Original Article

EFFECT OF NEO-MTA AND HYDROXYAPATITE NANO-PARTICLES ON PROLIFERATION AND OSTEOGENIC DIFFERENTIATION OF APICAL PAPILLA STEM CELLS (SCAPS) (IN VITRO STUDY)

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Abstract

Objective: The aim of the study was to evaluate the effect of Neo-Mineral Trioxide Aggregate and hydroxyapatite nanoparticles on the proliferation and osteogenic differentiation of apical papilla stem cells (SCAPs)

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.

• All tested biomaterials have the ability to induce osteogenic differentiation and proliferation of SCAP.

• Nano Neo MTA biomaterial has increased ability for differentiation of SCAP to osteoblasts.

• Nano NeoMTA biomaterial has increased ability for proliferation of SCAP proved by upregulated cell viability

Introduction:

(ERP) is a biological procedure to replace diseased, damaged, or missing structures including dentin, cells of the pulp-dentin complex and root structures to restore the normal physiologic functions of the pulp-dentin complex. Delivering and stimulation of the mesenchymal stem cells into root canal systems via different approaches during the regenerative procedures are necessary to attain a successful treatment. Because of their anti-inflammatory ability, these stem cells may survive in inflammatory situations and continue to play a critical role during the re-apexogenesis of developing roots arrested by periapical diseases (1).

Stem cell's origin is the mesenchyme, in vitro, they undergo multiline age differentiation and have magnificent clonal expansion capacity (2). Different stem cells' sources have been identified including stem cells from human exfoliating deciduous teeth (SHED), dental pulp stem cells (DSPSCs), and stem cells from the apical papilla

(SCAP). In 2006, SCAP were first discovered and isolated from the apical papilla tissue of incompletely developed tooth (3).

Evidence is supporting the hypothesis that SCAP appear to be the source of primary odontoblasts that are responsible for the formation of root dentin, whereas DPSCs are likely the source of replacement odontoblasts. Conservation of these stem cells when treating immature teeth may allow continuous root development (4).

SCAP have the characteristics of expression of MSCs markers, self-renewal, proliferation, migration, differentiation, and immunosuppression. Moreover, SCAP are capable of differentiating to various lineages of cells, such as osteogenic, odontogenic, neurogenic, adipogenic, chondrogenic, and hepatogenic cells, which a promising source for stem cell-based therapy (5)

Biomaterials used in endodontic always come in direct contact with the pulp and periapical tissues. Therefore, they should be biocompatible and have no adverse effect on differentiation and proliferation of stem cells present in the area.

Among multiple promising bioactive materials NeoMTA was developed with similar properties to MTA. MTA is indicated for perforation repair, root-end filling and other treatment modalities because of its physicochemical properties and biocompatibility. MTA is composed of Portland cement (PC) and contains 53.1% of tricalcium silicate, 22.5% of dicalcium silicate, and bismuth oxide (Bi_2O_3) as radio opacifier. Bi_2O_3 has been reported to promote discoloration in contact with sodium hypochlorite solution (6).

NeoMTA 2 is the second generation of root and pulp treatment materials whose predecessor was NeoMTA Plus. NeoMTA Plus is a new calcium silicate-based cement for root filling. Studies showed that Neo MTA Plus have adequate radiopacity, hydration and produce calcium hydroxide, which is important to induce mineralized tissue formation(6).Both materials are composed of a new tricalcium silicate based material, with tantalum oxide (Ta_2O_5) as a radiopacifying agent instead of bismuth oxide to overcome its well-known discoloration potential(7).

Due to recent introduction of Neo MTA 2 to the market, its effects on dental pulp cells are still not fully understood.

(HAp) is a significant biomaterial in the health care industry. Its chemical and mineral phases are analogous to those of natural bone and hence, its usage in the field of dentistry and orthopedics has been explored. Properties like osteoconductivity and osteoinductivity enhance bone regeneration and make hydroxyapatite an important material in tissue engineering, and its biocompatibility leads to its use as bioactive coating over implants. Nano-level hydroxyapatite has been investigated and demonstrated as having a good impact on cell biomaterial interaction(8).

Therefore, evaluation of the effect of Neo MTA 2 and nano-level hydroxyapatite on osteogenic differentiation and proliferation of SCAPs was thought to be of value

Materials and methods:

1. Materials:

Table 1: Materials and manufacture

Materials	**Manufacture**
1- Nanohydroxyapatite (NHAP)	**Nano gate, Cairo, Egypt**
2- Neo mineral Trioxide Aggregate (NeoMTA)	**Avalon Biomed, Texas, USA**
3- Dulbecco's Modified Eagle Medium (DMEM)	**Gibco, Thermosientific, Germany**

4- Penicillin G sodium, streptomycin and amphotericin B (PSA)	**Gibco, Thermosientific, Germany**
5- Fetal bovine serum (FBS)	**Gibco, Thermosientific, Germany**
6- Osteogenic Differentiation Medium (OM)	**Gibco, Thermosientific, Germany**
7- ALP assay kit	**Sigmaldrich, st Louis, MO**
8- Spectrophotometer	**Bio-Tek Instrument Inc., Winooski, VT, USA**
9- Rabbit Anti- RANKL	**Invitrogen; ThermoFisher Scientific, Hilden; Germany**
10- The Vybrant® MTT Cell Proliferation Assay Kit	**Cat no: M6494 (Thermo Fisher, Germany).**
11- Goat Anti-Rabbit secondary antibody-Alexa Flour 488	**Invitrogen; ThermoFisher Scientific, Hilden; Germany**

Summary of Materials and Methods:

Preparation and characterization of the materials:

A: Nano Neo Mineral Trioxide Aggregate (Neo MTA 2)

B: Nanohydroxyapatite (NHAP)

C: Dental Mesenchymal Stem Cells from Apical Papilla (SCAPs)

1- Samples collection and isolation

2- Culture protocol

3- Characterization of SCAPs

Sample preparation and Classification

Methods of Evaluation

A: Evaluation of Osteogenic Differentiation

Alkaline Phosphatase Assay

Receptor activator of nuclear factor kappa-B ligand (RANKL)

B: Evaluation of Proliferation

1- Cell Counting and cell viability" Trypan blue" by Hemocytometer

2- MTT Assay

Preparation and characterization of the materials:

Nano Neo MTA 2:

A.1. Preparation:

The material composition according to the manufacturers' information is Tricalcium silicate (Ca_3SiO_5), dicalcium silicate (Ca_2SiO_4), tantalum oxide (Ta_2O_5), and minor amounts of calcium sulfate ($CaSO_4$) and tricalcium aluminate ($Ca_3Al_2O_6$)

In order to prepare the material in nanoform, the Neo-MTA powder was milled by using ball mill machine (planetary-ball-mill-pm-400) for 10h, speed 350rpm and 3min intervals.

A.2 Characterization:

Transmission electron microscope (TEM):

The transmission electron microscope (TEM) imaging was performed by HR-TEM, which is JOEL JEM-2100 high resolution transmission electron microscope operating at an accelerating voltage of 200 kV equipped with Gatan digital camera Erlangshen ES500.

Material Properties:

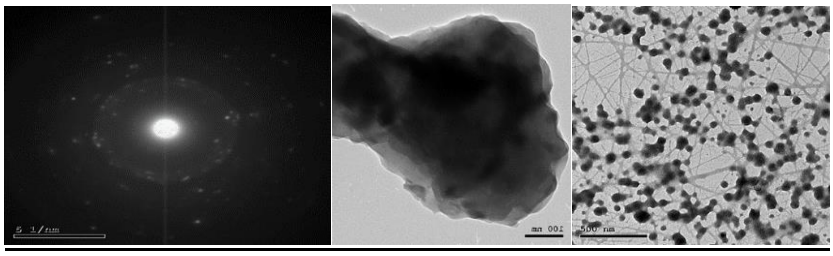
Appearance (Color): White to gray.

Appearance (Form): Powder.

