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Nano Hydroxyapatite & Mineral Trioxide Aggregate Efficiently Promote Odontogenic Differentiation of Dental Pulp Stem Cells

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Abstract

BACKGROUND: There has been an urge to shift from conventional therapies to the more promising regenerative strategy since conventional treatment relies on synthetic materials to fill defects and replace missing tissues, lacking the ability to restore the tissues' physiological architecture and function.

AIM: The present study focused on the assessment of the role of two commonly used biomaterials namely; mineral trioxide aggregate (MTA) and nano hydroxy-apatite as promoters of odontogenic differentiation of dental pulp stem cells (DPSCs).

METHODS: DPSCs were isolated, cultured in odontogenic media and divided into three groups; control group, MTA group and nanohydroxyapatite group. Odontogenic differentiation was assessed by tracing genes characteristic of different stages of odontoblasts via qRT-PCR. Calcific nodules formation was evaluated by Alizarin red staining.

RESULTS: Results demonstrated that both MTA and nanohydroxyapatite were capable of enhancing odontogenic differentiation of DPSCs.

CONCLUSION: Nano hydroxyapatite was found to have a higher promoting effect. However, in the absence of an odontogenic medium, MTA and nanohydroxyapatite could not enhance the odontogenic differentiation of DPSCs.

Introduction

Regenerative and cell-based therapies represent a promising alternative to conventional therapies to maintain pulp vitality and to avoid the more extensive treatment dictated by extraction or endodontic therapy. Mineral Trioxide Aggregate (MTA) and nano hydroxyapatite are promising materials which have wide clinical and regenerative uses. Different studies performed on MTA, showed that it plays an important role in regenerative endodontics, mainly through the activation of cementoblasts and the formation of cementum [1]. MTA was also reported to play a role in stimulation of the odontogenic differentiation of stem cells [2] and the induction of stem cell proliferation with excellent biocompatibility [3]. Another material that is widely used is nano hydroxyapatite, due to its particle size that is close to the apatite crystals present naturally in human mineralised tissue which is useful when used as a scaffold material in tissue engineering [4]. It also presents superior properties regarding enhancing stem cells proliferation and differentiation into osteoblasts resulting in bone formation [5]. Moreover, nano hydroxyapatite plays a remarkable role in guided tissue regeneration to enhance bone regeneration in the field of periodontology [6].

In this study, the role of MTA and nano hydroxyapatite as promoters of odontogenic differentiation is assessed on dental pulp stem cells.

Material and Methods

Dental pulp stem cells isolation and culture

Human dental pulp tissues were obtained from three impacted third molars. Teeth were collected from patients aged from 16–26 years. Extraction of teeth was performed in the department of Oral Surgery Department at Ain Shams University clinics under the approval of its ethics committee. The extirpated pulp tissues were minced into small pieces and digested with 2mg/mL collagenase type I (Serva) fa or 30 minutes at 37°C. Cell suspensions were cultured in 6-cm dishes in high-glucose Dulbecco modified Eagle medium (Lonza, Belgium) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) at 37°C in 5% CO_2. Passaging was performed when adherent cells reached 70% confluence.

Induction of odontogenic differentiation

To induce odontogenic differentiation, DPSCs from the third passage were seeded into 6-well plates at a density of $1x10^5$/well. Odontogenic differentiation was performed for 21 days by culturing DPSCs in odontogenic differentiation medium containing DMEM medium supplemented with 15% FBS, 10 mol/L b-glycerophosphate (Sigma-Aldrich), 0.2 mmol/L ascorbate-2-phosphate (Sigma-Aldrich), and 100 nmol/L dexamethasone (Sigma-Aldrich). DPSCs were divided into three groups according to the media and biomaterial used as follows; 1) Controls; positive controls were grown in odontogenic media with 15% FBS whereas negative controls were grown in DMEM with 15% FBS. 2) MTA group; which was supplied with MTA powder (Angelus, Brazil) and odontogenic medium (as above mentioned). For MTA preparation, a concentration of 0.02 mg/ml was implemented according to previous protocols [7]. Briefly, 0.02 mg MTA was dissolved in 1ml of odontogenic media and vortexed until suspended. The suspension was then left to settle for 10 minutes followed by 24 hours incubation in 37°C and 5% CO_2 to extract the bioactive contents of MTA. The resultant supernatant from this preparation was used to treat cells every other day with for 3 weeks. Finally, nano hydroxyapatite group; which comprised nano hydroxyapatite (Sigma–Aldrich, UK) at a concentration of 10 µg/mL suspended in odontogenic media [8]. For all groups media was changed three times a week for 21 days.

