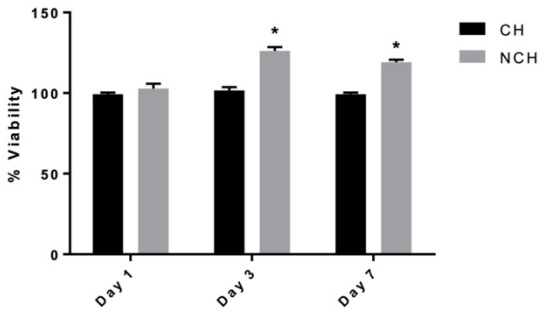

Fig. 3. MTT assay on day 1, day 3 and day 7.

Alizarin red assay

On day 14, Alizarin red staining was performed to determine the formation of mineralized nodules (Figure 4a) in the NCH, CH, and the positive control group (Osteo group). Quantitative analysis (Figure 4b) showed that hPDLSCs cultured with NCH-conditioned discs had the highest stain absorbance, reflecting the highest degree of mineralization potential that was statistically significant in comparison to all other groups ($p< 0.05$).

ALP activity

The kinetic profile of the ALP assay showed that hPDLSCs cultured with NCH-conditioned discs had the highest rate of p-NP over time. Statistical analysis of the slope of reaction in each group (Figure 4C), indicating the rate of ALP enzyme activity, confirmed that NCH-conditioned discs provided the most effect on increasing ALP activity, demonstrating its ability to accelerate the osteogenic differentiation potential of hPDLSCs, this was significantly higher from other groups ($p<0.05$).

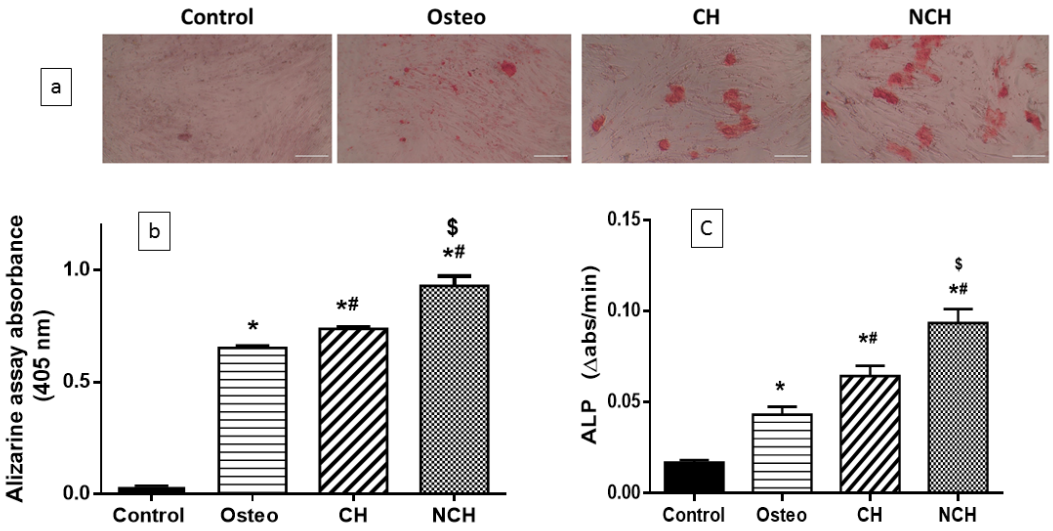

Fig.4. **a. ALZ red staining of hPDLSCs after 14 days of culturing in osteogenic medium. Magnification 100x, scale bar 250 µm, b. Absorbance values after of Alizarin Red S staining after solubilization, c. ALP activity after 14 days of osteogenic differentiation.**

PCR results

Results showed that hPDLSCs cultured with CH or NCH-conditioned discs possessed higher expression of the osteo/odontogenic genes as well as the CEMP1 cementogenic gene ($p<0.05$) compared to the Osteo and the control groups on day 14 of osteogenic induction of the hPDLSCs. The expression of the same respective genes was significantly higher in the NCH group compared to the CH group, with a 1.5-fold increase in the expression of RUNX2 and OPG, 1.3-fold increase in the expression of CEMP1, and 2.5-fold increase in the expression of DSPP (Figure 5).