Shape (TEM): spherical like shape

Avg. Size (TEM): 100±20 nm

Fig. 1: Shows the TEM images of the Neo-MTA2 nanoparticles

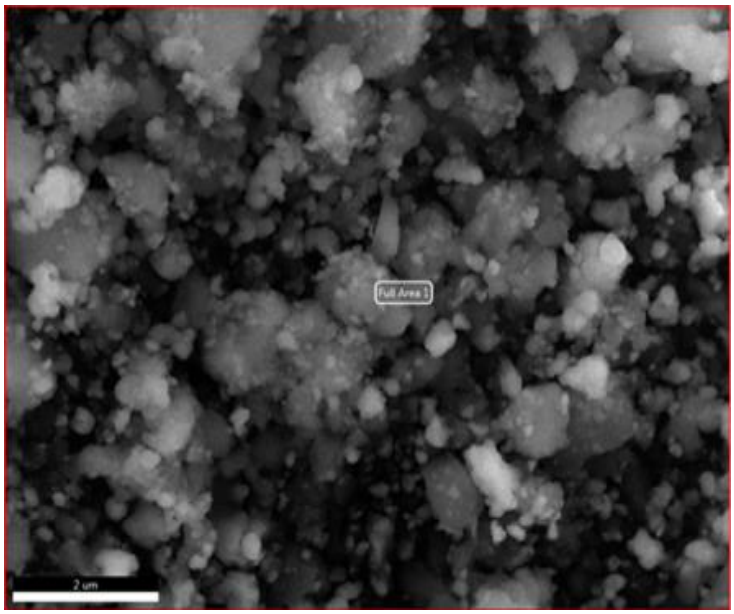


SEM/EDX

The morphology was determined by Field Emission scanning electron microscope (SEM)equipped with energy-dispersive spectroscopy (EDS) accessories (FESEM, Quattro - Thermos scientific, USA). (7) (52)

Fig. 2: Scanning electron microscope with energy-dispersive X-ray analysis (SEM-EDX) evaluating the chemical composition(spectra) and the element distribution (elemental mapping) of NeoMTA nanoparticles. (a) SEM micrograph of Neo MTA nanoparticles (b) figure illustrating the EDS plots with the correspondent peaks detected.

(a)



(b)

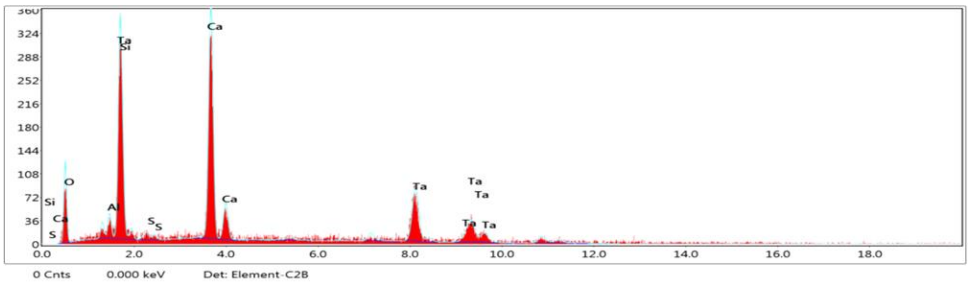


Table 2: EDX Elements Analysis of NeoMTA 2:

Element	Weight %	Atomic%	Error %
O K	40.08	71.85	13.19
AlK	2.55	2.71	16.91
SiK	0.85	0.87	39.31
S K	0.79	0.71	51.55
CaK	26.98	19.31	5.13
TaL	28.75	4.56	9.85

A. Nano Hydroxyapatite (powder):

B.1 Preparation:

Hydroxyapatite nanoparticles has been prepared by wet chemical precipitation method according to Xu et al (53)2004regardingthe next equation:

$$10Ca(NO_3)_2 + 6(NH_4)_3PO_4 + 8NH_4OH \longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 20NH_4NO_3 + 6H_2O$$

Calcium nitrate and ammonium phosphate were separately dissolved in aqueous solution. Calcium nitrate solution was dropped slowly into the ammonium phosphate solution with stirring and heating at 70◦C. The pH value of solution was kept between 10 and 12 by adding ammonium hydroxide. The apatite precipitation in water solution was treated hydrothermally in an autoclave at 140◦C under 0.3 MPa for 3 h. After treatment, the apatite precipitate was freeze dried at -50◦ C for 48 hours to make NHAP (Calcium Phosphate Hydroxide Ca10(PO4)6(OH) 2powder). The powder was used for further characterization.

B.2 Characterization:

TEM:

Transmission electron microscopy(54)(TEM JOEL JEM-2100 operating at 200 kV equipped with Gatan digital camera Erlangshen ES500) showed that NHAP white powder exhibited needles shape(54)with a particle size ranging from 150 ± 30 nm length and with 25±5 nm width (Figure. 3)

XRD:

The crystalline phases of the synthesized powders were determined using X-ray diffraction (performed using XPERT-PRO Powder Diffractometer system, with 2 theta (20^O - 80^O), with Minimum step size 2Theta: 0.001, and at wavelength (Kα) = 1.54614^O).

The XRD pattern (Figure. 4) of NHAP showed sharper peaks which indicate better crystallinity. The existence of 2θ peaks confirms the formation and presence of Ca10 (PO4)6(OH)2. The peak positions were matched exactly with primitive lattice with` the major crystalline phaseCa10 (PO4)6(OH)2. The results of XRD analysis obtained in the present investigation are in good agreement with the reported results of Bouyer et al., 2000(55).

Fig. 3: TEM images from NHAP indicating a needle-like crystals of an average length 150 ± 30 nm. The scale bar represents 100nm

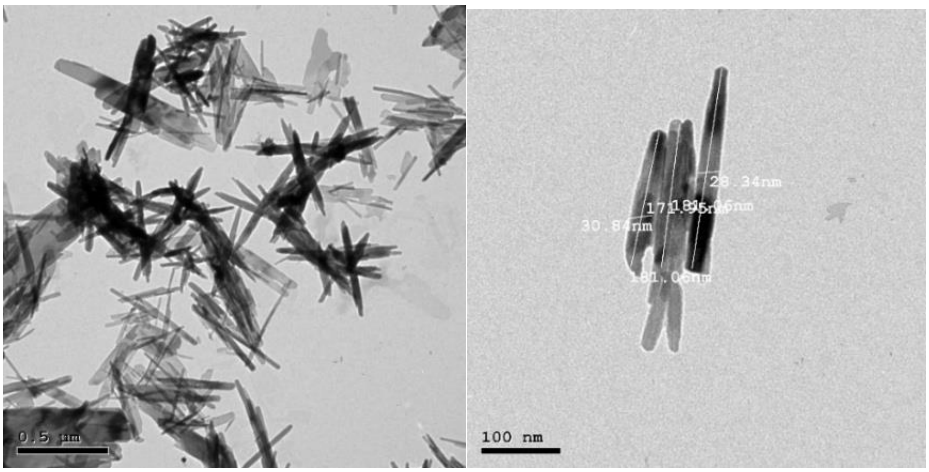
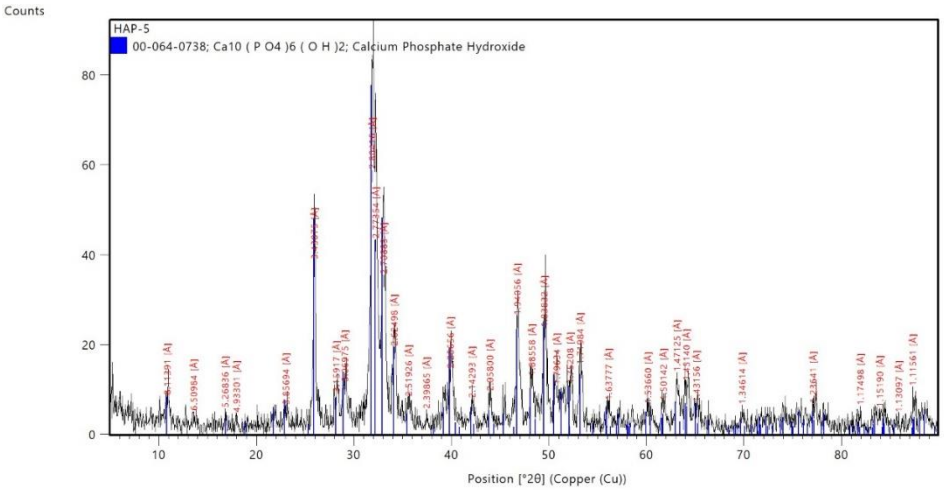


Fig. 4: XRD Pattern for synthesized NHAP showing sharper peaks indicating better crystallinity.



