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Evaluation of stem cell differentiation medicated with calcium phosphate nanoparticles in chlorohexidine paste

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Abstract

Background Our present study aimed to evaluate the effect of calcium phosphate nanoparticles in chlorohexidine paste on the odontogenic differentiation of human dental pulp stem cells. The human dental pulp stem cells (HDPSCs) were examined for viability and proliferation via trypan blue stain test and MTT assay. This was followed by evaluating the alkaline phosphatase assay and detecting the Dentin matrix protein (DMP-1) marker via immunofluorescence staining to confirm the odontogenic differentiation.

Results In viability testing, nano calcium phosphate in Chlorohexidine (CHX) and nano calcium hydroxide showed no significant difference regarding trypan blue staining and MTT assay. Moreover, DMP-1 marker detection revealed a higher expression with nano calcium hydroxide compared to nano calcium phosphate in CHX, while ALP showed no significant difference.

Conclusion Based on the analysis conveyed, it can be concluded that nano calcium phosphate in CHX can induce the proliferation and differentiation of HDPSCs.

Keywords HDPSCs, Calcium phosphate, DMP-1, ALP

Background

Regeneration has recently been proposed as an alternative treatment option for conventional endodontic therapy. It is considered an ideal treatment to maintain tooth homeostasis, prevent reinfection, fractures, and preserve tooth longevity. Regenerative Endodontic Procedures (REP) including vital pulp therapy maneuvers have the potential to increase root length, thicken the root wall, and achieve apical closure. The biological concept of regenerative endodontics involved the triad of stem cells, scaffold platforms, and signaling molecules (Lopes et al. 2021).

Many clinical studies suggest that the natural healthy tissues are the most favorable substitution for lost tissues. These healthy tissues could be biotechnologically obtained from the miraculous stem cells. The human pulp dentin complex is the pretended tissue to be regenerated from the dental pulp stem cells (DPSCs). These stem cells are considered odontogenic progenitor cells with self-renewal and differentiation potentials. This capability could be induced by several bioactive materials which provide ions that activate the DPSCS to differentiate into a mineral-producing cell. Bioactive endodontic materials in the nano-scale exhibit enhancement in the surface-to-volume ratio, facilitating the delivery of growth factors that guide the migration, proliferation, and differentiation of stem cells. The gold standard essential bioactive materials used in regenerative procedures are calcium hydroxide and mineral trioxide aggregate.

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Calcium phosphates also possess high bioactive capability due to the release of calcium and phosphate ions which promote the cascade of mineral-producing cell differentiation (Akhlangi et al. 2015).

In a study observing wound healing, calcium phosphate nanoparticles in chlorohexidine paste was applied and showed the healing capability of the wound from 90 to 95% in 10 days (Viswanathan et al. 2016). The promising results of calcium phosphate nanoparticles in chlorohexidine paste on wound healing push us to investigate its capability in dental regeneration. Accordingly, our present study aimed to evaluate the effect of calcium phosphate nanoparticles in chlorohexidine paste on the odontogenic differentiation of HDPSCs in comparison with calcium hydroxide in the nano form as it is considered the gold standard intracanal medicament (Omaia et al. 2021).

Methods

Material preparation and characterization

Calcium phosphate nanoparticles in chlorohexidine paste

The synthesis of calcium phosphate nanoparticles [#918601] was obtained by Nano Gate company, (Cairo, Egypt). The manufacturer added 0.7 g of calcium L-lactate, 0.5 g of sodium dihydrogen phosphate, 100 mg of sodium hydroxide and mixed them with 30 mL of ultrapure deionized water. The sample was stirred for 30–60 min and then it was centrifuged for 3–5 min at 4000 rpm. The supernatant was discarded, and the particles were collected. The preparation of the pre-mixer was done by adding 50 mL of gelatin solution, 25 ml of 0.4 mg/L chlorhexidine solution, and 1 g of calcium phosphate nanoparticles were taken in a 100 mL flask, and it was mixed for 1 h. The final pH was adjusted to 6.5–7.5 using 0.1 N sodium hydroxide solution (Viswanathan et al. 2016). Transmission Electron Microscopy (TEM) was performed on JEOL JEM-2100, (JEOL Ltd., Japan) high-resolution transmission electron microscope (Fig. 2). (Kumar et al. 2016).

Nano calcium hydroxide

The nano-particulate Ca (OH)2 [#MB-302001] was obtained in the form of white powder and purchased from the Nano Gate company, (Cairo, Egypt). TEM was performed on JEOL (JEM-2100) high-resolution transmission electron microscope (Fig. 3). The methodology in preparation was carried out according to (Mohamed et al. 2019) who used the same particle size for regenerative procedures.

