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Osteogenic differentiation and proliferation of apical papilla stem cells using nanoparticles of Neo MTA and bioactive glass

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ABSTRACT

Objective: The aim of this study was to evaluate the osteogenic differentiation ability and proliferation of apical papilla stem cells using nanoparticles of Neo MTA and bioactive glass.
Methods: Neo MTA and bioactive glass 45S5 nanoparticles were prepared and characterized using a transmission electron microscope and X-ray diffraction. Apical papilla stem cells were harvested from freshly-extracted fully-impacted wisdom teeth, cultured, and characterized using flow cytometric analysis. Tested nanomaterials were mixed and samples were classified into four equal groups as follows; Negative control group: SCAP with Dulbecco's modified eagle's medium, Positive control group: SCAP with inductive media, First experimental group: Neo MTA nanoparticles with SCAP, Second experimental group: Bioactive glass nanoparticles with SCAP. Osteoblastic differentiation was assessed using an alkaline phosphatase assay and RANKL expression using specific polyclonal antibodies by fluorescence microscope. The proliferation of SCAP was assessed using cell count and viability of Trypan Blue in addition to an MTT assay.
Results: Isolated SCAP showed a non-hematopoietic origin. Neo MTA showed the highest ALP concentration followed by bioactive glass nanoparticles, and negative control. Bioactive glass nanoparticles showed the highest H score for RANKL protein expression followed by Neo MTA, and negative control. Bioactive glass nanoparticles showed the highest viable cell count.
Conclusions: SCAP isolation is achievable from extracted fully impacted immature third molars. Both tested nanobiomaterials have the ability to induce osteogenic differentiation and proliferation of SCAP.

1. Introduction

Stem cells, growth factors, and scaffolds are the three major components of regenerative endodontic procedures. (Yan et al., 2014). Several types of dental stem cells can now be isolated. Apical papilla stem cells are located at the apical part of immature teeth (Sonoyama et al., 2008). Osteogenic differentiation of apical papilla stem cells and the formation of osteoblast and osteoblast-like cells have been demonstrated in addition to the formation of new hard tissue (Sequeira et al., 2018; Nada and El Backly, 2018).

Bioactive glass (BG) has the ability to modify osteoblastic gene expression in a way that properly controls cell proliferation and differentiation (Sun, 2007; Xynos et al., 2000). Applying the BG in a nano-sized particles, 45S5 BG, has dramatically increased its osteoconduction, osteoinduction properties and allowed for its use in tissue engineering (MačKović et al., 2012; El-Gendy et al., 2013).

Neo MTA has been recently launched into the market with the basic composition of tricalcium silicate, dicalcium silicate, calcium sulfate, calcium sulfate, tricalcium aluminate, and tantalum oxide as a radiopacifier. Neo MTA was shown to release calcium ions and hydroxyl ions following the hydration reaction which promotes the formation of hard tissues (Lozano-Guillén et al., n.d..). In addition to its different uses recommended by the manufacturer as a pulp capping material, root end filling material, root repair material, it has the potential to be used also a scaffold owing to its superior biologic characteristics based on its bioceramic nature (Camilleri, 2015; Siboni et al., 2017).

Up to our knowledge, the effect of Neo MTA nanoparticles and bioactive glass nanoparticles on the osteogenic differentiation and proliferation of stem cells of the apical papilla has not yet been evaluated. Therefore, the aim of the current study was to evaluate the effect of

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Neo MTA nanoparticles and bioactive glass nanoparticles on the osteogenic differentiation and proliferation ability of stem cells of the apical papilla. The null hypothesis tested is that there is no significant difference between the Neo MTA nanoparticles and bioactive glass nanoparticles on the osteogenic differentiation and proliferation of stem cells of the apical papilla.

2. Methodology

2.1. Preparation of nanomaterials

2.1.1. Bioactive glass 45S5 nanoparticles

The sol–gel method was adopted to prepare bioactive glass 45S5 nanoparticles from a colloidal solution of 45S5 composition (45 mol% SiO_2, 24.5 mol% CaO, 24.5 mol% Na_2O and 6 mol% P_2O_5) (Nanogate, Cairo, Egypt). Ceramic powder was produced from the gel after heating (Vafa et al., 2021).

2.1.2. Neo MTA nanoparticles

Milling of the Neo MTA powder (NuSmile Ltd. "Avalon Biomed", Houston, TX, USA) was performed using ball mill machine for 10 h at a speed of 350 rpm and 3 min intervals to obtained nanoparticles of Neo MTA.