•C: Stem cells from apical papilla (SCAPs):

The following methodology was adopted from Amirikia et al [16], Kang et al [5], Fehrmann et al [56]

This study was approved by the research ethics committees Faculty of Dentistry Ain Shams University with approval number (FDASU-Rec IM112105). All experiments were performed in accordance the committee guidelines of the stem cells experiment. For this in vitro testing, SCAP was isolated and cultured in stem cell unit, Global lab, Cairo, Egypt.

C.1. Patient recruitment:

- Three healthy patients aged between 17 and 18 years were scheduled for wisdom teeth extraction in the Oral and Maxillofacial Surgery Department of Ain Shams University.

- After gaining verbal and written informed consent (appendix) from these patients, the extraction was done. - Three fully impacted sound immature third molars (incomplete root) were used in the study.

- The extracted teeth were immediately rinsed with sterile PBS (PH 7.4) and transferred in transfer solution [PBS + 10000 U penicillin/streptomycin + preservative media (1% dimethyl sulfoxide)] until being transferred to the laboratory for isolation of stem cells. (Figure 5a&b)

Fig. 5: Photograph showing: a) extracted teeth in a transfer solution; b) extracted teeth in a petri dish showing the apical papilla; c) the dental tissue minced into small pieces; d) the cell pellet after tissue digestion.

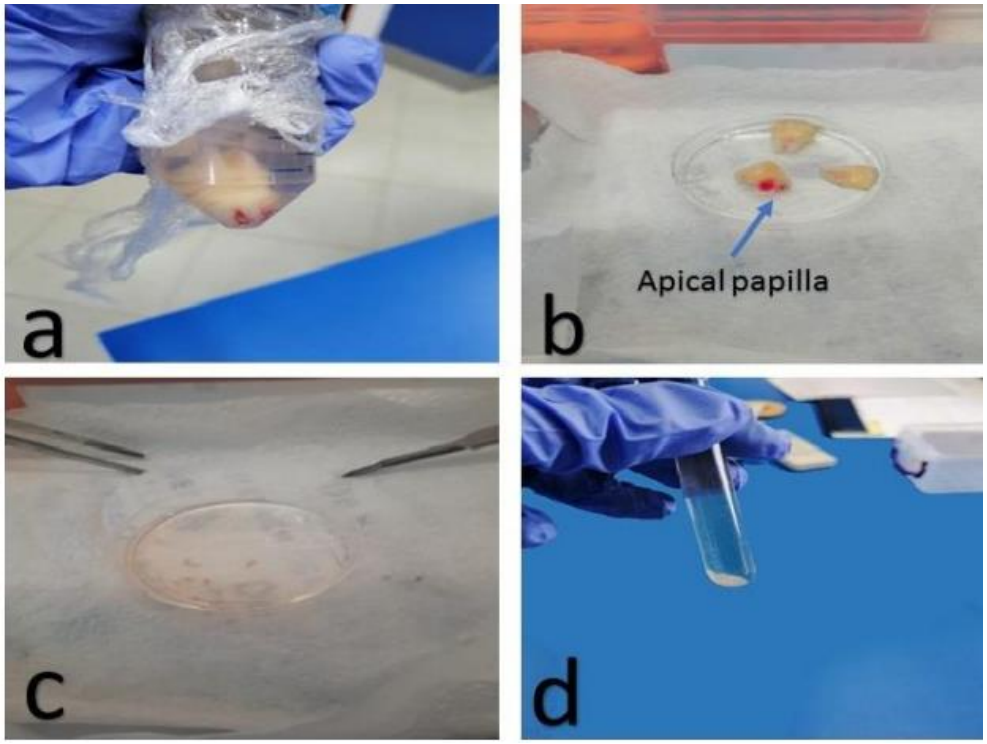
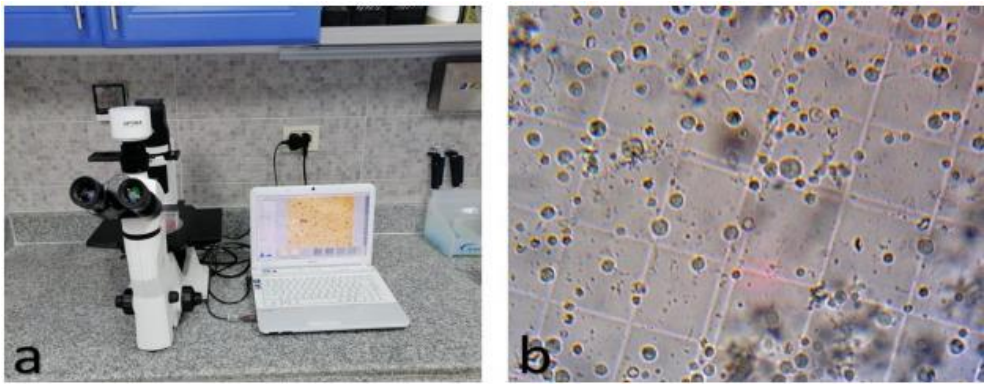


Fig. 6: Photograph showing: a) inverted phase contrast microscope; b) phase contrast photo of isolated stem cells after 24 hours culture.



C.2. Isolation of Stem cells of the apical papilla (SCAP): -

Apical papilla (soft tissue loosely attached to the apices of immature permanent teeth) was detached with a pair of forceps.

- SCAP were isolated from this tissue using enzymatic digestion method by the following protocol:

▪ The dental tissue was minced into small pieces approximately, $2mm^3$ diameters in Petri dish containing PBS (pH 7.4) and antibiotics.

▪ The tissue was digested in a solution of collagenase type I and dispase with continuous gentle agitation (in water bath and shaker) for 1 hour at 37°C.

▪ After digestion, the cell pellets were harvested by centrifugation. (figure5c&d) ▪ Cells passed through a cell strainer to obtain single cell suspension of SCAP.

▪ Cells were seeded in T- flask 75 cm3, in complete culture media [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 (25 µg) (PSA)]. Flasks were incubated at 37 °C in an atmosphere of 5% CO2.

▪ Isolated stem cells were monitored for infection and growth every 24 hours using inverted phase contrast microscope (figure 6).

C.3. characterization of SCAP: -

Characterization was done using flow cytometric analysis.

- The immunoassaying stains [CD45-PC5 (Phycoerythrin Cynin), CD44-FITC (fluorescein Isothiocyanate) and CD73-PE (phycoerythrin)] are used to stain the SCAP cells.

- The cells are suspended in PBS and the count is adjusted to 106 /ml. After centrifugation at 800xg for 10 minutes, the supernatant was discarded and the cell pellet is washed two times with PBS.

- Five µL of each antibody was added directly onto the cell pellet. - To avoid intense autofluorescence signals emerged from higher number of cells, only one antibody is used per tube.

- Cells were incubated at 4°C for 45 minutes, washed, and resuspended in binding buffer.

- Finally, cells were gated based on their monoclonal antibody staining, and the data was processed for flowcytometric analysis.

- The Navios software (Beckman Coulter) was used to analyze flowcytometry data.

C.4. Culture Protocol of SCAP:

- Stem cells that were isolated from apical papilla was cultured in T-flask 75 cm3, in complete culture media.

- Flask was incubated at 37°C in an atmosphere of 5% CO2. The media was changed every 24 hours.

- At 3rd passage (passage= 70%-80% confluency), cells were harvested.

- The harvested cells were cryopreserved in 1% DMSO and FBS and stored in -80°C for further analysis

D. Samples preparation and classification:

D.1. Preparation of mixed Nano NeoMTA2

- In order to determine the safe concentration of Nano NeoMTA2 to be applied on cells, EC50 test (half maximal effective concentration) was done.

- The EC50 was calculated using the Graph pad prism software 9.

- The Half maximal effective concentration (EC50) of Nano NeoMTA2 (N. NeoMTA2) to induce a halfway response between baseline and maximum response after exposure for 72 hours was calculated.

- A serial dilutions of N. NeoMTA2 were prepared by dissolving in 1ml of PBS to obtain five concentrations including; 50, 25, 12.5, 6.25, 3.12, and 1.56µg/ml.

Proper sonication was done to obtain homogenous suspension

- The samples were sterilized under UV for 30 minutes.