Sample collection and stem cell isolation

For this in-vitro testing, the dental pulp tissue was obtained from surgically extracted fully impacted immature (incomplete roots) 3rd molars from three healthy patients 18–20 years old at the maxillofacial surgery department, Faculty of Dentistry, Ain Shams University. Donors gave written informed consent according to the guidelines of the ethics committee of the faculty of dentistry, Ain Shams University. The HDPSCs were then isolated and cultured in Global labs, (Cairo, Egypt). An aseptic technique was followed during the procedure, the extracted tooth was placed in a tube containing 50 ml of tooth storage solution, phosphate buffered solution (PBS), with 100 U/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The crown of the tooth was then separated from the root using a fissure bur. The pulp tissue was removed from the canal using a small, barbed broach file. The collected pulp tissue was transferred into a sterile culture dish containing PBS and the antibiotic cocktail. The pulp tissue then was minced into pieces 2–4 mm using sterile surgical scissors. The tissue was digested using an enzyme solution containing collagenase type I and dispase and incubated for 1 h at 37 °C. 3 mL of the complete growth medium Alpha Modification of Eagle's Medium (α-MEM), supplemented with 15% FBS (fetal bovine serum), and 1% antibiotic/antimycotic (100 IU/mL of penicillin, 100 μg/mL streptomycin) was then added to stop the enzyme digestion. The tissue clumps were passed through a 70 μm cell strainer to obtain s single cell suspension and discard the supernatant. The obtained cells were then seeded in a Minimum Essential Medium (MEM) containing 10% fetal bovine serum and 1% of penicillin/streptomycin (100 U/mL penicillin and 100 g/mL streptomycin) and were incubated at 37 °C and CO_2 incubator until 70–80% confluence. The obtained cells were cryopreserved in 1% DMSO and FBS and stored at − 80 °C for further analysis (Kumar et al. 2016).

Characterization of isolated HDPSCs by flow cytometry

Flow cytometry was performed to determine the expression levels of the cell surface markers. The following antibodies were used CD45-PC5, CD44-FITC, and CD73PE. The cells were rinsed with PBS then 0.05% trypsin was added to it, the suspension was then incubated at 37 °C until cells were lifted. The suspension was then centrifuged for 6–7 min at least at 1300 rpm. Single-cell suspension (0.5^{x} 10^6/tube) was prepared and incubated with each antibody separately for 1 h. at 4 °C. The cells were then washed three times with PBS to remove unbound antibodies and finally resuspended with 300 μL PBS. The cells are analyzed using flow cytometer (Beckman Coulter MoFlo flow cytometer) after washing them.

The isolated HDPSCs were characterised using multiparametric flowcytometric analysis. The cells were labelled with three monoclonal antibodies: CD45-PC5,

CD44-FITC, and CD73PE. After sample processing, the data was analyzed using flowcytometry, and cells were gated using monoclonal antibody labelling (Ramos et al. 2016).

Experimental groups

The HDPSCs (4.5×10^5cells/well) were seeded on six-well plates and cultured until the cells reached 80% confluence and incubated at 37 °C and 5% CO_2 for 72 h.

- Group I: Triplicate of HDPSCs cultured with 5%calcium phosphate nanoparticles in 2% chlorohexidine paste.
- Group II: Triplicate of HDPSCs cultured with 20%nano calcium hydroxide.
- Group III: Positive control triplicate 2 mL of odonto-osteogenic differentiation medium (α-MEM containing 10% FBS, 100 μmol/L L-ascorbic acid, 10 mmol/L β-glycerophosphate, and 10 nmol/L dexamethasone).
- Group IV: Negative control triplicate HDPSCs were cultured in Dulbecco's Modified Eagles Medium (DMEM) media to serve as negative control (DMEM-NC) cells (no differentiation potential).

Evaluation of the HDPSCs proliferation

Cell counting using trypan blue solution

Trypan blue solution (cGMP, Grand Island, New York) was prepared in PBS at a concentration of 0.4 g/mL. For the study of cell viability of the ex-vivo grown mass of dental pulp stem cells, TB solution was added at the 1:1 ratio, the mixture was kept in an incubator for 2 min at 37 °C. The cells in triplicates were loaded on a hemocytometer and observed under a phase-contrast microscope at 400X magnification. The live cells remained unstained, whereas the nuclei of dead cells appeared blue, as TB is a membrane-permeable solution that enters dead cells and stains the nuclei to blue, whereas the viable cells remain unstained (Luke et al. 2020). The total number of viable cells per ml of aliquot is obtained by multiplying the number of viable cells by 2(dilution factor of trypan blue) while the total number of cells per ml aliquot is obtained by adding the total number of viable cells and dead cells multiplied by 2 (Fig. 5).