Real time RT-PCR analysis for odontogenic gene expression

After 3 weeks of odontogenic induction of the 3 groups of cells (nano hydroxyapatite, MTA and control groups). Total RNA was isolated using PureLink RNA Min Kit (Invitrogen, http: //www.invitrogen. com). All RNA samples were checked for purity using a ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA). Total RNA samples were reverse transcribed into cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) and quantitative real time PCR was performed using 1 µg of cDNA and Maxima SYBR Green qPCR master mix (Thermo Fisher) in a LightCycler® 480 Instrument (Roche life science). Real-Time Quantitative Polymerase Chain Reaction Analysis Differential expressions of five odontogenic genes; Alkaline Phosphatase (*ALP*), Osteopontin (*OPN*), *RUNX2*, Osteocalcin (*OCN*) and *collagen1* was carried out. The primer sequences are provided in (Table 1). Samples were run twice. The raw data were then analyzed with the Relative expression software tool (REST) using the automatic cycle threshold (Ct) setting to assign baselines and thresholds for the Ct determination. Delta Ct ($\Delta\Delta$CT) values were used for this analysis. The relative expressions (REs) of the sample genes were calculated using the $\Delta\Delta$CT method and GAPDH was used as the internal control or housekeeping gene. q RT-PCR experiments were carried out at least three times. Data were presented as the average values ± SEM (standard error of the mean). Statistical significance was analyzed with paired Student t-test. Significance levels or P-values are indicated in the figure legends.

Table 1: Primer sequences for quantitative real-time PCR analysis

Gene	Forward	Reverse
Runx2	5'-AAGTGCGGTGCAAACTTTCT-3'	5'-TCTCGGTGGCTGCTAGTGA-3'
Osteocalcin	5'-TCA CAC TCC TCG CCC TAT TG-3'	5'-TCG CTG CCC TCC TGC TTG-3'
ALP	5'-AGC TGA ACA GGA ACA ACG TGA-3'	5'-CTT CAT GGT GCC CGT GGT C-3'
Collagen1	5'-ACC GCC CTC CTG ACG CAC -3'	5'- GCA GAC GCA GAT CCG GCA G-3'
Osteopontin	5'- AAGGCGCATTACAGCAAACACTCA	3'- CTCATCGGACTCCTGGCTCTTCAT
GAPDH	5'- ACCACAGTCCATGCCATCAC	3'- TCCACCACCCTGTTGCTGTA

Alizarin Red Staining

To assess in vitro mineralization, cells were washed twice with phosphate-buffered saline, fixed with 4% paraformaldehyde (Sigma- Aldrich) for 1 hour, washed with deionized H_2O, and stained with 1% Alizarin Red S (Sigma-Aldrich) for 20 minutes. They were then rinsed 3 times with deionized H_2O, the mineralized nodules were observed under inverted light microscope (Leica, 6000B-4) using Suite V3 (Leica).

Results

Isolation of human dental pulp stem cells (DPSCs)

Human dental pulp stem cells (DPSCs) were successfully isolated from pulps of extracted third molars. The cultured DPSCs were observed on a regular basis using an inverted light microscope (Figure 1). After 24 hours of isolation, DPSCs began to develop processes assuming a spindle and stellate shape which is the typical appearance of mesenchymal stem cell and attached to the bottom of the culture dish. Approximately ten days follow ing t, the initial isolation, cells reached 70% confluence. At this stage, cells were passaged to passage three where differentiation was initiated.