- The SCAPs were cultured for 72 hours with the different drug concentrations and cells cultured in Dulbecco's Modified Eagle Medium (DMEM) serve as negative control.
- At the end of incubation, cells were harvested and counted by Trypan blue dye, then the EC50 was calculated by linear regression analysis.
- In order to assess the dose associated response of N. NeoMTA2 on the proliferation index of SCAPs, we conduct an MTT assay and the response of different concentrations was normalized to cells co-cultured in DMEM medium.

D.2. Preparation of Mixed NHAP:

- For NHAP preparation, a concentration of 10 ug/ml was implemented according to previous protocols. [57] [58]
- The tested nanomaterial was mixed according to manufacturer instructions.
- In brief, a concentration of 10 µg from nanohydroxyapatite was dissolved in 1ml of PBS (phosphate buffered saline).
- Proper sonication was done to obtain homogenous suspension.
- The samples were sterilized under UV for 30 minutes.

D.3. Osteogenic Differentiation Medium preparation: -

Osteogenic Medium (OM) consists of (optimized MSC Osteogenic Differentiation Basal Medium cell media, Mesenchymal Stem Cell Qualified Fetal Bovine Serum, Penicillin-Streptomycin, Glutamine, Ascorbate, β-Glycerophosphate, and Dexamethasone) [16] was used for assessment of osteogenic differentiation potential of SCAP.

D.4. Grouping composites of Nanomaterials and SCAP: -

- Cells were seeded with the corresponding nanomaterials as a scaffold and each experimental condition was carried out in triplicate for each material and analyzed in three independent experiments according to the study design as follows: [59]

- SCAPs cultured in previously prepared Nano Neomta (7.86 ug/ml).
- SCAPs cultured in previously prepared Nanohydroxyapatite (NHAP 10 ug/mL)
- Positive control group (OM-PC): SCAPs cultured in odontogenic differentiation medium (OM).
- Negative control group (DMEM-NC): HDPSCs cultured in DMEM media.

➢ Plates were incubated at 37 °C and 5% CO2 for 72 hours.

➢ Each experiment was carried out in triplicate for each group and analyzed in three independent experiments.

Follow up of the effect of different composites on SCAPs was done using LABOMED inverted phase contrast microscope.

E. Evaluation:

E.1 In Vitro Osteogenic Differentiation of SCAP: -

For osteoblastic differentiation, SCAP were cultured in OM seeded at 4.5×10^5 cells/well. The OM media represents the positive control cells (OM-PC).

- SCAP were cultured with various biomaterials for testing their differentiation potential. The culture condition was similar to the PC cells.

- SCAP were cultured in DMEM media to serve as negative control (DMEM-NC) cells (no differentiation potential).

- Finally, Plates were incubated at 37°C and 5% CO2 for 72 hours, and then different tests for differentiation and proliferation potentials was applied.

E.1.a. Assessment of Alkaline phosphatase activity in differentiated cells:

The following methodology was adopted from Gong et al [60]

- The activity of alkaline phosphatase (ALP) was measured in SCAP using enzymatic dephosphorylation of para-nitrophenol phosphate (p-NPP) to para-nitrophenol (n-NP) by ALP assay kit.

- The cells were seeded in 24-well culture plates and incubated at 37oC with 5% CO2 for 72 hours in specific media according to the experiment design.

- The assay was performed by adding 100 µL of each p-nitrophenol standard and 50 µL of each test sample to a 96-well microtiter plate. - The AMP-substrate buffer was then added to each of the test samples. - After incubation at 37oC the absorbance was measured immediately at 405 nm using a spectrophotometer using an ELx800 absorbance microplate reader.

- A standard curve of absorbance versus concentration was generated and used to determine the ALP activity (U/L).

E.1.b. Assessment of RANKL protein expression in SCAP using Immunofluorescence Staining:

The following methodology was adopted from Wang et al [61], Magri et al [15], Gabbai-Armelin et al [62]

- Cells from different groups were harvested and cultured for 24 hours on cover slips, and examined for the expression of RANKL for SCAP cells using specific polyclonal antibody.

- Cells were fixed with warm 4% formaldehyde.

- The cells were immune-stained with Rabbit Anti- RANKL, incubated overnight at 4 °C.

- The cells were washed with PBS and incubated with Goat Antirabbit IgG H&L secondary antibody-Alexa Flour 488.

- The slide was covered with Prolong Gold Antifade Reagent and mounted overnight at room temperature.

- The specimens were immediately examined or stored at 4°C protected from light for long term storage.

- The microscopic examination was performed by Fluorescence microscope. The IF staining intensity was scored according to a fourtier 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 0-300. The mean value of H-score was calculated.

E.2. In Vitro proliferation of SCAP:

E.2.a. Cell counting & cell viability 'Trypan blue' by Hemocytometer:

The following methodology was adopted from Saberi et al [17], Martin-Piedra et al [63], Paolo Di Nardo et al [64], Piccinini et al [65]

The cells were seeded in 96-well culture plates in and incubated at 37 °C with 5% CO2 for 72 hours in specific media according to the experiment design. The cells were counted by hemocytometer to estimate the total number of cells according to the following protocol (figure 7).

1- Adding 10 μl of the harvested cells to the automated hemocytometer.

2- The chamber was then placed in the inverted microscope under 10X objective.

3- The cells in the large, central gridded square ($1mm^2$) were counted, and were multiplied by 104 for the estimation of the total number of cells per ml.

4- 0.1 ml of trypan blue solution in buffer were added to 0.1 ml of cells

5- The samples were loaded on hemocytometer and examined under low magnification to estimate the number of dead cells.

6- The cells were counted and the dead cells were measured to estimate the number of viable cells as follow: % of viable cell = [1.00 – (number of blue cells ÷ Number of total cells)] × 100 7- The number of viable cells per ml of culture estimated by the formula: Number of viable cells × 104 × 1.1 = cells/mL culture.

Fig. 7: Isolation and cell pellet after digestion

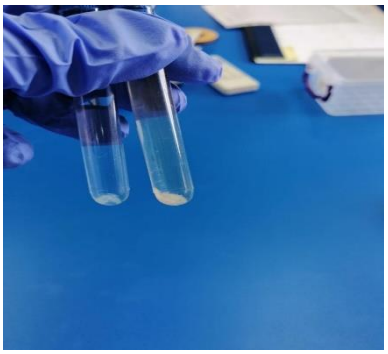
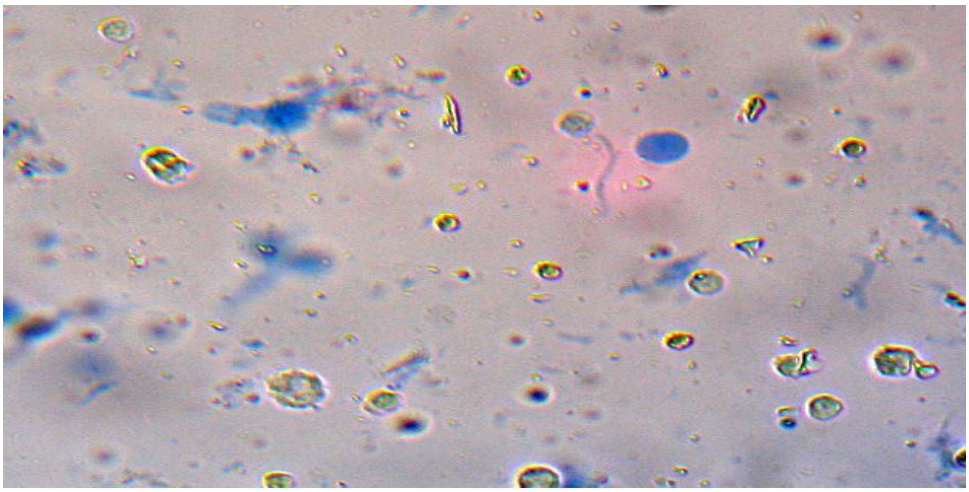


Fig. 8: Phase contrast photo of isolated SCAP stained with trypan blue.



E.2.b. Assessment of cell viability by cell proliferation assay (MTT):

The following methodology was adopted from Hameed et al [66], Doyon et al [67]

- The cell cytotoxicity assay was performed using the Vybrant® MTT Cell Proliferation Assay Kit.