Cell proliferation by (MTT) assay

Cell proliferation was measured using an MTT assay kit (Thermo Fisher, Germany). For conducting the experiment, suspensions of HDPSCs at a concentration of 2×10^4 cells/well were seeded into 96-well plates in DMEM supplemented with 10% FBS and incubated for 24 h, at 37 °C and 5% CO_2. Then the culture media was replaced with the medications according to the experiment design (OM for PC, DMEM for NC, 5% nano calcium phosphate in 2% chlorohexidine and 20% nano calcium hydroxide). The tested materials were added directly in a paste form, then the cells were incubated for 72 h at 37 °C and 5% CO_2. After 72 h, MTT solution (1 mg/ml) was added to each well and incubated for 4 h. Finally, the MTT solution was removed and 100 μL of sulphur dodecyl sulfate-hydrochloric acid (SDS-HCL) was added to each well, and the optical density was measured at 570 nm on a multi-well plate reader. Background absorbance of the medium in the absence of cells was subtracted (Fig. 1). (Woo et al. 2015).

Evaluation of the HDPSCs differentiation

Assessment of alkaline phosphatase activity of differentiated HDPSCs after 72 h

Alkaline phosphatase activity was measured for each triplicate of the experimental groups in the supernatant of differentiated cells after 72 h. with an ALP kit (Sigma, U.S.A.). Briefly, cells in a 96-well plate were washed with 0.01 PBS three times, then lysed in 50 mL Lysis Buffer and incubated at 4 °C overnight. Then 50 mL alkaline buffer solution and 50 mL stock substrate solution were added and mixed well. Fifteen minutes later, 110 mL of 0.5 NaOH was added to stop the reaction. The optical density (OD) values were obtained at 405 nm. The ALP assay was repeated at least three times. A standard curve

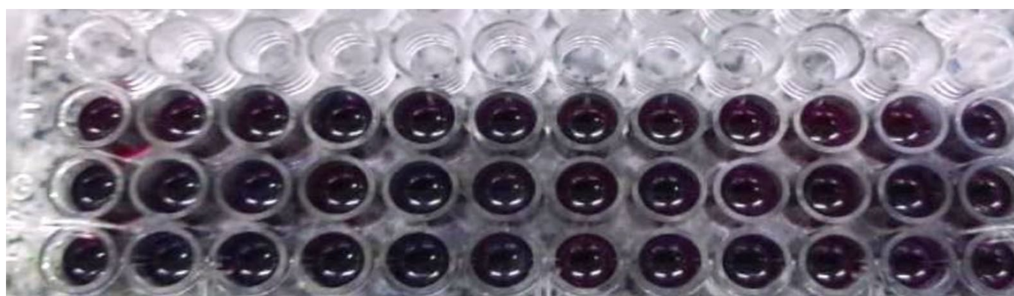

Fig. 1 MTT assay, well plate optical density indication for cell viability

of absorbance versus concentration was generated and used to determine the ALP activity (U/L) (Lv et al. 2016).

Immunological assessment of differentiated HDPSCs

Cells were seeded on poly-L-lysine-treated glass coverslips (8000 cells/well). After reaching 30% confluence. Monolayers were fixed with paraformaldehyde (4%), permeabilized, and incubated over a night at 4 °C with primary antibody: anti-DMP-1 (Sigma-Aldrich, U.S.A.). It was prepared in a blocking buffer at 1: 50 dilution. Goat anti-rabbit IgG biotinylated antibody was added at room temperature, the samples were washed, and peroxidase coupled streptavidin (Thermo Fisher Scientific, Germany) was added. The slide was covered with Prolong Gold Antifade Reagent (Abcam, U.S.A.) and mounted overnight at room temperature. The specimens were immediately examined or stored at 4 °C and protected from light for long-term storage.

The microscopic examination was performed by a LABOMED Fluorescence microscope (LX400, cat no: 9126000, labo-America, Inc, U.S.A.) (Fig. 6).The Immunofluorescence staining intensity was scored according to a four-tier system: 0, no staining; 1+, weak; 2+, moderate; and 3+, strong. In brief, the H-score of each sample was calculated as the sum of each intensity (0–3) multiplied by the percentage of positive cells (0–100%). The score ranged from 0 to 300. The median value of the H-score was also calculated (Inagaki et al. 2015).

Results

Characterization of the nanomaterials by TEM

Calcium phosphate nanoparticles

The well-crystallized Nano calcium phosphate particles were needle or rod-like, and their dimensions were approximately (110 ± 10) nm in length and (25 ± 5) nm in width, (Fig. 2).

Nano calcium hydroxide

The Nano-sized Ca(OH)2 particles were cubic and spherical with the particle size of 15.57 ± 3.14 nm (Fig. 3).

Characterization of isolated HDPSCs by flow cytometry

The Beckman Coulter Navios software was used to analyze flow cytometry data. The histograms of logarithmic dot plots are shown in (Fig. 4). Multiparametric analysis using three unique markers was used to stain the isolated HDPSCs, as seen in flow cytometric images (CD44, CD73 and CD45). 76.1% of cells displayed double brilliant surface expression of CD44/CD73, compared to only 3.9% of cells that were double negative for both biomarkers Fig. 4a, according to the findings. Furthermore, 51.5 percent of the subjects were CD44+/CD45—Fig. 4b, but 76.1 percent were CD73+/CD45—Fig. 4c. To confirm the non-hemopoietic origin of stem cells, CD73 and CD44 cells were gated with CD45' hemopoietic stem cell marker. The data revealed that 22.0% Fig. 4b and 19.0% Fig. 4c of CD44 and CD73 positive cells were expressed respectively while the expression of CD45, indicated that the stem cells were recovered from a non-hematopoietic source.

