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EFFECT OF BIOACTIVE GLASS NANO PARTICULATES AND NANO HYDROXYAPATITE COATED BY CHITOSAN ON ODONTOGENIC DIFFERENTIATION AND PROLIFERATION OF HUMAN DENTAL PULP STEM CELLS

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ABSTRACT

This study aimed to evaluate effect of Nano hydroxyapatite coated by Chitosan (NHAP/CS) and bioactive glass nano particulates (nBG) on odontogenic differentiation and proliferation of human dental pulp stem cells (hDPSCs).

Methods: hDPSCs were seeded in direct contact with the corresponding Nano-biomaterials in 5 groups: Group (I): NHAP (10μg/ml), Group (II): NHAP/CS (10μg/ml), Group (III): nBG (500μg/ml). Group (IV): hDPSCs were cultured with osteogenic medium as a positive control while Group (V): hDPSCs were cultured with Dulbecco's Modified Eagle Medium (DMEM) only as a negative control. Odontogenic differentiation was evaluated based on mineralization related genes .Gene expressions were verified using alkaline phosphatase (ALP) assay and immunofluorescence staining of Dentin matrix protein (DMP-1). Proliferation of cells was evaluated via Trypan Blue staining test and methylthiazol tetrazolium (MTT) assay.

Results: All the tested Nano biomaterials exhibited favorable media for odontogenic differentiation and proliferation of hDPSCs. NHAP/CS showed the highest proliferation index via MTT, highest ALP concentration and highest DMP-1Immunofluorescence staining.

Conclusions: Compared to pure NHAP and a major biomaterial as nBG, NHAP/CS can induce odontogenic differentiation and proliferation of hDPSCs and could be considered as a good candidate for dentin-pulp regenerative procedures.

Keywords: Alkaline phosphatase, Dentin matrix, Immunofluorescence.

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INTRODUCTION

Regenerative endodontic methods have the potential for regenerating both pulp and dentin tissues. Direct pulp capping aims to seal the vital pulp tissue exposed by trauma or deep caries excavation to enable the pulp to heal. Pulp exposure mostly involves odontoblast destruction and fibroblast injury. A successful pulp-capping procedure requires a mild inflammatory reaction to stimulate recruitment and proliferation of hDPSCs from their vascular niche. These cells need to migrate and differentiate into mineralized tissue to form a hard-tissue barrier that will (re-)isolate the exposed pulp from the oral environment[1].

Bioactive glass shows osteoinductive behavior with bonding ability to soft and hard tissue due to a carbonated hydroxyapatite layer when biologically exposed[2]. Hydroxyapatite (HAP) is close to the natural apatite crystals present in human mineralized tissues is useful as a scaffold in tissue regenerations [3].

Chitosan has excellent non-toxic, antibacterial, antiviral, and antifungal properties. It is biocompatible, biodegradable and shows bio- adhesion[4]. The null hypothesis of this study was that incorporation of Chitosan polymer with NHAP would not affect the dental pulp stem cells differentiation and proliferation compared to the pure NHAP.

MATERIALS AND METHODS

I) Preparation and characterization of tested materials

A) Nano Hydroxyapatite (NHAP):

NHAP has been prepared by wet chemical precipitation method according to Xu et al[5]. Its chemical composition is $Ca_{10}(PO_4)_6(OH)_2$ powder. Transmission electron microscope (*TEM* JOEL JEM-2100 showed that NHAP exhibited needles shape with a particle size ranging from 25±5 nm width and 150 ± 30 nm in length [6].

X-ray diffraction analysis (XRD) pattern (performed using XPERT-PRO Powder Diffractometer system) showed that NHAP had sharper peaks which indicate better crystallinity. The existence of 2θ peaks confirms the formation and presence of the crystalline phase $Ca_{10}(PO_4)_6(OH)_2$ [7].