Discussion

AAE guidelines recommend placing CH or antibiotic pastes inside root canals for 1-4 weeks for adequate disinfection. A review of in-vitro studies concluded that CH is preferred over antibiotic pastes prior to REP in regards of viability, proliferation, and differentiation of MSCs. [17]

Thus, we investigated the effects of CH and NCH used as ICM during REP on the viability, and hard tissue differentiation potential of hPDLSCs. Exploring such effects is important because they are assumed to be involved in periodontal regeneration and healing of apical periodontitis. [18,19]

Although disinfection is a key aspect of root canal treatment, a biological balance between disinfection and SC survival is crucial. Komabayashi et al. [20] reported that the particle size of most CH preparations is larger than that of dentinal tubules, Therefore, its penetration s is limited. Production of NCH enabled better and deeper drug penetration into dentin, resulting in greater efficacy. [21] Our results showed that both CH and NCH were cytocompatible to hPDLSCs, with dentin discs treated with NCH being associated with significantly higher cell viability.

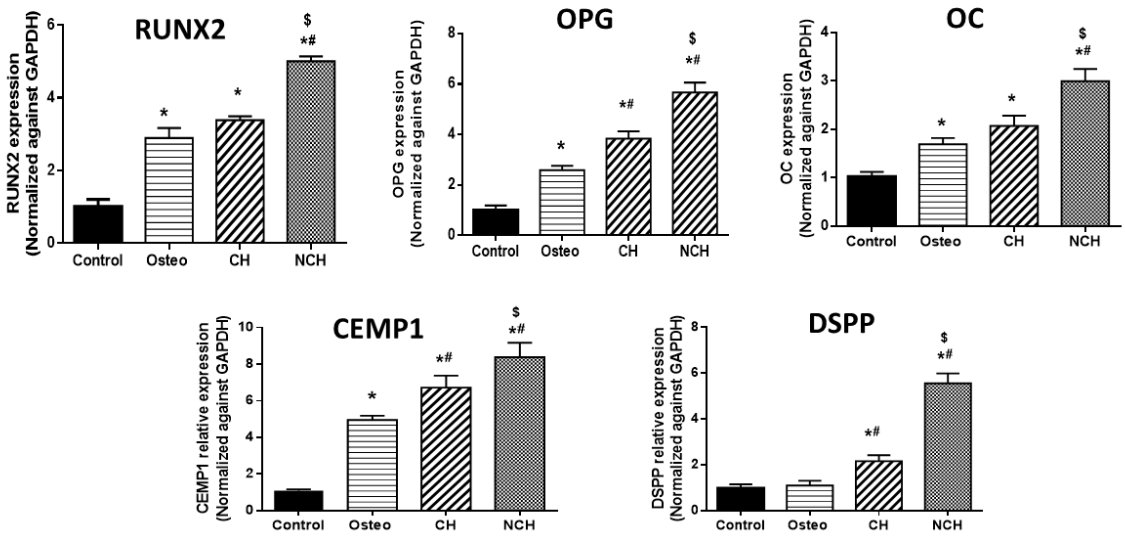

Fig. 5. Osteo/odontogenic and cemetogenic markers expression after 14 days of hPDLSCs osteogenic differentiation.