Cell count of HDPSCs by trypan blue dye

The mean and standard deviation of all groups regarding cell count, dead cells, and viable cells were presented in Table 1. Cell count in group II was significantly the highest, while group IV was significantly the lowest. Group I and III were between the other groups with insignificant differences between them. Dead cells in group IV were

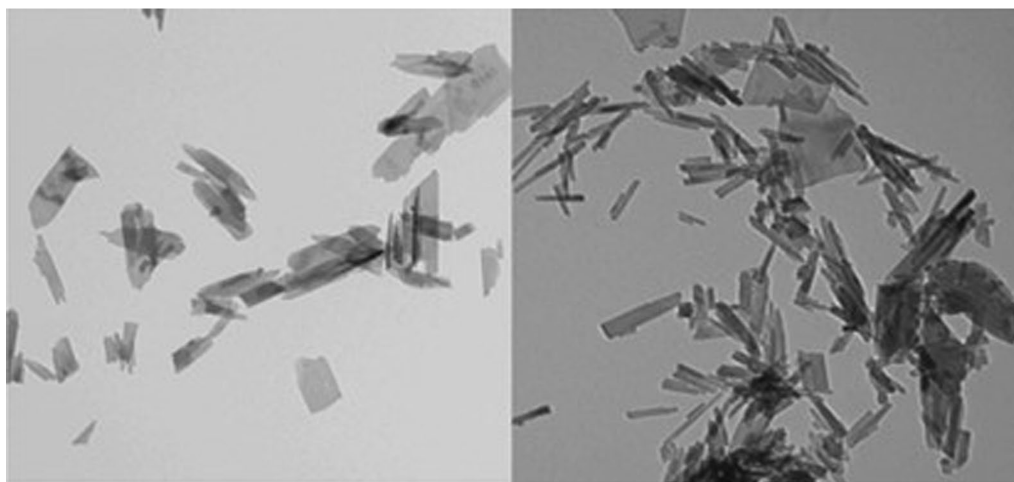

Fig. 2 TEM showing nano calcium phosphate particles indicating needle-like crystals with width (25 ± 5) nm and length (110 ± 10) nm

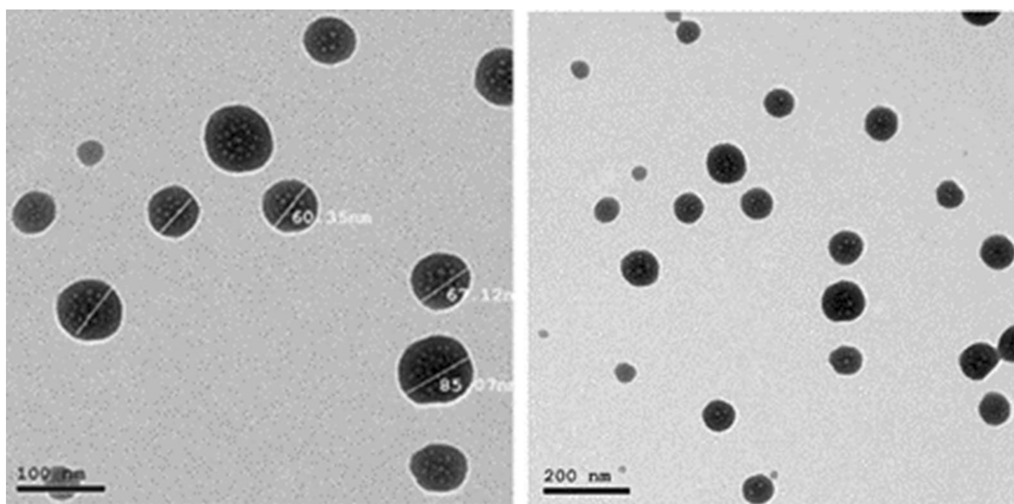

Fig. 3 TEM of nano calcium hydroxide particles indicating spherical and cubic shape with 15.57 ± 3.14 nm size