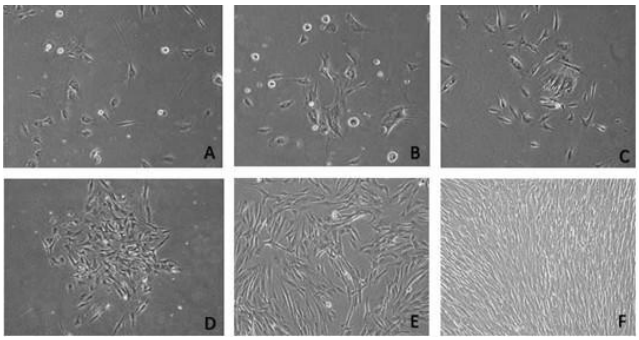

Figure 1: Isolation and morphological observation of human dental pulp stem cells (DPSCthe s) be the fore induction of differentiation by phase contrast microscopy; (A) DPSCs after 24 hours from isolation; (B) Isolated DPSCs with different morphological appearances following isolation; (C) DPSCs assuming colonies on day 3 of isolation; (D) DPSCs on day 5 of isolation showing increase in colony size with increase in cell number; (E) DPSCs a week after isolation; cells are approaching confluence; (F) DPSCs reaching 80% confluence 10 days after isolation (Magnification 100X)

Successful induction of odontogenic differentiation of DPSCs

After applying the odontogenic medium for differentiation, DPSCs were observed on a regular basis for morphological alterations. During the first week after induction, DPSCs which have reached confluency began to change their spindle-shape developing into rounded cells which gathered to form clusters. Rounded aggregates of DPSCs appeared as a result of cell migration chiefly from the periphery toward the centre of the plates (Figure 2).

On day 21, staining with Alizarin Red was done. Orange-red nodules indicating the beginning of mineralisation was demonstrated (Figure 3B).

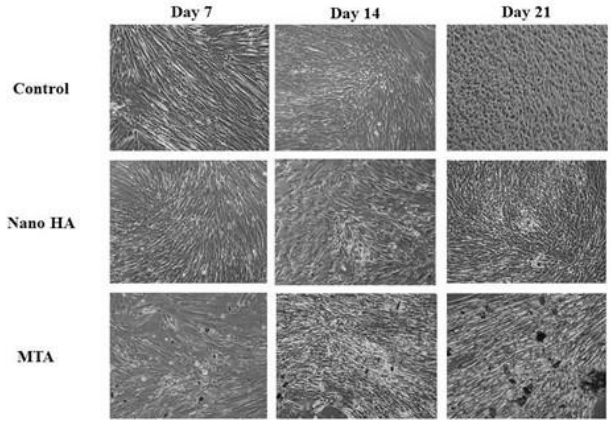

Figure 2: Morphological observation of DPSCs during odontogenic differentiation; Photomicrographs illustrating the morphological appearance of DPSCs while cultured either in odontogenic differentiation media supplemented with nano-hydroxyapatite (NanoHA) or MTA over a p,period of 21 days. DPSCs gradually transformed from spindle /stellate shape into a more rounded polygonal morphology. However, there was an apparent gradual decrease in cell number in the MTA group (Magnification 100X)

MTA and nano hydroxyapatite promoted odontogenic differentiation

Gene expression and statistical analysis were performed to assess the differential expression of five odontogenic markers in cells differentiated with either MTA or nano hydroxyapatite compared with control counterparts. The studied genes were ALP, *OPN, RUNX2, OCN,* and *collagen1* genes (Figure 3A)**.**