This favorable cellular behaviour can be attributed to the biological properties of the medications used, having positively charged powder particles that adheres to the negatively charged dentin, as well as the release of hydroxyl ions from prepared pastes that create an alkaline microenvironment. An alkaline pH has been reported to regulate various processes related to the function and survival of mesenchymal stem cells, including enzymatic activities and intracellular signaling.[22] This effect is expected to be more profound with NCH due to its increased surface area and reactivity. Another deciding factor that promoted hPDLSCs viability on dentin discs treated with either material was the relatively low drug concentration used to treat dentin (1 mg/ml). Ruparel et al.[23] reported that 1 mg/ml of drug concentration maintains the highest survival rate of stem cells, while Labban et al.[24] reported that CH below 2.5 mg% is nontoxic. Additionally, low concentrations of CH induced higher (pERK) expression which indicates PDLSC proliferation. [25] This agrees previous studies reporting increased cell survival of MSCs exposed to CH [26], or L929 murine fibroblasts exposed to CH and NCH.[27] However, our results partially agree Pudgee et al. [28] who reported significantly less survival of primary human dental pulp cells cultured for 1, 3, 5 , and 7 days with NCH in comparison to conventional CH.

The differentiation capability of hPDLSCs was assessed by three assays: cell mineralization, ALP activity, and examining gene markers expression. [29,30]

RUNX2 is considered to be the master-switch of osteoblast differentiation. [31,32] OC modulates energy use and control bone remodeling. [16] It is worthy to mention that ALP is an early differentiation marker of osteoblasts, while OC is a relatively late one.[33] OPG is critical in inhibition of bone resorption. [34] CEMP-1 protein is primarily associated with cementogenesis, while DSP is a major protein component of dentin and plays a key function in dentinogenesis.

Results of this study showed that hPDLSCs seeded on NCH-treated discs showed significantly more mineralization and a higher alkaline phosphatase activity. This agrees Mahran et al. [35] who reported that human dental pulp stem cells (hPDLSCs) co-cultured with NCH had significantly higher ALP activity.

Our results also showed that hPDLSCs seeded on NCH-treated discs had an increased expression of the gene markers DSPP, CEMP1, RUNX2, OPG and OC ($p<0.05$). This concurs Chen et al. [36] who reported similar effects for CH through the MAP-Kinase pathway, and Youssef et al. [37] who described ALP upregulation and increased expression of DSPP by DPSCs treated with CH. Another study by Han et al. [38] who reported an increased expression of OPG and inhibition of osteoclast activity by CH. Moreover, Paula-Silva et al. [39] reported that CH promoted cementogenesis.

Although in-vitro biologic assays are simpler, faster, repeatable, and consume small amounts of materials that have been evaluated. Future studies should consider the impact of the long term application of these materials on the fatigue life of treated teeth, being already immature and often compromised by caries or trauma. [40]

Conclusion

According to our findings, we conclude that NCH can be a valuable alternative to CH as an ICM during REPs.

References

[1] Saber SE. Tissue engineering in endodontics. J Oral Sci. 2009;51(4):495-507.

[2] [Regenerative Endodontics - American Association of Endodontists. Available online: https://www.aae.org/specialty/clinicalresources/regenerative-endodontics. Accessed 11 March 2024.

[3] Saber SM, Alfadag AMA, Nawar NN, Plotino G, Hassanien EE. Instrumentation kinematics does not affect bacterial reduction, post-operative pain, and flare-ups: A randomized clinical trial. Int Endod J. 2022;55(5):405-415.

[4] Fouad AF. The microbial challenge to pulp regeneration. Adv Dent Res. 2011;23(3):285-289.

[5] Silva EJ, Accorsi-Mendonça T, Almeida JF, Ferraz CC, Gomes BP, Zaia AA. Evaluation of cytotoxicity and up-regulation of gelatinases in human fibroblast cells by four root canal sealers. Int Endod J. 2012;45(1):49-56.

[6] Fahim MM, Saber SEM, Elkhatib WF, Nagy MM, Schafer E. The antibacterial effect and the incidence of post-operative pain after the application of nano-based intracanal medications during endodontic retreatment: a randomized controlled clinical trial. Clin Oral Investig. 2022;26(2):2155-2163.

[7] Kontakiotis E, Nakou M, Georgopoulou M. In vitro study of the indirect action of calcium hydroxide on the anaerobic flora of the root canal. Int Endod J. 1995;28(6):285-289.