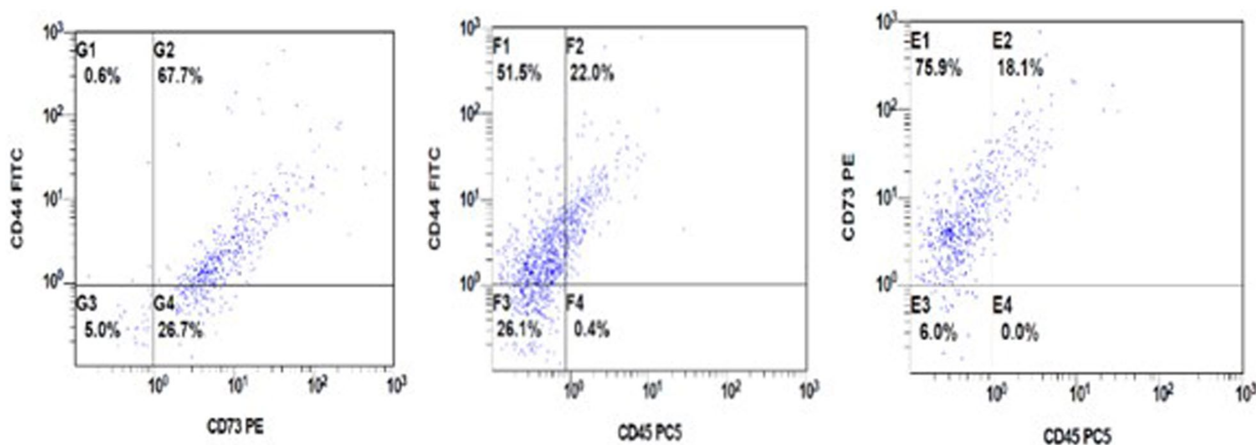

Fig. 4 A respective FCM dot-plot showing the gate protocol for HDPSCs. The HDPSCs were stained with stem cell markers (CD73, CD44, CD45). The CD73 and CD44 positive cells were gated cross-ponding to CD45

Table 1 Cell count by trypan blue dye of HDPSCS treated with different medications for differentiation

	Group I [N-Ca3(PO4)/CHX]		Group II [N-Ca (OH)2]		Group III [OM-PC]		Group IV DMEM [NC]		*p*-value
	M	SD	M	SD	M	SD	M	SD	
Cell count	6.60^{ab}	0.08	6.78^{ab}	0.18	7.20^{a}	0.09	6.19^{b}	0.57	0.02*
Dead cells	4.45^{a}	0.22	4.05^{a}	0.52	4.55^{a}	0.16	5.46^{b}	0.08	0.002*
Viable cells	6.60^{ab}	0.09	6.80^{ab}	0.17	7.21^{a}	0.12	6.32^{b}	0.55	0.03*

p: Probability level which is significant at $p \leq 0.05$ calculated for the tested groups in a raw

Means (M) with different superscript letters within the same horizontal row are significantly different

M Mean, *SD* Standard deviation

*$P \leq 0.05$

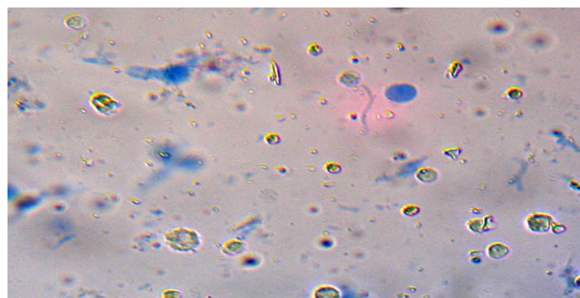

Fig. 5 HDPSCs stained with trypan blue solution

significantly the highest, while there was no significant difference between other groups. Viable cells in group II was significantly the highest, while group IV was significantly the lowest and there was no significant difference between other groups, (Fig. 5).

Cell proliferation assay (MTT) after 72 h of incubation of HDPSCs

The mean and standard deviation of all groups regarding cell proliferation assay (MTT) after 72 h of incubation was presented in Table 2. Group II and group I were significantly the highest with no significant difference between them while group IV was significantly the lowest.

Table 2 MTT assay after 72 h

	OD at 570 nm		
	M	**SD**	***p* value**
Group I[N-Ca3(PO4)/CHX]	1.46^{b}	0.06	0.0001*
Group II [N-Ca (OH)2]	1.56^{b}	0.10	
Group III [OM-PC]	1.17^{a}	0.16	
Group IV [DMEM-NC]	0.83^{c}	0.08	

Incubation of HDPSCs treated with different medications for differentiation

p: Probability level which is significant at $p \leq 0.05$

Means (M) with different superscript letters within the same vertical column are significantly different

M Mean, *SD* Standard deviation

$^{*}P \leq 0.05$

Table 3 Alkaline phosphatase assay after 72 h

	Concentration (U/L)		
	M	**SD**	***p*-value**
Group I [NCa3(PO4)/CHX]	72.24^{ab}	9.79	0.01*
Group II [N-Ca (OH)2]	81.16^{a}	11.01	
Group III [OM-PC]	61.05^{ab}	8.28	
Group IV [DMEM-NC]	50.26^{b}	6.81	

Incubation of HDPSCs treated with different medications for differentiation

p: Probability level which is significant at $p \leq 0.05$

Means (M) with different superscript letters within the same vertical column are significantly different

M Mean *SD* Standard deviation

$^{*}P \leq 0.05$

Alkaline phosphatase assay after 72 h of incubation HDPSCs

The Mean and standard deviation of all groups regarding Alkaline phosphatase assay after 72 h incubation were presented in Table 3. Group II was significantly the highest followed by group I and group III with no significant difference between them. Group IV was significantly the lowest.