Characterization of all of the prepared nanoparticles was done using high-resolution TEM (JEOL Ltd., Tokyo, Japan) and XRD (X'PERT-PRO Quantify, Almelo, Netherlands) with 2 thetas (10°–70°), with a scanning speed of 1°/min and minimum step size 2Theta: 0.001 at wavelength (Kα) = 1.54614° (Vuong Bùi, 2017) as shown in Fig. 1.

2.2. Stem cells harvesting and culture

2.2.1. Patient recruitment

Stem cells of the apical papilla were harvested and cultured from freshly extracted wisdom teeth of three patients after obtaining an informed consent (ethical approval number: FDASU-Rec IM112105). The three patients were medically fit and aged between 17 and 18 years, scheduled for wisdom teeth extraction for reasons not related to the current study.

2.2.2. SCAP Isolation

Apical papilla was dissected, and SCAP were isolated using enzyme digestion method, cells were seeded, incubated, and passaged for three times following the methodology detailed by Mahmoud et al. (Mahmoud et al., n.d.). Inverted phase contrast microscope (Labomed, New York Microscope company, NY, USA) was used to check for growth and/or contamination The harvested cells were cryopreserved and stored in −80 °C for further analysis.

2.2.3. SCAP characterization

Flow cytometric analysis was performed using the protocol published earlier on Navios software (Beckman Coulter, Pasadena, California, USA) (Mahmoud et al., n.d.) using immunoassaying stains CD45-PC5 (Phycoerythrin Cynin), CD44-FITC (fluorescein Isothiocyanate) and CD73-PE (phycoerythrin) to stain SCAP.

2.2.4. SCAP Culture

Cells cultured in complete culture media and harvested after the third passage. The harvested apical papilla stem cells were cryopreserved at −80 °C for further analysis (Mahmoud et al., n.d.).

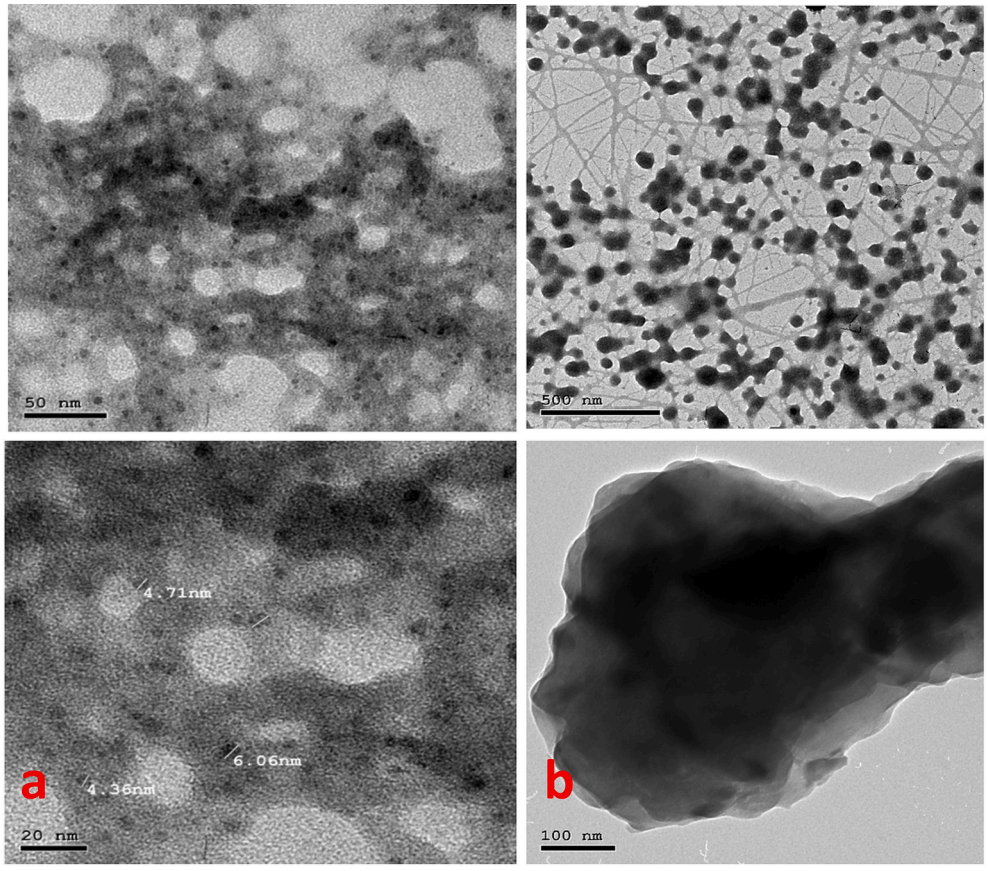

Fig. 1. Transmission electron microscopic image of a) Bioactive glass 45S5 nanoparticles, b) Nano Neo MTA nanoparticles.