[8] Saber Sel-D, El-Hady SA. Development of an intracanal mature Enterococcus faecalis biofilm and its susceptibility to some antimicrobial intracanal medications; an in vitro study. Eur J Dent. 2012;6(1):43-50.

[9] Al-Hassan Abdo Heidar S, Saber SE, Abdel-Aziz Eissa SAL, El-Ashry SH. Antibacterial potential of nanoparticulate intracanal medications on a mature E. faecalis biofilm in an ex vivo model. Ital Endod J 2020 Oct;34(2):100–9.

[10] Martin G, Ricucci D, Gibbs JL, Lin LM. Histological findings of revascularized/revitalized immature permanent molar with apical periodontitis using platelet-rich plasma. J Endod. 2013;39(1):138-144.

[11] Elashiry MM, Raafat SN, Tay FR, Saber SM. Effect of rapamycin on human periodontal ligament stem cells that have been exposed to sodium hypochlorite. Life Sci. 2023;329:121989.

[12] Saber SM, Gomaa SM, Elashiry MM, El-Banna A, Schäfer E. Comparative biological properties of resin-free and resin-based calcium silicate-based endodontic repair materials on human periodontal ligament stem cells. Clin Oral Investig. 2023;27(11):6757-6768.

[13] Bakr MM, Shamel M, Raafat SN, Love RM, Al-Ankily MM. Effect of pulp capping materials on odontogenic differentiation of human dental pulp stem cells: An in vitro study. Clin Exp Dent Res. 2024;10(1):e816.

[14] Sayed M, Mahmoud EM, Saber SM, Raafat SN, Gomaa SM, Naga SM. Effect of the injectable alginate/ nano-hydroxyapatite and the silica/ nano-hydroxyapatite composites on the stem cells: a comparative study. Journal of Non-Crystalline Solids. 2023;610:122327.

[15] Rady D, Albar N, Khayat W, Khalil M, Raafat S, Ramadan M, et al. Evaluation of