B) Nano Hydroxyapatite coated by Chitosan(NHAP/CS)

It was prepared by using in situ hybridization method described by Nikpour et al 2014(8). Transmission electron microscope (*TEM* JOEL JEM-2100) showed that NHAP exhibited needles shape with a particle size 10:25 nm width and 100 ± 20 nm in length[6].

XRD analysis pattern showed that NHAP/CS with sharper peaks indicating better crystallinity[7].

C) Bioactive Glass Nano particulates(nBG):

45S5 nBG was synthesized by sol-gel method according to Bui 2017[9]. Transmission electron microscope showed that nBG exhibited white spherical particle shape with a particle size 20:50 nm in width and 4.30 ±2nm in length.

XRD analysis pattern showed that nBG exhibited major crystalline phases $Na_6Ca_3Si_6O_{18}$.

II) Cell culture

This study was approved by the ethics committee of faculty of dentistry, Ain Shams University with approval number FDASU-Rec IM122113. hDPSCs were obtained as preserved cell line from global research Labs, Cairo. Egypt. The cells were cultured in (T75) flasks 75 cm^3 in a complete supplemented medium **Gibco Dulbecco's Modified Eagle Medium** (DMEM) containing 10% fetal bovine serum (FBS) and 1% of penicillin G sodium (10.000 UI), streptomycin (10 mg) and amphotericin-B (25µg) (PSA) (Gibco, Thermo-scientific, Germany).

Flasks were incubated at 37 °C in an atmosphere of 5% CO_2 until reached 70% confluence. Cells from third passage of culture were used for differentiation and proliferation.[10].

III) Samples Preparation and Classification:

Each given weight of the tested materials (10μg of NHAP, 10μg of NHAP/CS and 500μg of nBG) were dissolved in 1 ml of PBS and vortexed until being suspended. Then hDPSCs were seeded in direct contact with the corresponding Nano-biomaterials in 5 groups:

Group (I): Nano Hydroxyapatite (10μg/ml)

Group (II): Nano Hydroxyapatite coated by Chitosan (10μg/ml)

Group (III): Bioactive Glass Nano particulates (500μg/ml)

Group (IV): Positive control group; hDPSCs were cultured with odontogenic/osteogenic medium (Inductive Medium) (**Gibco, Thermo-scientific, Germany**) consists of optimized MSC Osteogenic Differentiation Basal Medium Cell Media, Mesenchymal Stem Cell-Qualified Fetal Bovine Serum (FBS) **(Gibco, Thermo-scientific, Germany)**, Penicillin-Streptomycin, Glutamine, Ascorbate, β-Glycerophosphate, and Dexamethasone.

Group (V): Negative control group of hDPSCs were grown with DMEM **(Gibco, Thermo-scientific, Germany)** only**.**

These groups were observed while hDPSCs were seeded at 4.5 × 10^5 cells/well (6-well plates) and incubated at 37ºC and 5% CO_2 for 72 hours. Each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments.

A) Evaluation of Odontogenic Differentiation:

1. Alkaline phosphatase activity assessment (ALP Assay)[12]:

The Alkaline phosphatase (ALP) activity was measured after 72 hours of culturing with tested groups in supernatant of differentiated cells using an **ALP assay kit (Sigma-Aldrich, St Louis, MO, USA)** with *p*-nitrophenyl phosphate as substrate. AMP-substrate buffer was added to each test sample and incubated at 37ºC for 30 minutes. The m absorbance was measured at 405 nm using a spectrophotometer (**Elx800; Bio-Tek Instruments Inc., Winooski, VT, USA).**

2. Immunofluorescence Staining Assessment for DMP protein expression [13]:

The hDPSCs cells were cultured for 24 hours on coverslips, examined for the expression of **DMP** using specific polyclonal antibody, fixed at warm 4% formaldehyde, immune-stained with **anti-DMP (Fine Biotech, Wuhant; China),** primary antibody **(Invitrogen; Thermo Fisher Scientific, Hilden; Germany)** and incubated overnight at 4 °C. They were washed with PBS and incubated with Goat Anti-Rabbit IgG and L secondary antibody-Alexa Flour 488 (**Invitrogen; Thermo Fisher Scientific, Hilden; Germany).** The specimens were immediately examined using **Fluorescence microscope (*LABOMED, PA, USA*).**