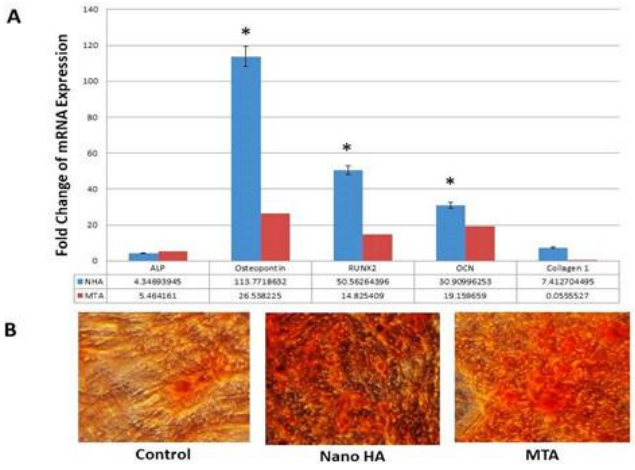

*Figure 3: Evaluation of odontogenic differentiation capacity of DPSCS cultured under different conditions; (A) Expression of genes characteristic of odontogenic differentiation; Alkaline Phosphatase (ALP), Osteopontin, RUNX2, Osteocalcin (OCN), Collagen1 in nano-hydroxyapatite (NHA) and MTA groups. GAPDH was used as housekeeping gene. The data were analysed by qRT–PCR and compared with positive control, i.e. cells cultured in odontogenic media only. Results are presented as mean ± SEM of three independent experiments (n = 3). *P < 0.05; (B) Alizarin Red staining was used to determine mineral nodule formation on day 21 in all groups*

Using q RT-PCR**,** we detected the expression of all studied genes in all groups although the quantities varied. *OPN*, *RUNX2* and *OCN* were

overexpressed in all groups with significantly higher expression in groups supplemented with nano hydroxyapatite. *Collagen1* was showed higher expression in Nano hydroxyapatite group than in MTA but with no statistical significance, whereas *ALP* was the only gene that illustrated increased expression MTA group.

It is worthy to note that both MTA and nano hydroxyapatite added to DMEM only without odontogenic supplements were not able to induce odontogenic differentiation (Supplementary Figure 1).