Table 1
Mean ± SD and p-values of ALP concentration of all tested groups.

Adjuvant medium	OM [PC]	NNMTA (7.9 µg /mL)	NBG (500 µg/mL)	DMEM [NC]
Mean ± SD	77.68 ± 1.28 [c]	87.45 ± 1.5 [d]	69.86 ± 0.98 [b]	55.81 ± 1.6 [a]
P value	<0.001			

*Groups having different letters are significantly different from each other.

Table 2
Mean ± SD and p-values of H-score for RANKL protein expression by IF assay for all groups.

Adjuvant medium	OM [PC]	NNMTA (7.9 µg /mL)	NBG (500 µg/mL)	DMEM [NC]
Mean ± SD	84 ± 1.55 [b]	82 ± 2.54 [b]	156 ± 4.14 [c]	18 ± 1.53 [a]
P value	<0.001			

* Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

2.3. Sample preparation and classification

All tested nanomaterials were mixed with 1 ml of PBS, then sonicated for proper homogenization of the suspension followed by ultraviolet sterilization for 30 min. The samples were classified into four equal groups and testing was done following ISO 10993-5 standard as follows:

- **Negative control group "DMEM [NC]":** SCAP with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermosientific, Germany).
- **Positive control group "OM [PC]":** SCAP with inductive media, osteogenic differentiation medium (OM) (Gibco, Thermosientific, Germany).
- **Group I "NNMTA":** Neo MTA [EC50 7.864 µg/ml] with SCAP.
- **Group II "NBG":** Bioactive Glass Nanoparticles [NBG 500ug/mL] with SCAP.

2.4. Samples evaluation

For osteoblastic differentiation, six-well plates were used to culture stem cells of the apical papilla OM seeded at 4.5×10^5 cells/well. Plates were incubated for a period of 72 h at 37 °C and 5 % CO_2. The activity of ALP was measured using enzymatic dephosphorylation by ALP assay kit (Sigma-Aldrich, St. Louis, Missouri, USA). For testing the expression of RANKL for SCAP, the cells were examined using specific polyclonal antibody (Invitrogen, Thermo Fisher Scientific, Hilden, Germany) by fluorescence microscope (Labomed Fluorescence microscope LX400, cat no: 9126000; New York Microscope company, NY, USA). H-score of each sample was calculated as the sum of each intensity multiplied by the percentage of positive cells.

Regarding evaluation of the proliferation, the SCAP were stained by trypan blue and counted by hemocytometer to estimate the number of dead cells. The MTT assay was performed using the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen, Thermo Fisher Scientific, Hilden, Ger many). Cell viability was determined by measuring the optical density at 570 nm on an ELx800 spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).

2.5. Calculation of EC50 of nano Neo MTA to induce effective proliferation on human stem cells of apical papilla

In order to determine the safe concentration of Nano Neo MTA to be applied on cells, the half maximal effective concentration (EC50) test was calculated using the linear regression analysis. The best fit EC50 was (7.86 µg/ml). Therefore, the safe concentration determined and used in this study was (7.864 µg/ml).

2.6. Statistical analysis

Mean and standard deviation values of each group were calculated. Shapiro-Wilk's and Levene's tests were used to test for normality of the data. One-way ANOVA test was run followed by Tukey's post hoc test as the data was normally distributed. The significance level was set at $p < 0.05$. Statistical analysis was performed with Statistical package for Social Science software.

3. Results

3.1. SCAP characterization

The observed results of the characterized SCAP, revealed that the cells showed double bright surface expression of CD44/CD73 and failed to express CD45, indicating a non-hematopoietic origin.

3.2. SCAP differentiation

NNMTA showed the highest ALP concentration (87.45 ± 1.5) followed by OM, NBG, and DMEM-NC as shown in the Table 1. RANKL expression results are shown in Table 2 and Fig. 2 where NBG showed the highest H score followed by OM, NNMTA, and DMEM-NC.

3.3. SCAP proliferation

NBG showed the highest viable cell count as shown in Tables 3. NNMTA showed the highest viable count also using the MTT assay although the difference was not statistically significant from that of NBG as shown in Table 4.