A. Evaluation of Cell Proliferation:

***1. Trypan blue Staining*[14]:**

10μL of the harvested hDPSCs cells were added to the hemocytometer **(ThermoFisher Scientific, Hilden; Germany)**. The chamber was placed in the inverted microscope under 10X. The cells in the large, central gridded square (1mm^2) were counted, and were multiplied by 10^4 for the estimation of the total number of cells per ml. 0.1 ml of trypan blue solution in a buffer (Figure 1).

2. Methylthiazol Tetrazolium (MTT) Assay [15]:

Proliferation of hDPSCs was assessed by (MTT) assay using **MTT Cell Proliferation Assay Kit (The Vybrant, cat no: M6494, Thermo Fisher,**

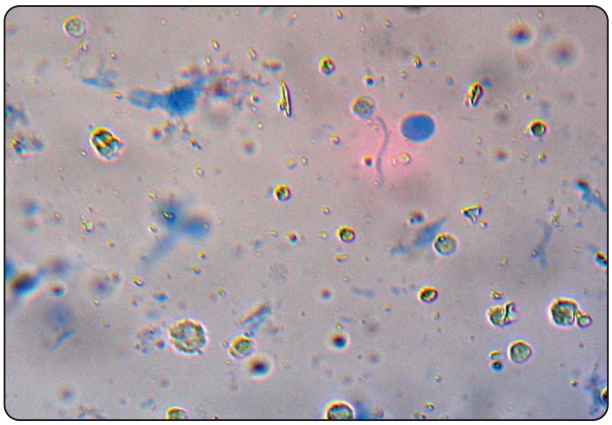

Fig. (1): Trypan Blue Test for cell counting and viability.

Germany). The Differentiated hDPSCs (8×10^3 cells per well) were seeded in 96-well culture plates and incubated at 37 °C with 5% CO_2 for 72 hours in specific media (OM for PC, DMEM for NC, and specific tested compounds). Finally, the MTT solution was removed and 100 µL of Sodium dodecyl sulfate-HCL (SDS-HCL) was added to the wells. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer **(Elx800 Bio-Tek Instruments Inc., Winooski, VT, USA)** .

Statistical analysis:

Results were analyzed using Minitab 19 program **(Minitab® LLC.PA,USA),** one-way analysis of variance (ANOVA) and Tukey Honestly Significant Difference (HSD). All data are expressed as mean ± standard deviation .$P \leq 0.05$ was considered as the level of significance**.**

RESULTS:

I. Results of Odontogenic Differentiation evaluation

A. *ALP Assay*

Alkaline phosphatase enzyme analysis result presented in (Figure .2) . Group II showed the statistically significant highest value of odontogenic cell differentiation using ALP assay while group V showed the statistically significant lowest value of ALP concentration. Groups III and IV showed no statistically significant difference between each other. There is statistically significant difference between them and I, II and V groups.

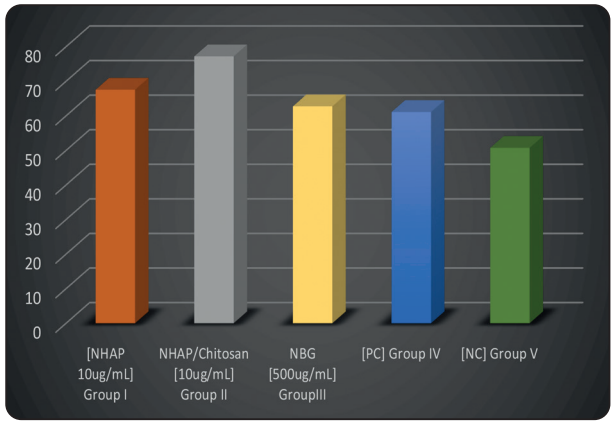

Fig. (2): Bar chart showing the differentiation potential "ALP activity" of the different compounds on hDPSCs after incubation for 72 hours .