- The cells (8×103 cells per well) were seeded in 96-well culture plates in and incubated at 37°C with 5% CO2 for 72 hours in specific media according to the experiment design.

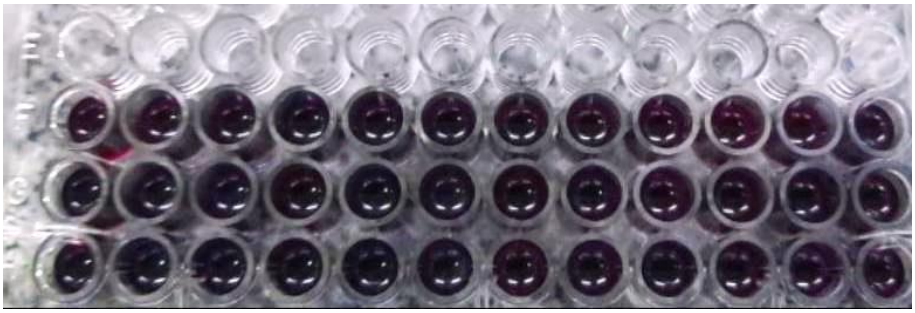
- Then 100µL of media was removed and replaced by new media.

- 20µL of MTT solution (1 mg/mL) was added to each well.

- The plates were incubated at 37°C and 5% CO2 for four hours. (Figure 2)

- Finally, the MTT solution was removed and 100 µL of SDS-HCL (Sodium Doucdyle Sulphate-HCL) was added to the wells. Cell viability was determined by measuring the optical density at 570 nm on a spectrophotometer. (Figure 9)

Fig. 9: Photomicrograph of 96-well plate with culture cells of different groups and MTT reagent. Showing assessment of cell viability by cell proliferation assay (MTT).



IV: Statistical analysis:

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (SPSS 24 for windows; SPSS Inc, Chicago). All graphs were plotted by GraphPad Prism Software 8.4.2 (San Diego, US).

1. Descriptive statistics:

- Mean, Standard deviation (±SD) and range for parametric numerical data.

- Frequency and percentage of non-numerical data and cross-tabulation.

Test of normality was done by using **Shapiro-Wilk test**, and the result confirmed that the data was normally distributed and so, we have decided to use **ANOVA test** and **post-Hoc test.**

2. Analytical statistics:

- **ANOVA test** was used to assess the statistical significance of the difference between more than two study group means.

- **Post-Hoc test**: Post hoc tests are an integral part of ANOVA. After the use of ANOVA to test the equality of at least three group means, statistically significant results indicate that not all of the group means are equal. However, ANOVA results do not identify which particular differences between pairs of means are significant. Post hoc tests were used to explore differences between multiple groups means while controlling the experiment-wise error rate.

- **P-value: level of significance**, a p-value ≥ 0.05: means statistically insignificant, p-value

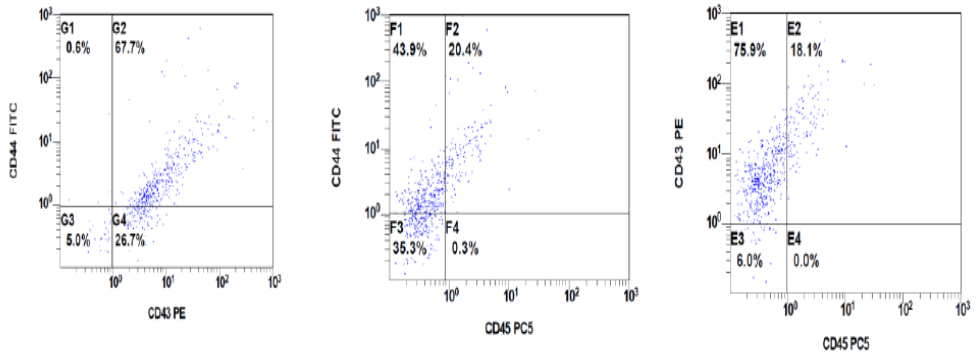
Results;

1. Characterized SCAP:

The observed results revealed that 67.7% of cells showed double bright surface expression of CD44/CD73 in contrast to only 5% of cells were double negative for both biomarkers. In addition, 26.7% were positive for CD73

and 0.6% express CD44. In order to confirm the non-hemopoietic source of stem cells, the CD73 and CD44 cells were gated with CD45 "hemopoietic stem cell marke". The obtained results revealed that 43.9% and 75.9% of the CD44 and CD73 positive cells; respectively didn't express CD45, which confirm that the isolated stem cells are isolated from non-hematopoietic source. (Figure 10)

Fig. 10: Characterization of SCAPs cells using multiparametric analysis: a representative FCM dot-plots showing the gate protocol for SCAPs cells. The SCAPS stem cells were stained with stem cell markers (CD43, CD44 and CD45). The CD43 and CD44 positive cells were gated in crossponding to CD45.



2. The effect of the tested materials on SCAP differentiation:

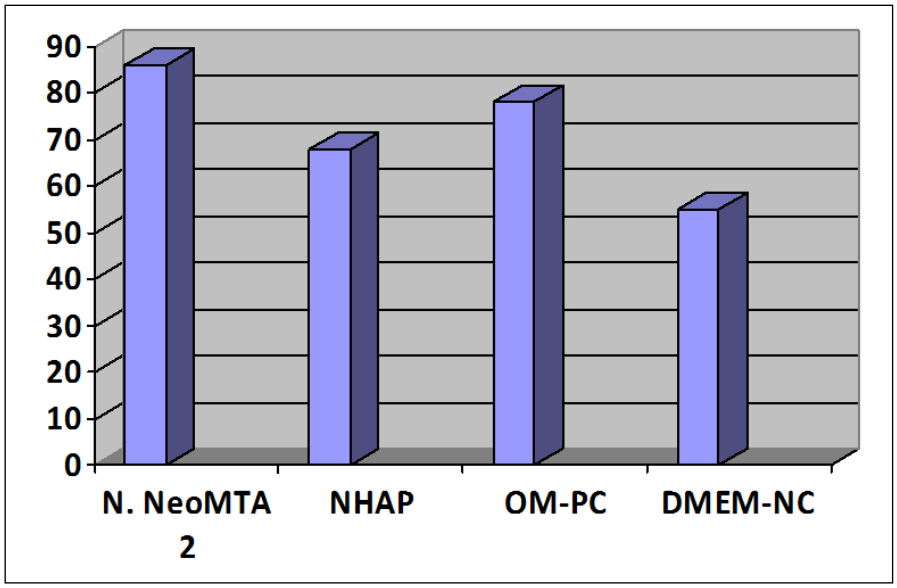
2.a. Alkaline phosphatase enzyme activity (ALP): [Table 3, Figure 11].

NeoMTA2 showed the highest concentration of Alp (86.2 ±0.1) followed by NHAP (68.72 ±0.06) and DMEM-NC showed the lowest concentration (55.12 ±0.03). There was a statistically significant difference between all tested groups.

Table 3: Alkaline phosphatase assay after 72 hours incubation of SCAPs treated with10µg/mL of NHAP and 7.9µg/mL of N. NeoMTA2 compounds for differentiation.

	N. NeoMTA2 [7.9µg/mL]	**NHAP [10µg/mL]**	**[OM-PC]**	**[DMEM-NC]**
Mean ±SD	86.2 ±0.12[a]	68.72 ±0.06[c]	77.99 ±0.05[b]	55.12 ±0.03[d]
P-value			<0.001	

Fig. 11: Alkaline phosphatase assay after 72 hours incubation of SCAPs treated with 10µg/mL of NHAP and 7.9µg/mL of N. NeoMTA2 compounds for differentiation.



2.b. RANKL protein expression: [Table 4, Figure 12&13]

NeoMTA2 showed the highest H score (180.67 ±4.04) followed by NHAP (68±4) and DMEM-NC showed the lowest H score (18±1). There was a statistically significant difference between all tested groups

Table 4: Calculation of H-score for RANKL protein expression on SCAPs cells.

	N. NeoMTA2 [7.9µg/mL]	**NHAP [10µg/mL]**	**[OM-PC]**	**[DMEM-NC]**
Mean ±SD	164 ±4 [a]	68 ±3 [c]	84 ±1 [b]	18 ±1 [d]
P-value			<0.001	

Means that don't share same letter are significantly different.