Discussion

In the present study, we focused on two promising biomaterials, MTA and nano hydroxy-apatite, which have been known to play an imperative role in the field of dentistry. Evaluation of the effect of the MTA and the nano hydroxyapatite on the odontogenic differentiation potential of the DPSCs was done by tracing the expression levels of genes characteristic of odontoblastic differentiation markers including *ALP, OC, OPN, RUNX2* and *Collagen 1*. Primary pulp cells are known to express type I collagen, ALP, bone sialoprotein, and osteocalcin [2]. The expression of these markers relates to different stages during odontogenic differentiation. OPN was highly expressed in both groups with a significant increase in nano hydroxyapatite group. The role of OPN in dentinogenesis is well described in the literature. During reparative dentinogenesis, OPN expression was found to play an undeniable role in the differentiation of odontoblast-like cells during pulpal healing following tooth transplantation [9]. In accordance to our study, Kuratate M. et al., suggested that pulpal responses to MTA capping involve proliferation and migration of pulp stem cells followed by their differentiation into odontoblast-like cells where osteopontin played a triggering role in the initiation of the pulpal reparative process [10]. Similarly, RUNX2; which is a key factor that is essential for odontoblastic differentiation, was highly expressed in both MTA and nanohydroxyapatite supplemented media. In accordance to our study, Matsumoto S. *et al.* proved that in the presence of MTA, the expression levels of RUNX2 in C2C12 cells (cell line obtained from RIKEN Cell Bank, Japan) were significantly increased indicating odontoblastic differentiation [11]. Furthermore, in accordance to our study, Mohamed *et al*. concluded that nano hydroxyapatite promoted odontogenic differentiation of DPSCs [12]. Also, Liu H-C et al. found that DPSCs seeded on nano-hydroxyapatite/collagen/Poly (L-lactide) could undergo odontogenic and osteogenic differentiation evident by the expression of OCN, COL 1 and ALP [13]. Next, we assessed the expression level of OCN. OCN has been known to be expressed only during the later stages of odontoblasts cytodifferentiation during tooth development. OCN was also detected in odontoblasts and their processes within the extracellular matrix at the maturation stage of enamel formation. It was shown that OCN is produced by human odontoblasts and determine the expression pattern of DSPP in human teeth [14]. Our results showed that OCN was highly expressed in both MTA and nanohydroxyapatite supplemented with odontogenic media indicating odontogenic differentiation of the DPSCs. The role of ALP has been suggested to be implicated in early mineralisation. In our study, ALP was expressed both in MTA and nano hydroxyapatite groups. However, its expression in both groups was less than other genes studied. This finding is by Alliot-Licht B. et al., and Zhang W. et al., who revealed that ALP activity gradually increased after reaching its peak expression on the day and then declined [15] [16]. In accordance to our study, Min K-S. et al. found that DPSCs treated with MTA showed up-regulation of m-RNA expression levels of ALP and OCN which confirmed successful odontogenic differentiation of the DPSCs [17]. ALP also participates in dentin formation and was found to be highly expressed by mature odontoblasts; its absence indicates defective dentin mineralisation [18]. According to our results of genes tracing, it was noted that nano-hydroxyapatite showed higher levels of odontogenic genes expression in comparison to MTA supplemented with odontogenic medium suggesting a higher odontogenic differentiation potential of the nano hydroxyapatite. This increased differentiation potential might be attributed to the difference in their chemical composition and surface topography. Nano hydroxyapatite composition is similar to crystals present in dental hard tissues (calcium and phosphates) and has special biological and physicochemical properties [19]. Also, nanoscale of hydroxy-apatite had been shown to positively affect the adhesion and differentiation of stem cells and has excellent biological properties compared to their larger micron-structured counterparts which are attributed to increased surface reactivity [20]. Successful odontogenic differentiation of DPSCs was further confirmed by Alizarin red staining assay. Positive colorimetric changes were observed in both MTA and nano hydroxy-apatite groups as well as in the positive control group, which indicated calcific nodules formation. In accordance with our study, Jung J-Y. et al., suggested that MTA plays a role in differentiation of DPSCs into odontoblasts as evidenced by the alizarin red staining of calcified nodules [21]. Moreover, Woo S-M. et al., found that calcium ions released from MTA has a major impact on the odontoblastic differentiation of DPSCs and enhancement of mineralized nodule formation [22]. In accordance with our experiment, Liu H-C. et al., found that sporadic nodules-shaped islands after alizarin red staining as a result of the differentiation of DPSCs into odontoblast and osteoblast when nano hydroxyapatite was used as a scaffold [13]. In our study, MTA extract was used to induce odontogenic differentiation of DPSCs after 24 hours of incubation of MTA powder in

odontogenic media. By this method, we aimed to avoid direct contact of MTA with DPSCs and thus simulating the clinical conditions achieved when MTA is used for direct pulp capping. However, it was observed that MTA exerted an observable decrease in cell viability during culture, despite using a very low concentration. The concentration used was according to Hakki SS. et al., which was described as the least cytotoxic concentration capable of inducing differentiation [7]. It is worthy to mention that our results revealed that both MTA & nano hydroxyapatite were not sufficient to induce odontogenic differentiation by their selves. This was clearly evident, from the minimal expression of *ALP, OCN, OPN, RUNX2* and *collagen1* exhibited by MTA and nano hydroxyapatite added to DMEM media in the absence of odontogenic differentiation supplementation. In conclusion, it was observed that both MTA and nano hydroxyapatite in the presence of odontogenic medium could enhance the odontogenic differentiation of the DPSCs. It was also clear that nano hydroxyapatite possess higher odontogenic differentiation potential than MTA, evidenced by higher fold increase in the expression of most of the odontogenic genes studied. These data may be useful in future studies to promote odontogenic differentiation of DPSCs and may be useful in designing regenerative therapies for dentin.

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