B. *Immunofluorescence Staining for DMP protein expression (Figure 3,4):*

Group II showed the statistical significant highest value among tested groups while group V showed the statistical significant lowest value. The results showed a statistically significant difference between all tested groups.

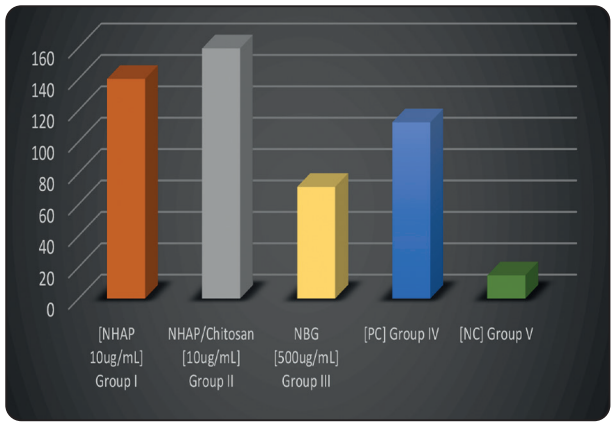

Fig. (3): Bar chart showing the fluorescence intensity DMP positive hDPSCs after incubation for 72 hours.

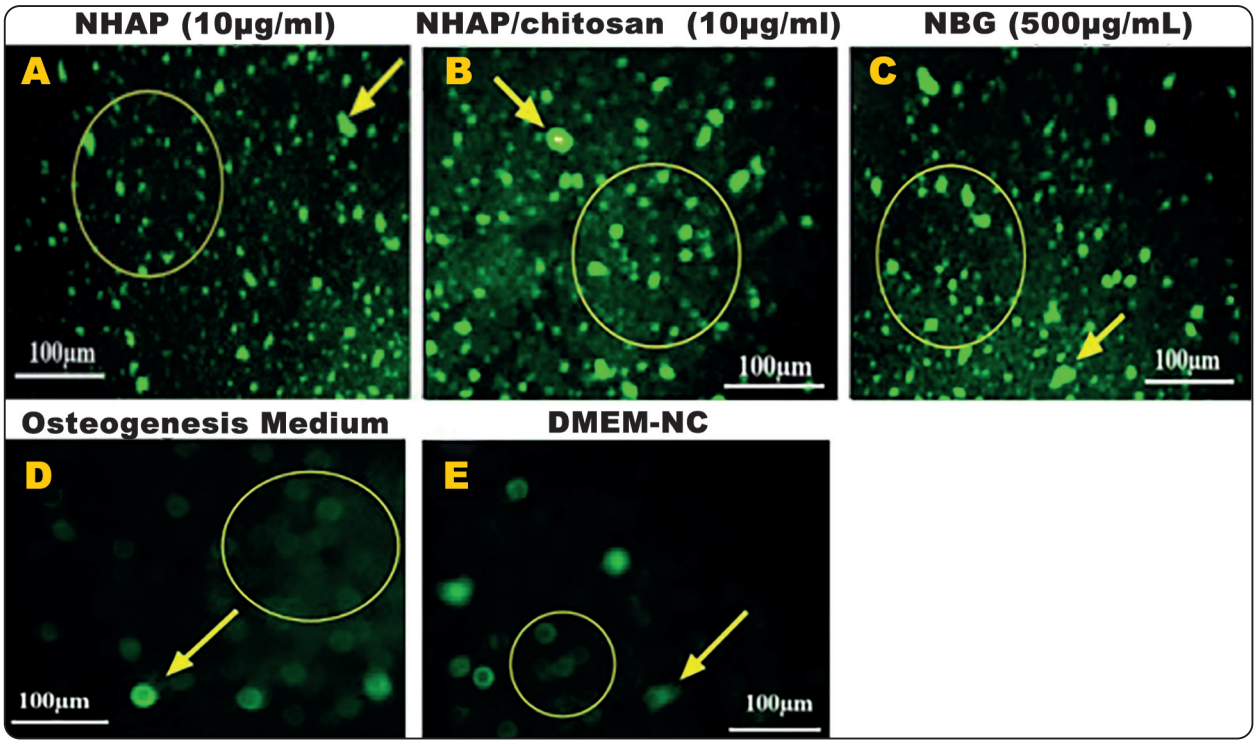

Fig. (4) Photomicrographs for immunofluorescence staining for DMP expression for tested groups: An Alex Fluor 688 anti-rabbit IgG secondary antibody was used for detection. Fluorescence was examined by immunofluorescence microscope (LABOMED; USA). (A.B&C): Groups I, II& III showed a merged large colony of odontoblasts which dense homogenous membranous and nuclear expression of DMP. (D): Group IV showed increased number of odontoblast colonies which are presented with dense homogenous expression of DMP (++). (E): Group V showed a few collection "small colonies" of cells with a homogenous faint expression of DMP with dim fluorescence intensity (+), the expression was localized to the cell membrane and nucleus .DMP (green). The magnification power is 10x.The yellow circles highlight the odontoblast colonies, yellow arrow: pointed to the intensity of DMP expression.