Fig. 12: H-score for RANKL protein expression on SCAPs cells.

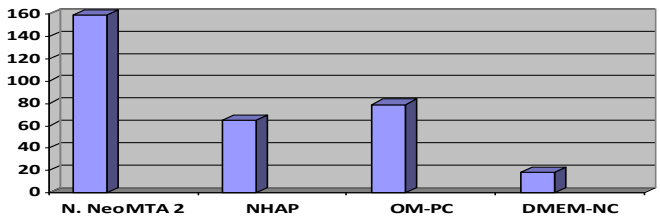
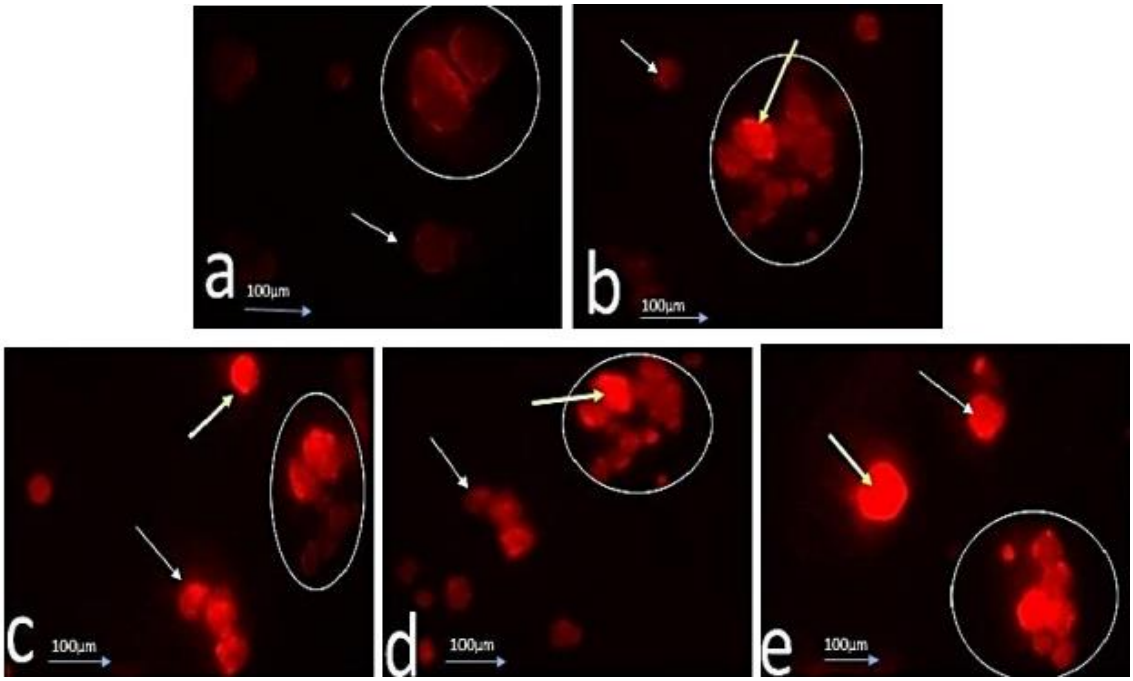


Fig. 13: Photomicrograph showing expression of RANKL protein in differentiated SCAP, the photos were captured by LABOMED Immunofluorescence microscopes. (a): Negative control cells shows small colonies of cells that showed a homogenous faint expression of RANKL, the expression was localized to the cell membrane. (b): Positive control cells with increased number of osteoblasts like colonies which are presented with dense homogenous expression of RANKL. However, the SCAP cultured with NHAP (c), NeoMTA2(d), showed a merged large colony of osteoblast like cells with dense homogenous membranous and nuclear expression of RANKL. The magnification power is 10x.The white circles highlight the osteoblast like colonies, white arrow: membranous expression of RANKL, yellow arrow: dense nuclear expression of RANKL.



3. The effect of the tested materials on proliferation potentials of SCAP (viability):

3.a. Trypan blue results: **[Table 5 and Figures 14]**

- **Dead cells**

A statistically significant difference was found between (DMEM-NC) and each of (OM-PC), (NHAP 10ug/mL) and (N.Neo MTA2) where (p<0.001).

While no statistically significant difference was found between (OM-PC) and each of (NHAP 10ug/mL) and (N.Neo MTA2) where (p=0.913) and (p=0.928).

Also, no statistically significant difference was found between (NHAP 10ug/mL) and (N.Neo MTA2) where (p=0.999).

The highest mean value was found in (DMEM-NC), while the least mean value was found in (NHAP 10ug/mL).

- **Viable cells**

A statistically significant difference was found between (N.Neo MTA2) and each of (OM-PC), (NHAP 10ug/mL), (DMEM-NC) where (p<0.001).

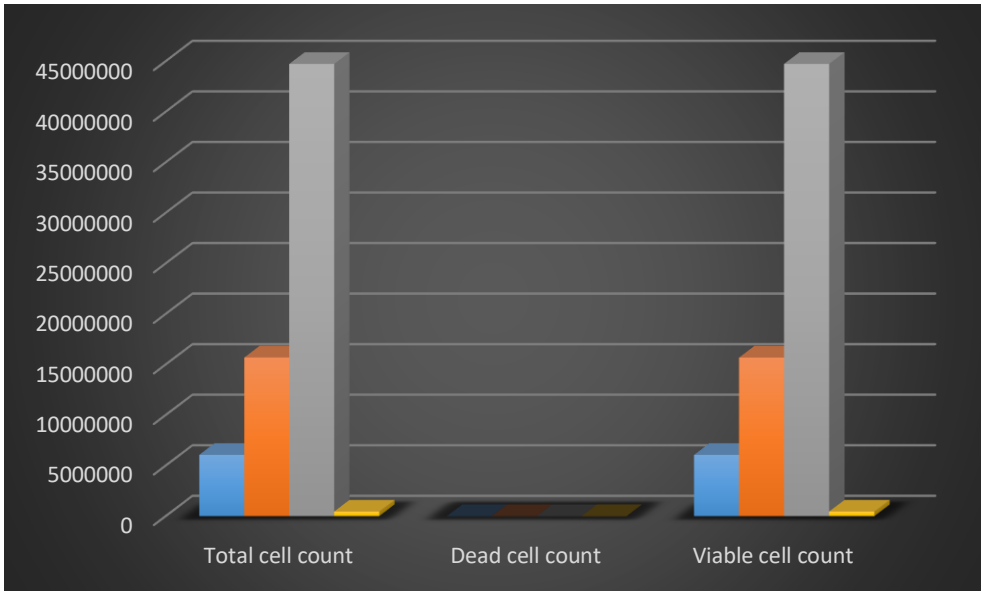
No statistically significant difference was found between any other groups.

The highest mean value was found in N. Neo MTA2 (mean: $3.51x10^{7}$), followed by cells co-cultured in NHAP (mean: $7.69x10^{6)}$), while the least mean value was found in (DMEM-NC).

Table 5: Cell count by Trypan blue dye of Stem cell of Apical papillae (SCAP) co-cultured with NHAP and N. NeoMTA2 compounds for differentiation.

	N. NeoMTA2 [7.86 ug/mL]	**NHAP [10ug/mL]**	**OM-PC**	**DMEM-NC**
Cell count [mean]	$3.51x10^{7\,a}$	$7.69x10^{6\,b}$	$6.38x10^{6\,c}$	$2.39x10^{6\,d}$
Dead cells [mean]	$2.59x10^{3\,c}$	$1.64x10^{3\,d}$	$1.60x10^{4\,b}$	$2.91x10^{5\,a}$
Viable cells	$3.51x10^{7\,a}$	$7.69x10^{6\,b}$	$6.36x10^{6\,c}$	$2.09x10^{6\,d}$

Fig. 14: Cell count by Trypan blue dye of Stem cell of Apical papillae (SCAP) co-cultured with NHAP and N. NeoMTA2 compounds for differentiation



3.b. MTT assay results: [Table 6 and Figure 15].

Both NHAP and N. NeoMTA2 have a significant potential to stimulate the proliferation of SCAPs after 72 hours incubation ($p<0.0001$). The ANOVA test revealed a high significant difference between the tested compounds ($p<0.0001$).