4. Discussion

Endodontic management of nonvital immature teeth is quite challenging owing to the thin dentinal wall, compromised crown/root ratio, and the ability to seal the apical root end properly and three-dimensionally (Chueh et al., 2009).

The presence of mesenchymal stem cells have been demonstrated related to immature teeth in addition to mature ones. The origin of such mesenchymal stem cells is believed to be the apical papilla, bone, PDL, and/or granulomas (Miller et al., 2018).

Apical papilla stem cells are derived from an embryonic neural crest-like tissue, located at the root end of immature teeth. In contrast to other isolated types of stem cells, apical papilla stem cells demonstrate impressive odontogenic differentiation and proliferation in addition to massive dentinogensis (Sonoyama et al., 2008; Lei et al., 2011).

Under favorable conditions, MTA can stimulate the proliferation and differentiation of apical papilla stem cells. Bioactive glass has shown

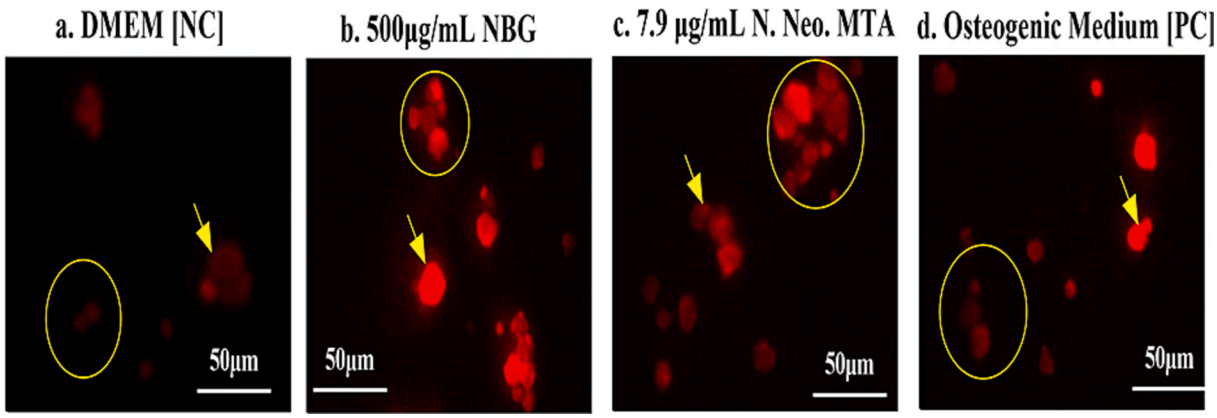

Fig. 2. Photomicrograph showing expression of RANKL protein in differentiated SCAP, the photos were captured by LABOMED Immunofluorescence microscopes. a) discreate of cells with homogeneous faint expression of RANKL with dim fluorescence intensity (+) was found in the negative control cells "UM." The expression was localised to the cell membrane. b) NBG. c) Neo MTA; both experimental groups revealed a merged big colony of odontoblasts with dense homogeneous membrane and nuclear RANKL expression. d) positive control showed increased number of odontoblast colonies with dense homogeneous RANKL expression (++) and an H-score of 84. The magnification is 10X. The odontoblast colonies are highlighted by the yellow circles, and the yellow arrow indicates RANKL expression in the membranous and nuclear compartments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Total, dead, viable cell counts and mean ± SD values for tested groups.

	Total Cell Count	Dead Cell Count	Viable Cell Count
OM [PC]	$60.73 \times 10^5 \pm 27.9 \times 10^5$ [c]	$7.34 \times 10^5 \pm 1.54 \times 10^5$ [b]	$60.42 \times 10^5 \pm 8.28 \times 10^5$ [c]
N. NeoMTA (7.9 µg /mL)	$44.73 \times 10^5 \pm 32.4 \times 10^5$ [b]	$1.37 \times 10^5 \pm 0.19 \times 10^5$ [a]	$44.7 \times 10^5 \pm 0.029 \times 10^5$ [b]
NBG (500 µg/mL)	$198.75 \times 10^5 \pm 56.9 \times 10^5$ [d]	$18.9 \times 10^5 \pm 6.37 \times 10^5$ [c]	$195.5 \times 10^5 \pm 47.26 \times 10^5$ [d]
DMEM [NC]	$4.65 \times 10^5 \pm 1.11 \times 10^5$ [a]	$4.12 \times 10^5 \pm 3.6 \times 10^5$ [a]	$4.33 \times 10^5 \pm 0.34 \times 10^5$ [a]
P value	<0.001	0.004	<0.001

*Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

Table 4
Mean ± SD and p-values of viability test (MTT assay).