II. Evaluation of hDPSCs Proliferation:

A. *Trypan blue Staining:*

Regarding viable cell count, Group II showed highest cell count while group V showed lowest cell count. There is no statistically significant difference between groups I, VIV and V. There is no statistically significant difference between group II and group III (Figure.5).

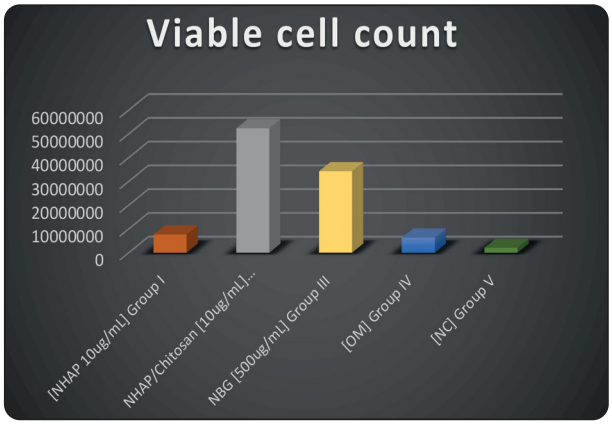

Fig. (5): Bar chart showing the number of viable cells of hDPSCs co-cultured in each tested compound after incubation for 72 hours.

B. MTT Assay:

Group II showed high cell proliferation index and group III also showed high cell proliferation index .Group V showed the lowest cell proliferation index.

Groups I and IV showed statistically significant difference with the other three tested groups, there is no statistically significant difference between

each other. Groups II and III showed statistically significant high difference with the other three tested groups, there is no statistically significant difference between each other. Group V showed statistically significant difference with the other groups. (Figure .6, 7).

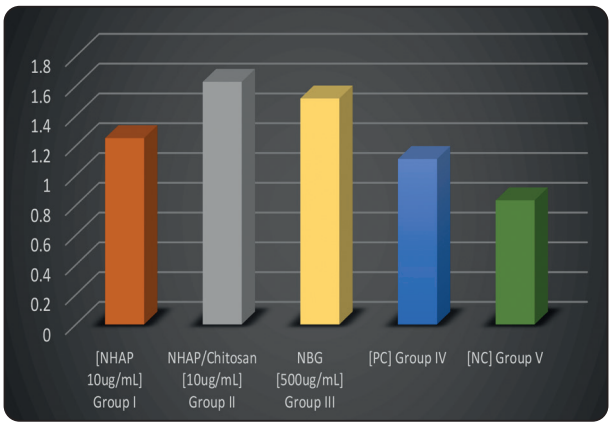

Fig. (6): Bar chart showing the proliferation index "viability" of hDPSCs after incubation for 72 hours via MTT assay.