Assessment of DMP-1 expression in HDPSCs using immunofluorescence staining

The mean and standard deviation of all groups regarding DMP-1 protein expression was presented in Table 4 and Fig. 6. Group II was significantly the highest followed by group I and III with insignificant difference between them while group IV was significantly the lowest.

Discussion

Stem cells derived from dental pulp tissue have been investigated in many studies to be used as a treatment element to regenerate biological tissues and to replace the traditionally existing root canal therapy (Hu et al. 2019). HDPSCs which are present within the "cell-rich zone" of the dental pulp serves as a source of primitive cells that are able to replace damaged cells at the site of injury, it also shows the ability to be induced to secret dentine-like matrix by stimulating dentinogenesis in HDPSCs and promoting their proliferation, differentiation, and mineralization in vitro (Kim et al. 2019).

Since the extracellular matrix and mineral crystals in dental tissue have nanoscale patterning, nanotechnology could be useful in improving the properties of the materials that are involved in repairing these tissues (Huang et al. 2016). Endodontic medications in the nanoscale showed enhanced antibacterial effect while maintaining their cytotoxicity, another beneficial achievement was the increase in the potency without the need to increase the volume (Khaled Hassan Abd El Bary et al. 2019). So, in this study, we attempted to obtain medication with a maximized beneficial effect by developing a paste of calcium phosphate nanoparticle mixed with chlorhexidine and tested its effect on HDPSCs, and evaluated the ability of these primitive cells to differentiate into (Odontoblast like cells) mineral-releasing cells that could be used in tooth repair and regeneration.

Calcium phosphates (CaPs) are the most extensively used bone substitutes in bone tissue engineering because they imitate the bone mineral with perfect biocompatibility. It can act as a vehicle for delivering growth factors

Table 4 Assessment of DMP-1 protein expression in HDPSCs using immunofluorescence staining

	HDPSCs stained with DME		
	H-score		*p*-value
	M	SD	
Group I [N-Ca3(PO4)/CHX]	134.00[a]	11.37	0.0001*
Group II [N-Ca (OH)2]	164.00[b]	13.92	
Group III [OM-PC]	110.00[a]	9.34	
Group IV [DMEM-NC]	15.00[c]	1.27	

p: Probability level which is significant at $p \leq 0.05$

Means (M) with different superscript letters within the same vertical column are significantly different

M mean, *SD* Standard deviation

*$P \leq 0.05$

and drugs in tissue engineering. The calcium phosphate nanoparticles easily disintegrate in acidic pH, thereby releasing calcium ions into the inflamed tissue (Viswanathan et al. 2016).In addition, the self-setting and the moderate compressive strength even in loading conditions suggest that CaPs are superior to pure calcium hydroxides, it also shows higher cell viability and lowers inflammatory mediation in periodontal ligament cells when compared to other sealers. The reduction in the particle size has been reported to produce a substantial decrease in the setting time and acceleration in the hardening of the CaPs. Furthermore, the different topography of the CaPs have been shown to significantly affect the behavior of osteoblast-like cells, showing a slight decrease in the proliferation rate but with substantially improved differentiation activity (Perez et al. 2013). Loading these CaPs on a paste such as chlorhexidine may enhance its properties. This may be explained by the ability of CHX-derived products to rapidly kill a broad spectrum of bacteria and fungus, it also inhibits biofilm formation. Chlorhexidine exhibits positively charged molecules that are easily bound to negatively charged cell surfaces causing the cell surface rapture on the interface with osmosis and attacking the cytoplasmic membrane leading to cell death (Viswanathan et al. 2016).

The particle size and shape of the calcium phosphate nanoparticles and nano calcium hydroxide were analyzed using the Trans electron microscope (TEM) imaging. Accordingly, the calcium phosphate nanoparticles were prepared with average size of (25 ± 5) nm width and (110 ± 10) nm length. This is supported by (Hu et al. 2007) who used Calcium phosphate with higher crystallinity and stated that it significantly promoted the proliferation of bone marrow mesenchymal stem cells more than amorphous calcium phosphate. In addition, crystallized calcium phosphate as hydroxyapatite yielded higher rates of differentiation of BMSCs to osteoblasts than amorphous calcium phosphate. The particle size of the nanosized Ca(OH)2 particles was found to be 59.82 ± 19.62 nm (41.01–86.87 nm). This comes in agreement with (Khaled Hassan Abd El Bary et al. 2019) who used nano-sized calcium hydroxide paste in studying its effect regarding the regenerative procedure.