dental pulp stem cells response to flowable nanohybrid dental composites: A comparative analysis. PLoS ONE 2024;19(5): e0303154.
[16] Saber S, Raafat S, Elashiry M, El-Banna A, Schäfer E. Effect of Different Sealers on the Cytocompatibility and Osteogenic Potential of Human Periodontal Ligament Stem Cells: An In Vitro Study. J Clin Med. 2023;12(6):2344.
[17] Rahul M, Tewari N, Mathur V, et al. In-vitro Evaluation for Effects of Intracanal Medicaments on Viability, Proliferation, and Differentiation of Stem Cells From Apical Papilla - A Systematic Review. Eur Endod J. 2022;7(3):167-177.
[18] Panduwawala CP, Zhan X, Dissanayaka WL, Samaranayake LP, Jin L, Zhang C. In vivo periodontal tissue regeneration by periodontal ligament stem cells and endothelial cells in three-dimensional cell sheet constructs. J Periodontal Res. 2017;52(3):408-418.
[19] Shamel, M., Raafat, S., El Karim, I. et al. Photobiomodulation and low-intensity pulsed ultrasound synergistically enhance dental mesenchymal stem cells viability, migration and differentiation: an invitro study. Odontology (2024). https://doi.org/10.1007/s10266-024-00920-6.
[20] Komabayashi T, D'souza RN, Dechow PC, Safavi KE, Spångberg LS. Particle size and shape of calcium hydroxide. J Endod. 2009 Feb;35(2):284-7.
[21] Roy A, Bhattacharya J. Synthesis of Ca(OH)2 nanoparticles by wet chemical method. Micro & Nano Letters. 2010;5(2):4.].
[22] Monfoulet LE, Becquart P, Marchat D, et al. The pH in the microenvironment of human mesenchymal stem cells is a critical factor for optimal osteogenesis in tissue-engineered constructs. Tissue Eng Part A. 2014;20(13-14):1827-1840.
doi:10.1089/ten.TEA.2013.0500
[23] Ruparel NB, Teixeira FB, Ferraz CC, Diogenes A. Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. J Endod. 2012;38(10):1372-1375.
[24] Labban N, Yassen GH, Windsor LJ, Platt JA. The direct cytotoxic effects of medicaments used in endodontic regeneration on human dental pulp cells. Dent Traumatol. 2014;30(6):429-434.
[25] Ji YM, Jeon SH, Park JY, Chung JH, Choung YH, Choung PH. Dental stem cell therapy with calcium hydroxide in dental pulp capping. Tissue Eng Part A. 2010;16(6):1823-1833.
[26] Althumairy RI, Teixeira FB, Diogenes A. Effect of dentin conditioning with intracanal medicaments on survival of stem cells of apical papilla. J Endod. 2014;40(4):521-525.
[27] Dianat O, Azadnia S, Mozayeni MA. Toxicity of calcium hydroxide nanoparticles on murine fibroblast cell line. Iran Endod J. 2015;10(1):49-54.
[28] Pugdee K, Klaisiri A, Phumpatrakom P. The viability of human dental pulp cells and apical papilla cells after treatment with conventional calcium hydroxide and nanoparticulate calcium hydroxide at various concentrations. Saudi Dent J. 2023;35(8):1000-1006.
[29] Sudo K, Kanno M, Miharada K, et al. Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in vitro are present in most primary fibroblast-like cell populations. Stem Cells. 2007;25(7):1610-1617.
[30] Li J, Zhang F, Zhang N, et al. Osteogenic capacity and cytotherapeutic potential of periodontal ligament cells for periodontal regeneration in vitro and in vivo. PeerJ. 2019;7:e6589.
[31] Bruderer M, Richards RG, Alini M, Stoddart MJ. Role and regulation of RUNX2 in osteogenesis. Eur Cell Mater. 2014;28:269-286.
[32] Komori T. Regulation of Proliferation, Differentiation and Functions of Osteoblasts by Runx2. Int J Mol Sci. 2019;20(7):1694.
[33] Malaval L, Modrowski D, Gupta AK, Aubin JE. Cellular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. J Cell Physiol. 1994;158(3):555-572.
[34] Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997;89(2):309-319.
[35] Mahran AH, Fahmy SH, Ibrahim SS. Evaluation of Stem Cell Differentiation Medicated With Calcium Phosphate Nanoparticles in Chlorohexidine Paste. Bulletin of the National Research Centre. 2023; 47(1).
[36] Chen L, Zheng L, Jiang J, et al. Calcium Hydroxide-induced Proliferation, Migration, Osteogenic Differentiation, and Mineralization via the Mitogen-activated Protein Kinase Pathway in Human Dental Pulp Stem Cells. J Endod. 2016;42(9):1355-1361.
[37] Youssef AR, Emara R, Taher MM, et al. Effects of mineral trioxide aggregate, calcium hydroxide, biodentine and Emdogain on osteogenesis, Odontogenesis, angiogenesis and cell viability of dental pulp stem cells. BMC Oral Health. 2019;19(1):133.
[38] Han B, Wang X, Liu J, et al. Influence of calcium hydroxide-loaded microcapsules on osteoprotegerin and receptor activator of nuclear factor kappa B ligand activity. J Endod. 2014;40(12):1977-1982.
[39] Paula-Silva FW, Ghosh A, Arzate H, et al. Calcium hydroxide promotes cementogenesis and induces cementoblastic differentiation of mesenchymal periodontal ligament cells in a CEMP1- and ERK-dependent manner. Calcified Tissue International. 2010;87(2):144-157.

[40] Haridy MF, Ahmed HS, Kataia MM, et al. Fracture resistance of root canal-treated molars restored with ceramic overlays with/without different resin composite base materials: an in vitro study. Odont. 2022;110:497–507.