DISCUSSION:

Regenerative endodontics may offer an alternative method to save teeth that may have compromised structural integrity. Recently, the role of stem cells for hard tissue formation has considerably increased attention of researchers as these cells can be a possible, fascinating source of stable differentiated cells, capable of inducing hard tissue formation [16].

hDPSCs have been chosen in this study due to their easy surgical access, their differentiation ability and interactivity with biomaterials [18] . According to **D'aquino et al** [18],these cells are multi-potential cells, with moderate proliferation rate and can be used in dentin-pulp regeneration or bone regeneration. NHAP coated by chitosan was prepared with 10-25 nm width and 100± 20nm length. This is supported

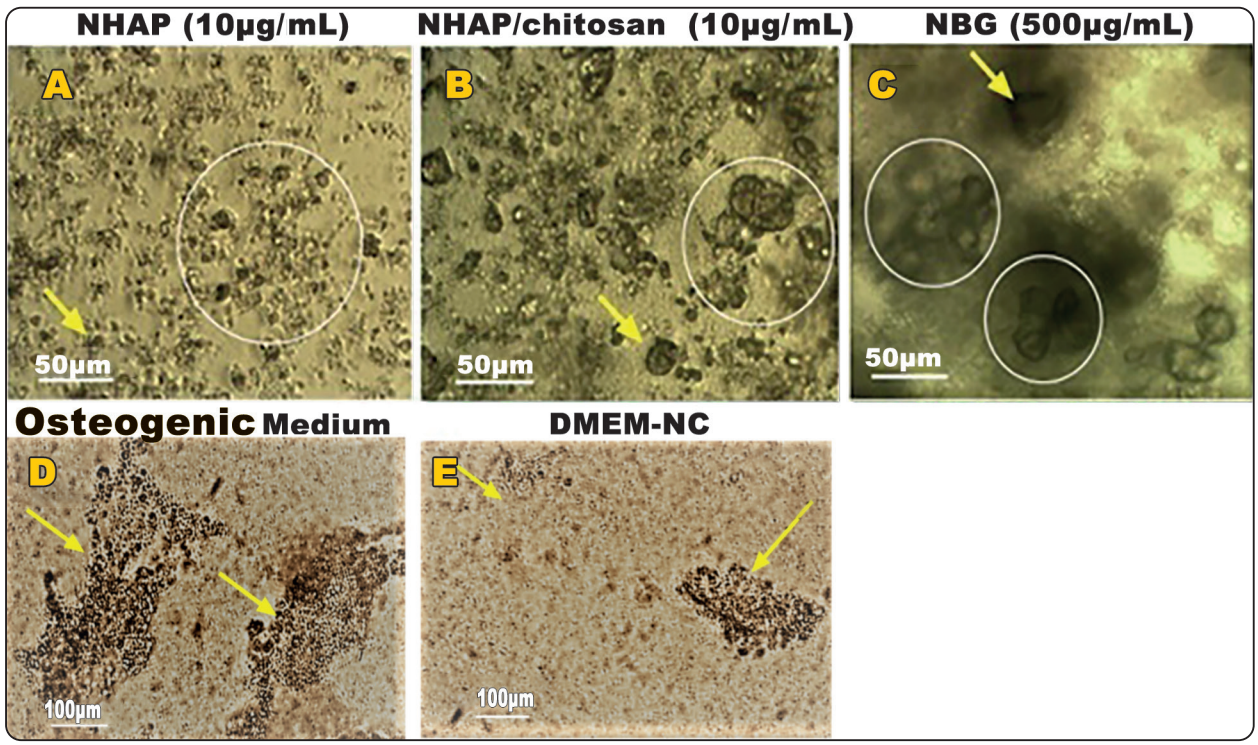

Fig (7) Photomicrographs for cell proliferation (MTT assay): hDPSCs after co-cultured with three different compounds, the images were captured by Labomed Vega Digital Camera (Labomed, USA). (A,B&C): Groups I ,II & III showed marked increase in cell count, large sized cells and low nuclear cytoplasmic ratio .In contrary,(D): hDPSCs cultured (Group IV) showed a large dense colony of cells .While (E): Group V showed discrete small colony of cells. The magnification power is 10x. The white circles highlighted the cell count. Yellow arrows pointed to the nuclear cytoplasmic ratio.

by **Yang et al** [19] who used NHAPs with a diameter of about 20nm. They demonstrated that hMSCs incubated with smaller-sized NHAP (S50 and S100) have higher differentiation rate compared with that treated with S150. They concluded that the efficiency of osteogenic differentiation of hMSCs was dependent on the size of NHAPs. NHAPs have been tested at a concentration of 10 μg/ml according to **Yang and colleagues**[19] and **Remya et al**[20]**.** These studies showed that NHAPs does not induce cytotoxicity from 10μg/ml up to 800 μg/ml, and concluded that cell viability decreased with increasing concentration. NHAP/ Chitosan (Group II) with a coating ratio 70/30 have been prepared and tested according to **Szatkowski and colleagues**[21]. According to **Lieder et al**[4],Chitosan coating have various advantages beside its antibacterial efficacy; it promotes cell attachment and supports the formation of the natural extracellular matrix (ECM), critically aiding in bone regeneration[4].