The characterization of HDPSCs by detecting the surface molecular markers of the antibody present on the stem cells was performed using the by flow cytometric analysis. The HDPSCs possess mesenchymal stem cell markers which are usually targeted for detection. Each Cluster of Differentiation (CD) marker is conjugated with a different dye in order to be directed to different gates according to each dye. The cultured cells were allowed to pass single cell at a time. The markers that were detected are CD73-APC, CD45-APC, CD44-PE. CD45 are usually used to assist in the identification of hematopoietic stem

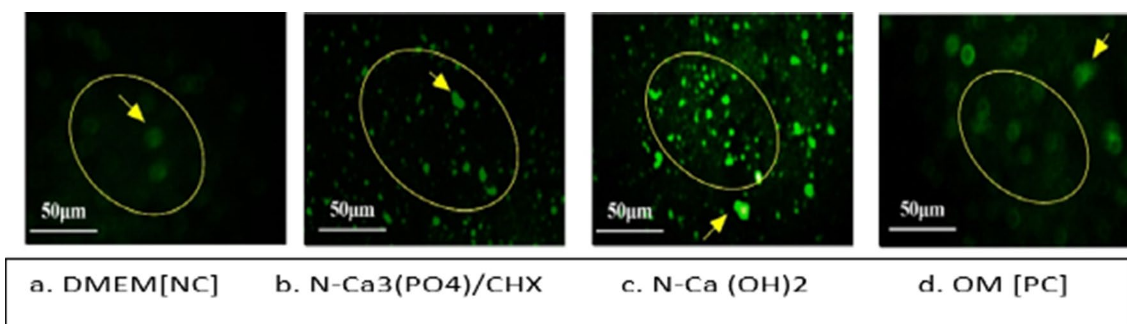

Fig. 6 Expression of DMP1 protein expression in differentiated odontoblasts by immunofluorescence **a** a discrete of cells with a homogeneous faint expression of DMP with immunofluorescence intensity (+) was found in the negative control cells The expression was localized to the cell membrane. **b**, **c** HDPSCs co-cultured with N-Ca3(PO4)/CHX, N-Ca(OH)2 revealed a merged big colony of odontoblasts with dense homogeneous membrane and nuclear DMP expression. **d** HDPSCs were co-cultured in an Osteogenic medium (OM), resulting in an increased number of odontoblast colonies with faint homogeneous DMP expression (++) and an H-score of 110. The magnification is 10X. The odontoblast colonies are highlighted by the yellow circles, and the yellow arrow indicates DMP expression in the membranous and nuclear compartments

cells in several species including humans, while CD44, CD73 are considered a human mesenchymal stem cell marker that when detected indicates that the stem cell is of human origin. The obtained stem cells were positive to CD44 and CD 73. Unfortunately, there is no distinct marker characterizing HDPSCs because they are a heterogeneous and mixed population (Ramos et al. 2016).

The extracted third molars were chosen to be the source of HDPSCs due to their easy surgical access and high efficacy of stem cell extraction from the pulp tissue. 5%nano calcium phosphate in 2% chlorohexidine paste concentrations according to (Viswanathan et al. 2016, Ahmed et al. 2021) and 20% nano calcium hydroxide according to (Mohamed et al. 2019) were added on the HDPSCs along with a positive group containing the HDPSCs with the osteo-odontogenic differentiation medium and a negative group containing the HDPSCs and DMEM media. A basic property of HDPSCs is that they form mineralized tissues, with odontoblast differentiation only induced under specific conditions. Osteo-odontogenic media was applied to induce odontogenic differentiation (Graziano et al. 2008) (Fang et al. 2012). All groups were experimented to reveal their ability for odontogenic differentiation.

Testing the influence of nano calcium phosphate in chlorohexidine on viability and proliferation was done using the trypan blue dye exclusion test and MTT assay. Cell proliferation and viability are important parameters for studying the biological effect (such as differentiation) of nanoparticles. In the current study, HDPSCs have been cultured with the tested medications for 72 h. the viable and dead cells were counted by trypan blue dye. The results revealed that the cell viability was high in nano calcium hydroxide and nano calcium phosphate in chlorohexidine group with no significant difference. Regarding viability, HDPSCs cultured with nano calcium hydroxide showed the highest viability followed by the nano calcium phosphate in chlorohexidine paste with no significant difference between them. These results came in line with (Dianat et al. 2015) where they found that the cytotoxicity of nano calcium hydroxide decreased after 72 h, indicating more cell viability. (Li et al. 2019) also found that the nano-hydroxyapatite (crystalline calcium phosphate) with nanoparticles approximately 20 showed higher mesenchymal DPSCs viability after incubation for 72 h. compared to other np HAP of different sizes. Moreover, the negative control group containing HDPSCs with DMEM showed lower cell count and viability compared to the positive control group as the odontogenic differentiation medium containing alpha modified essential medium is enriched with more non-essential amino acids and vitamins and other predominant nutritional elements than DMEM. This comes in agreement with (Patel et al. 2018) who found that α-MEM was superior to DMEM for in-vitro generation of Adipose-Derived Mesenchymal Stem Cells regarding cell count and viability.