A statistically significant difference was found between (DMEM-NC) and each of (OM-PC), (NHAP 10ug/mL) and (N.Neo MTA2) where ($p=0.034$), ($p=0.007$) and ($p<0.001$).

Also, a statistically significant difference was found between (N.Neo MTA2) and each of (OM-PC), (NHAP 10ug/mL) where ($p=0.008$) and ($p=0.037$).

While no statistically significant difference was found between (OM-PC) and (NHAP 10ug/mL) where ($p=0.677$).

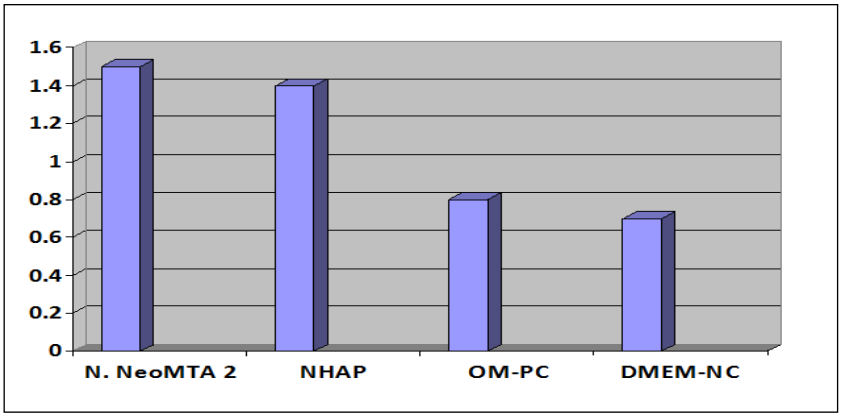
The highest mean value was found in (N.Neo MTA2), while the least mean value was found in (DMEM-NC).

Table 6: Cell proliferation assay (MTT) after 72 hours incubation of Stem cell of Apical papillae (SCAP) treated with NHAP and N. NeoMTA2 compounds.

	N.NeoMTA2	NHAP 10ug/ml	OM-PC	DMEM-NC
Mean±SD	**1.62±0.06** [a]	**1.29±0.14** [b]	**1.17±0.16** [b]	**0.83±0.08** [c]
P-value		**<0.001***		

Means that don't share same letter are significantly different.

Fig. 15: Proliferation index of NHAP and N. NeoMTA2 on SCAPs



Discussion:

Permanent immature non vital teeth present a distinctive clinical challenge for the endodontist. The major challenge in these teeth that even with proper disinfection and filling, the root canal dentinal wall still with minimal thickness making these teeth weak and liable to fracture. (68) Various treatment modalities have been employed to create hard tissue barrier at the apex, which includes non-vital pulp therapy with calcium hydroxide, apexification with mineral trioxide aggregate (MTA), pulp revascularization and regeneration. Numerous published case reports have revealed increased dentinal wall thickness, continued root development and apical closure, but there is still lack of sound scientific evidence regarding histological nature of the type of tissue. (69)

The traditional and most commonly used option for treatment of an immature necrotic is apexification with calcium hydroxide paste that have been reported to have a good outcome. However, there are certain limitations associated with this technique. The principal drawback is the long duration required for the formation of hard tissue apical barrier. This technique also tends to decrease the fracture resistance of the root dentine. Thus, there is always a possibility of root fracture before hard tissue formation. The second option uses an apical (MTA) plug to shorten overall apexification treatment time. Although, these treatment options still doesn't lead to increase root canal wall width or length sufficiently.(70)

The goal of the third option, pulp revascularization, is different than the first two options. The goals of revascularization are to stimulate apical root growth as well as to restore the blood circulation to the pulp space. In an immature necrotic tooth with an open apex, the apical pulpal tissue may remain vital and can act as a scaffold on which new tissue can grow upon. (71)

It has been shown that a substantial number of mesenchymal stem cells are transferred from the apical tissues during REPs in immature and mature teeth. These cells are hypothesized to be derived from the apical papilla but could also be sourced in various apical tissues including bone, the periodontal ligament, and granuloma. (17) More precisely, the apical papilla refers to the soft tissue that is loosely attached to the apices of immature permanent

teeth and can be easily detached with a pair of tweezers. There is a cell rich zone lying between the apical papilla and the pulp. The apical papilla is different from the pulp in terms of containing less cellular and vascular components than the pulp. [9]

Stem cells of the apical papilla (SCAP) are an important population of human mesenchymal stem cells in regeneration. They are derived from an embryonic neural crest like soft tissue, located at the apex of the incompletely developed root. Sonoyama et al. was the first who isolated and characterized these cells. SCAP have greater odonto/osteogenic capacity and outstanding dentinogenesis among the stem cells present in the periapical area and are ideal for dental tissue engineering. [72][73] Compared with dental pulp stem cells, SCAP exhibit a more pronounced population doubling capacity, an enhanced proliferation rate and mineralization potential indicating a more potent stem cell population [74]. They have the potential to proliferate and differentiate into osteoblast-like cells and odontoblast-like cell after culture in osteogenic inductive medium. [5]

Several studies have indicated that SCAP and human DPSC treated with MTA can differentiate to osteoblasts/odontoblasts and form bone-like or dentin-like tissues under suitable in vitro conditions. However, information regarding the effect of other biomaterials on SCAP is limited. [1][75]

Bioceramics have several favorable properties, including the ability to induce hard tissue formation and biocompatibility; they are also nontoxic, no resorbable, and unaffected by blood contamination. [18]

Hence, the aim of the present study was to investigate the effect of Neo MTA and NHAP on osteogenic differentiation and proliferation of stem cells of apical papilla.

In vitro assays have been widely used in the literature to characterize and establish a “biological profile” of new commercially available endodontic material before the analysis of their performance in animal models or clinical trials. [76]

Cell proliferation is the process of increase in the number of cells which occurs as a result of regulated cell growth and cell division. the proliferation potential of the SCAP affected by tested biomaterials used in this study assessed by counting the viable cells by trypan blue stain and MTT assay. [63]

Trypan Blue is recommended for counting viable cells. This dye can penetrate the cell membrane thus, it enters the cytoplasm of cells with compromised membranes (dead cells) to stain them blue. The live cells remain intact and can be distinguished from dead cells by their ability to exclude the blue dye. Hence during the evaluation of cell viability, it's preferable to stain cells with trypan blue dye for counting of dead and viable cells. [64][65]

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by living cells, which determines mitochondrial activity. The mitochondrial activity of the cells is reflected by the conversion of the tetrazolium salt MTT (yellow color) into formazan crystals (blue color), which can be solubilized for homogenous measurement. Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 540 and 720 nm. Since for most cell populations the total mitochondrial activity is related to the number of viable cells, this assay is broadly used to measure the in vitro cytotoxic effects of drugs on cell lines or primary patient cells. [77][78]

Stem cell differentiation involves the changing of a cell to a more specialized cell type, involving a switch from proliferation to specialization. This involves a succession of alterations in cell morphology, membrane potential, metabolic activity and signal responsiveness. In this study, alkaline phosphatase (ALP) enzyme activity assays and immunofluorescence (IF) assay were done to test SCAP differentiation into osteoblasts like cell.

Alkaline phosphatase activity as measured by reduction of p-nitophenol phosphate is a common marker of bone cell differentiation [79]. Osteoinductive biomaterials could stimulate stem cells to differentiate towards the osteogenic pathway. Alkaline phosphatase activity was important factors to determine the osteogenic differentiation of human MSC [80]. ALP is an early marker during the osteogenic differentiation, which can provide phosphate groups for subsequent hydroxyapatite deposition [81].