In the present study, the original nBG 45S5 was used in (Group III) with a mean particle size 20-50nm. This is in agreement with **Gong et al** [22] and **Sheng et al**[23] They recorded that these nano size particles enhanced cell proliferation and differentiation. A non-cytotoxic concentration 500μg/ml nBG was tested in this work. The used concentration came in agreement with **Gong et al**[22] who tested it in (1mg/ml) concentration on hDPSCs. In the same line, **Wang et al**[24] tested the same concentration but with BMSCs. **Ajita et al** [25] demonstrated that nBG was non cytotoxic up to 20mg/ml.

In the current experiments, the ability of nano BG 45S5 and NHAPs/CS to enhance (ALP) activity in hDPSCs was examined. This choice was preferred because it is considered as an early and commonly used repeatedly osteogenic/odontogenic differentiation marker. This interpretation came in agreement with **Gong et al** [22] **and Reilly et al** [26]**.** They reported that ALP can provide phosphate groups for subsequent hydroxyapatite deposition during mineralization. In order to confirm the odontogenic differentiation of hDPSCs, the marker of choice was DMP-1. DMP-1 was evaluated via immunofluorescence staining[27]. According to **Baldion et al** [28]**,** DMP-1 seemed to be one of the main non-collagenous proteins formed in the mineralized dentin matrix. DMP-1 was suggested to be the main phenotypic markers of odontoblasts. **Peng et al** [15] supported the use of DMP-1in odontogenic differentiation evaluation. In this work, trypan blue dye was used to ensure the proliferative power of cells used in regenerative medicine and tissue engineering protocols to guarantee their success. **Piedra et al** [14]**2014** reported that it is one of the most exclusive and extensively used cell viability methods that could determine the number of cells and their viability . MTT assay was used to confirm cell proliferation rate as it is one of the most commonly used viability and cytotoxicity test [15],[11]. Our study results revealed that all three tested materials exhibited favorable media for odontogenic differentiation and proliferation of hDPSCs.This came in agreement with **Gong et al** [22], **Szatkowski et al**[21] and **Yang et al** [19].