Adjuvant medium	OM [PC]	NNMTA (7.9 µg /mL)	NBG (500 µg/mL)	DMEM [NC]
Mean ± SD	0.93 ± 0.03 [a]	1.6 ± 0.03 [b]	1.56 ± 0.24 [b]	0.82 ± 0.02 [a]
Viability (%)	113.46 %	196.06 %	189.19 %	100.08 %
P value	<0.001			

*Groups having the same letter are not significantly different from each other, while those having different letters are significantly different from each other.

promising results when tested for their biologic effect on dental pulp stem cells and mesenchymal stem cells (Gong et al., 2014; Gough et al., 2004; Gholami et al., 2017; Yang et al., 2018; Houreh et al., 2017; Amir et al., 2014). The aim of the current study was to investigate the effect of nano Neo MTA and nano BG on osteogenic differentiation and proliferation of stem cells of the apical papilla.

Trypan Blue was used for counting the viable cells in the current study owing to its characteristic ability to stain only the dead cells following penetration of its cell membrane (Piccinini et al., 2017; Paolo Di Nardo et al., 2017). The MTT assay was used due to its capability to determine the mitochondrial activity (Meerloo and Cloos, 2011; Gomes-Cornélio et al., 2017). Alkaline phosphatase enzyme activity assays was used as a measure of SCAP differentiation into osteoblast-like cell as it is considered as a characteristic marker for bone-forming cell differentiation (Reilly et al., 2007). Immunofluorescence assay is considered as one of the most reliable tests that helps elaborate specific protein of interest through antigen–antibody reaction (Ryu, 2017). The RANK-L concentration is proportional to the number of osteogenic cells because it is deemed mandatory for its differentiation (Magri et al., 2017; Gabbai-Armelin et al., 2015).

SCAP characterization results of the current study comes in full agreement with Kang et al who also confirmed the non-hemopoietic origin of the stem cells by lack of CD45 expression (Kang et al., 2019).

Although the superior results of the NNMTA group regarding the ALP assay cannot be directly compared to other studies as none could be identified in the literature testing nano Neo MTA, this comes in full agreement with Yan et al. (Yan et al., 2014), Schneider et al. (Schneider et al., 2014), Hajizadeh et al. (Hajizadeh et al., 2018), and Du et al. (Du et al., 2020) who have shown MTA and different MTA-based biomaterials to positively enhance and stimulate odontoblastic and osteoblastic differentiation of SCAP.

The enhancement and stimulation of osteoblastic differentiation could be attributed to the presence of tantalum oxide in the composition. Tantalum oxide, a biomaterial which promotes the production of calcium hydroxide (Tanomaru-Filho et al., 2017). The increased levels of calcium ions will in turn help increase the expression of osteopontin and bone morphogenic protein-2, the mineralization promoting genes in the dental pulp stem cells (Wang et al., 2014).

On the other hand, NBG showed statistically significantly higher RANKL expression compared to the NNMTA group as well as the negative control group. This is consistent with Wang et al. (Wang et al., 2019) who tested BG on bone marrow stem cells. This could be simply

explained by the increased ion release, specifically calcium ions which attracts different cells.

The NBG group also showed significant effect on SCAP viability and osteogenic differentiation in comparison to the negative control group. This finding comes in agreement with Yang et al. (Yang et al., 2018) who tested it on mesenchymal stem cells. This could be explained based on the nanoparticle size which greatly affects its behavior in addition to the increased calcium ions release that increases cell mineralization.

Although the NBG showed better results for the MTT assay than the NNMTA group, the difference was shown to be non-statistically significant. This is a quite logic finding as both bioceramic-based nanoparticles possess excellent bioactivity due to the calcium ion release. Both tested bioceramic-based nanoparticles proved to enhance the osteogenic differentiation and proliferation of SCAP. This could improve the regenerative procedure in endodontics as osteogenic differentiation enhances lesion healing and laying down hard tissue structure which might be dentin-like.

5. Conclusion

Within the limitations of this in vitro study, it can be concluded that isolation of SCAP can be done from extracted fully impacted immature third molars. Both tested bioceramic-based nano-biomaterials can induce osteogenic differentiation and proliferation of SCAP.

Ethical statement

The research proposal was approved by the Faculty of Dentistry Ain Shams University ethical committee with approval number FDASU-Rec IM112105.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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

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