Immunofluorescence (IF) Assay is a microscopic method that can detect and visualize the proteins expressed in cells via antigen—antibody reaction. Immunofluorescence is a powerful approach for getting insight into cellular structures and processes using microscopy. Specific proteins can be assessed for their expression and location by immunofluorescence. IF experiment is based on Specific antibodies bind to the protein of interest and fluorescent dyes are coupled to these immune complexes in order to visualize the protein of interest using microscopy. [82]

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a member of the tumor necrosis factor (TNF) super family, it exists as either a membrane-bound protein or can be cleaved to form a secreted protein that still retains activity. RANKL is expressed on osteoblasts and binds the receptor RANK, which is produced on osteoclasts and their progenitors. [83][84]

RANK-L is a key factor for differentiation and activation of osteoclasts. Furthermore, the increase of the RANK-L concentration can be related to number of osteogenic cells, once this factor is expressed by osteoblasts. Higher immunoexpression of RANK-L could be combined with the increase of the osteoblasts and an attempt of degradation the material. [15][62]

During characterization of SCAP, like other MSCs, they express wide range of markers including CD13, CD24, CD29, CD44, CD49, CD51, CD56, CD61, CD73, CD90, CD105, CD106, CD166, NOTCH3, and vimentin. Meanwhile, SCAP are found to be negative for the expression of CD14, CD18, CD34, CD45, CD117, and CD150, indicating that they are not of hematopoietic origin. CD24 is considered a specific marker for SCAP, which is undetectable in other MSCs, including dental pulp stem cell. [3][5][85]

In this study, after SCAP characterization by Flowcytometry, the observed results revealed that 67.7% of cells showed double bright surface expression of CD44/CD43. In order to confirm the non-hemopoietic source of stem cells, the CD73 and CD44 cells were gated with CD45' hemopoietic stem cell marker". The obtained results revealed that 43.9% and 75.9% of the CD44 and CD73 positive cells; respectively didn't express CD45, which confirm that the isolated stem cells are isolated from non-hematopoietic source. This result come in agreement with Kang et al. [5]

The results of the total effect of Neo MTA, NHAP on osteogenic differentiation potential of SCAP in this study showed that, the highest ALP activity and RANKL protein expression were observed in Neo MTA group, however, the least number of cells was observed in NHAP group. There was significant difference detected between different groups.

Nano-scaled materials may exhibit very different physical, chemical and biological properties compared to micro sized materials of the same composition. Building biomaterials at the nanoscale level is very crucial for dental pulp regeneration. It allows the concentration of many different functions in a small volume and presents the advantage of increasing the quality of targeting while controlling the cost and delivery of the active molecules. These endodontic nanomaterials can be reservoirs of antibacterial and anti-inflammatory molecules. They also deliver growth factors guiding the migration, proliferation, and differentiation of the different pulp cells[86]

The present study was carried out to evaluate the effect of specific nano biomaterials NHAP and Nano NeoMta on osteogenic differentiation and proliferation of SCAPS. Therefore, we isolated SCAPs from young adult patient due to the regenerative ability of dental stem cells and bioactivity of the tested nano biomaterials also to correlate their efficacy on SCAPs against the plain cultured cells (negative control) or the supplemented cells with the odontogenic/osteogenic medium (positive control).

In the current experiments we examined the ability of nano NeoMTA and NHAPs to enhance alkaline phosphatase activity (ALP) in human MSCs as it is considered as an early and commonly used repeatedly osteogenic/odontogenic differentiation marker in multiple studies in agreement with **Gong et al., 2014**[60], **Reilly et al., 2007**[79] who declared in their studies that Alkaline phosphatase activity (ALP) was measured by reduction of p-nitrophenol phosphate as a common marker of bone cell differentiation. Multiple studies have suggested that alkaline phosphatase activity is enhanced when cells are grown on Nano HAP demonstrated by **Yang et al** [45]**2018**

After isolation and identification of SCAPS the study tried to expose those cells by two main different tested nano bioactive materials to test their osteogenic differentiation potential and proliferation by specific tests.

SCAPs cultured with nano NeoMTA2 showed highest intensity of dentin matrix protein expressed as biomarker for odontogenic differentiation.

The results of the present study came in accordance with Rodriguez et al 2021[7], who claimed that that NeoMTA2 promotes cell viability, cell migration, and cell attachment, and induces the odonto/osteogenic differentiation of hDPSCs without using chemical osteogenic inducers. They claimed that NeoMTA2 was associated with high release of calcium ions which stimulates hydroxyl apatite formation and release of alkaline phosphatase and bone morphogenic protein 2, which are important in the mineralization process.

However, there are limited studies about NeoMTA 2 and NeoMTA Plus which are new materials released to eliminate the disadvantages of MTA.

Previous studies showed that NeoMTA Plus, which contain Ta_2O_5, promote mineralization and the formation of reparative dentine bridges in vitro and in vivo. Tantalum oxide has been reported to promote the production of mineralized nodules necessary in the pulp repair process. [6]

The higher mineralization potential exhibited by NeoMTA 2 than his predecessor NeoMTA Plus may be related to the higher proportion of tantalite [7].

In our study, Nano NeoMTA 2 showed high proliferative potential on SCAPs after 72 hrs. This result came in contradiction with Rodriguez et al 2021[7] who claimed that after 72 hrs. of culture, the exposure of hDPSCs to the material eluates did not significantly affect mitochondrial metabolism. This might be due to different material preparation method and different culture conditions.

However, their results suggested that the material promoted cell viability and did not exhibit apparent cytotoxic effect after 48 and 72 hrs.

Unfortunately, the toxic effect of NeoMTA on SCAPs still remains unclear.

Tanomaru-Filho et al [6] researched the biocompatibility of the NeoMTA Plus, MTA Angelus and the tricalcium silicate cement containing the tantalum oxide with MTT test. They formed the materials in different dilutions and measured the cell viability after 24 h. According to the test results, they reported that NeoMTA Plus, MTA Angelus and the tricalcium silicate cement were biocompatible on hDPSCs.

Tomas Catala et al [30] researched the effects of the NeoMTA Plus, MTA Angelus and MTA Repair HP on dental pulp stem cells by using MTT test on days 1, 3 and 7. As a result they reported that there was a high level of cell proliferation and binding in all three materials stated.

Regarding nano NeoMTA group, its results showed significant effect on SCAP viability and osteogenic differentiation in comparison with the negative control group.

The second biomaterial selected for this work was NHAP have been reported by **Yang et al**[48] 2018 that NHAPs with a diameter of about 20nm promoted the type I collagen, OCN, and OPN expressions of rabbit mesenchymal stem cells. Also, they reported that NHAPs of all sizes had stimulatory effect on the osteogenic differentiation of hMSCs in vitro. The DMSCs incubated with smaller-sized NHAP (S50 and S100) seemed to have higher differentiation rate compared with that treated with S150, indicating that the efficiency of osteogenic differentiation of hMSCs was dependent on the size of NHAPs. Moreover, **Wang et al.** prepared HA nanospheres (~50nm in diameter) and HA nanorods (~50nm in length) through the induction of protein template and found that both nanoparticles could significantly enhance the osteoblastic differentiation of rat mesenchymal stem cells, especially the HA nanospheres. NHAPs have been tested at a concentration of 10 ug/ml according to **Yang and colleagues 2018**[48] and in agreement with **Remya et al., 2014**[34] as their MTT assay results indicated that NHAPs does not induce cytotoxicity from 10µg/ml up to 800 µg/ml, and amplified that cell viability decreasing with increasing concentration so our wok tried to use the safest concentration for NHAP.

Our results revealed that nanohydroxyapatite has the ability to stimulate osteogenic differentiation of SCAPs. In accordance to our study, **Mohamed et al.** [87] concluded that nano hydroxyapatite promoted odontogenic differentiation of DMSCs. **Liu H-C et al.** [88] 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. Also, **Yang et al.** [48] proved that HANPs of all sizes had stimulatory effect on the osteogenic differentiation of hMSCs in vitro reflected by increased ALP activity and enhanced expression of bone-related markers.

In our study, SCAPs cultured with Nanohydroxyapatite showed high proliferation rate after 72 hrs. This came in partial agreement with **Mohamed et al.** [47] who claimed that the tested material showed questionable inhibitory effects at long culture periods. Also, in accordance to our study, **Remya et al.** [34] revealed that the percentage viability of bone marrow mesenchymal stem cells (BMSCs) treated with low concentrations of HANPs (below 800ug/ml) were comparable to the negative control (cells alone) after 24 hrs.

Regarding nano hydroxyapatite group, its results showed significant effect on SCAP viability and osteogenic differentiation in comparison with the negative control group

Conclusions:

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.

• All tested biomaterials have the ability to induce osteogenic differentiation and proliferation of SCAP.

• Nano Neo MTA biomaterial has increased ability for differentiation of SCAP to osteoblasts.

• Nano NeoMTA biomaterial has increased ability for proliferation of SCAP proved by upregulated cell viability.

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