In order to verify the ability of the isolated HDPSCs to differentiate into odontoblast-like cells several investigations were carried out on different levels concerning the cell enzymatic activity, surface protein cell markers and the genetic pathway. The mineralization assessment was performed in our experiment via measurement of Alkaline phosphatase (ALP) activity as it plays an important role in the initial formation of mineralized tissues. In our study nano calcium hydroxide showed the highest (ALP) activity followed by nano calcium phosphate in chlorohexidine and positive control with no significant difference between them. (Lee et al. 2015) in their study of the effect of nano calcium phosphate on HDPSCs noted an increase in ALP activity compared to macro-sized particles thus, indicating that the odontogenic differentiation cascade is taking place. da Rosa et al. (2018) also reported a high ALP activity of adipose-derived mesenchymal stem cells in a nano-hydroxyapatite scaffold.

The Dentine Matrix Protein-1 mineralization marker was also evaluated along with the ALP as the differentiation of stem cells into odontogenic-like cells requires several markers to be detected. The DMP1 expression was detected using immunofluorescence staining (Ferro et al. 2014). Our experiment revealed that nano calcium hydroxide was significantly the highest to express DMP-1 followed by high expression of DMP-1 for HDPSCs treated with nano calcium phosphate in chlorohexidine and odontogenic medium-positive control with no significant difference between them. This comes in agreement with (Lee et al. 2015) who recorded that nano calcium phosphate promoted odontogenic differentiation of HDPSCs via DSPP and DMP-1 markers gene expression along with other markers. This increased differentiation potential might be attributed to the composition which is similar to crystals present in dental hard tissues (calcium and phosphates) and has special biological and physicochemical properties (Pettersson et al. 2017).

The NF-Kβ gene expression was evaluated since the odontogenic differentiation and inflammatory responses that are involved in dentinogenesis are signaled by several signaling pathways such as the p38 MAPK, and NF-Kβ. Understanding the cascade of cellular and molecular events underlying the repair and regeneration processes provides a reasonable new approach to targeted interaction between tooth tissue and bioactive molecules (da Rosa et al. 2018).

Conclusion

Within the limitations of this ex-vivo study, all tested biomaterials exhibited a favorable medium for proliferation and odontogenic differentiation of HDPSCs. Nano calcium phosphate in chlorohexidine paste showed the ability for proliferation of HDPSCs this was proved by upregulated cell viability while the differentiation of HDPSCs was evidenced by increasing ALP activity and DMP-1 stain. Further molecular study of the odontogenic genes would be more relevant to reinforce our findings.

Abbreviations

Abb.	Full term
AlP Assay	Alkaline phosphatase assay
Bcl2	B-cell lymphoma 2
BMP	Bone morphogenetic proteins
Ca(OH)2	Calcium hydroxide
CaPs	Calcium phosphates
CD44	Family of cell surface glycoproteins with isoforms generated by alternate splicing of mRNA. Important in epithelial cell adhesion to hyaluronate in basement membranes and maintaining polar orientation of cells; also binds laminin, collagen, and fibronectin
CD45	Leukocyte common antigen (LCA) expressed on all white blood cells (hematopoietic)
CD73	Member of a family of single-pass transmembrane sialomucin proteins that show expression on early hematopoietic and vascular-associated progenitor cells
CEM	Calcium enriched material
CHX	Chlorohexidine
DMEM	Dulbecco's Modified Eagles Medium
DMP	Dentin matrix protein
DMSO	Dimethyl sulfoxide
DPSCs	Dental pulp stem cells
DSPP	Dentin sialophosphoprtein
ECM	Extracellular matrix
EMD	Enamel matrix derivative
FAK	Focal adhesion kinase
FCM	Flowcytometry
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
HDPSCs	Human dental pulp stem cells
IC	Inhibitory concentration
MAPK	Mitogen activated protein kinase
MSCs/BMSCs	Mesenchymal stem cells/bone marrow MSCs
MTA	Mineral trioxide aggregate
MTT	Methyl-thiazole tetrazolium assay
NaOH	Sodium hydroxide
NC	Negative control
NF-Kβ	Nuclear factor-kappa beta
NSCs	Neural stem cells
OCN	Osteocalcin
OD	Optical density
OM	Odontogenic/osteogenic medium
OPN	Osteopontin
PBS	Phosphate buffered saline
PC	Positive control
REP	Regenerative endodontic procedure
TB	Trypan blue
TEM	Transmission electron microscope
TGF-β	Transforming growth factor-β
VPT	Vital pulp therapy
XRD	X-ray diffraction
α-MEM	Alpha Modification of Eagle's Medium

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Author contributions
SS performed the investigations. AH, SH, SS contributed in writing the original data including analysis and interpretation. AH, SH, SS performed the editing of the manuscript. All authors have read and approved the manuscript.

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Availability of data and materials
The data generated and analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This study was approved by the ethics committee of the faculty of dentistry, Ain Shams University, Cairo, Egypt with the approval no. (FDASU-Rec IR102209). All patients were informed about the use of their extracted teeth, and they all submitted a written informed consent before enrolment, which was carried out in accordance with the Declaration of Helsinki.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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