Focusing on odontogenic differentiation evaluation via ALP activity, NHAP/CS results showed the highest significant difference in comparison with pure NHAP or nBG. It could be attributed to presence of chitosan coating. This is in agreement with **Hosseinzade et al** [29] who noted that the positively charged chitosan leads to a beneficial interaction with adjacent tissue cells. They noticed an increasing osteogenesis and angiogenesis activities in NHAP/CS composite. **Leider et al**[4] also demonstrated that Chitosan promotes cell attachment and supports the formation of natural ECM . **Palazzo et al** [30]**,** confirmed that the NHAP/chitosan scaffolds can promote the osteogenic differentiation of hMSCs. This could be explained by chemical interaction occurred between NHAP and chitosan providing an

anchoring site for NHAP particles in its structure probably through its lateral amino and hydroxyl groups. This explanation was confirmed by **Kong et al** [31] that ALP activity increased with NHAP/CS composite. NHAP results regarding ALP activity showed no significant difference with OM group (group IV) but still significant in comparison to negative control group (group V). The results of the present work came also in agreement with **Yang et al** [19], recorded an increasing ALP activity with adding NHAP specially with smaller nanoparticle s50 and s100 without inductive medium. **Swarup et al**[32] supported our results through demonstration of ability of NHAP to produce complete dentinal bridges, favorable cellular and vascular response via histological analysis methods. On the other hand, the present work results came in contrast with **Hanafy et al** [3] who concluded that NHAP could not induce differentiation without presence of inductive medium. In contradiction to **Hanafy et al** [3], nBG (group III) showed high significant difference in odontogenic differentiation results in contrast with NC and other groups. The present study revealed that nBG 45S5 increased ALP activity. This is in agreement with **Mocqout et al**[27] and **Gong et al**[22]. Our results came also in agreement with **Mackovic et al** [17] who reported that increased cell activity caused by the use of nano-sized BG compared with the micro-sized ones. Regarding odontogenic differentiation evaluation via immunofluorescence staining for DMP-1 expression, our study results showed a statistically significant difference between all tested groups. NHAP/CS group showed the best and highest significant difference amongst all the tested materials in contrast with NC group. In agreement with our study, **Mohamed et al** [33] concluded that NHAP promoted odontogenic differentiation of DPSCs via highest mean value for DSPP and DMP-1 gene expression. The present work results revealed that nBG showed high statistically significant odontogenic differentiation via DMP-1 immunofluorescent staining than other tested groups but not than NHAP/CS. This is consistent with **Mocqout et al** [27] and **Gong et al** [22] who recorded increased DMP-1 immunofluorescent staining and upregulation of DMP-1 gene expression via qRT-PCR.

The present study results revealed that cell proliferation was markedly increased with NHAP /CS in comparison to all other groups. This is consistent with **Tondnevis et al**[34] who reported that after adding chitosan, the ability of cell attachment and cell proliferation exponentially enhanced. This was proved by representing proper surface morphology and topography beside the enhancement of surface tension and hydrophilicity of NHAP/ CS scaffold**.** In this work, NHAP increased cell viability significantly when compared to NC group. This is in agreement with **Remya et al** [20] who recorded that the viability percentage of BMSCs treated with lower concentrations of NHAP (10:800 μg/mL) was comparable to the negative control (cells alone)**..** On the other hand, **Mohamed et al** [35] reported that there was a decrease in cell counts, and viabilities in the NHAP supplemented cells but only in comparison to MTA or CEM. They attributed this effect due to the role of calcium silicate and other calcium compounds present in MTA and CEM .This persistently controlled the rate of soluble silicon and calcium ions released in the medium thus, promoting similar more cell survival and biocompatibility than NHAP. The null hypothesis of this research work was rejected as NHAP coated by Chitosan showed a significant increase in odontogenic differentiation and proliferation of hDPSCs when compared to pure NHAP.

CONCLUSION

Within the limitations of this in vitro study: The odontogenic /osteogenic differentiation potential and proliferation rate were significantly enhanced when hDPSCs were treated with NHAP coated by Chitosan biomaterial compared to pure NHAP and nBG.

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Conflicts of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article**.**

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