Differentiation of stem cells from apical papilla into neural lineage using graphene dispersion and single walled carbon nanotubes[3]

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SCAP differentiation into neural lineage using graphene dispersion and carbon nanotubes

ABSTRACT

Stem cell-based therapies are considered a promising treatment modality for many medical conditions. Several types of stem cells with variable differentiation potentials have been isolated from dental tissues, among them stem cells from apical papilla (SCAP). In parallel, new classes of biocompatible nanomaterials have also been developed, including graphene and carbon nanotube-based materials. The aim of the study was to assess whether graphene dispersion (GD) and water-soluble single walled carbon nanotubes (ws-SWCNT), may enhance SCAPs capacity to undergo neural differentiation. SCAPs cultivated in neuroinductive medium supplemented with GD and ws-SWCNT, separately and in combination, were subjected to neural marker analysis by real-time PCR (NF-M, ngn-2, β III-tubulin, MAP2) and immunocytochemistry (NeuN and β III-tubulin). GD, ws-SWCNT, and their combination, had neuro-stimulatory effects on SCAPs, as judged by the production of neural markers. Compared to cells grown in nanomaterial free medium, cells with GD showed higher production of B3T, cells with ws-SWCNT had higher production of ngn-2 and NF-M, while the combination of nanomaterials gave similar levels of both B3T and NF-M as the neuroinductive medium alone, but with the finest neuron-like morphology. In conclusion, GD and ws-SWCNT seem to enhance neural differentiation of stem cells from apical papilla.

Keywords: stem cells, apical papilla, neural induction, graphene dispersion, carbon nanotubes

INTRODUCTION

Stem cells (SCs), thanks to their ability to undergo differentiation into various cell types and great capacity for self-renewal, have a prominent role in regeneration of damaged tissues and/or organs. They could also bring under control some illnesses such as Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease and diabetes (1, 2).

Stem cells can be derived from embryonic tissues (embryonic stem cells, ESCs), various postnatal tissues (adult stem cells, ASCs), as well as by cell reprogramming (inducible pluripotent stem cells, iPSCs). ASCs of mesodermal origin are referred to as mesenchymal SCs (MSCs) and under permissive environment they show a remarkable capacity to differentiate into various cell lineages (3, 4). Owing to this differentiation potency, to immunosuppressive activity and high self-renewal capacity, MSCs are considered to be extremely valuable for clinical use.

Many dental tissues represent niches of MSCs and are becoming increasingly attractive in basic research and applicative regeneration studies due to their easy accessibility and absence of additional health risks for the donor. Apical papilla is a soft tissue at the apex of a not fully formed tooth, containing more than 95% of MSCs (stem cells from apical papilla or SCAP). Originating from neural crest, they express some early neural markers even without neural induction. They can be transformed into different cell types of neural lineage, therefore making them suitable for potential therapeutic applications in neurodegenerative diseases. Further exploration of their biological behavior is fundamental (5-9).

A growing number of nanomaterial-based scaffolds are being tested for their use in tissue engineering. Single wall carbon nanotubes (SWCNTs) are proposed as a promising material for neuro-regeneration, owing to their unique properties and biocompatibility (10-12), SWCNTs can

be added in neuronal cell cultures as strata or as colloidal aqueous solutions (CITs)(13, 14); the latter ws-SWCNTs are shown to enhance neurite outgrowth in neural cultures, or when applied on spinal cord injuries (9).

Graphene based nanomaterials (GBN), are also becoming increasingly popular in bioengineering, due to their biophysical properties along with their biocompatibility (15-18). Graphene improves cell adhesion during the differentiation process and promotes differentiation towards neurons more than towards glial cells. All reported data suggest that GBNs, and among them more specifically graphene oxide, may represent a superior nanostructured scaffold for neural differentiation and neuro-regeneration (19-23). While graphene oxide application in various stem cell differentiation settings has been widely studied, another potentially interesting GBN, the exfoliated liquid graphite in the form of colloidal dispersion of two-dimensional flakes (GD), has been rather ignored, though its biocompatibility and nontoxicity, along with cell proliferation support, have been demonstrated (24-28).

The aim of the present study was to explore SCAP potential to undergo neural differentiation in the presence of two types of nanomaterials (ws-SWCNT and GD), separately and in combination, i.e. to evaluate the neuro-stimulatory effects of the two nanomaterials, by means of cell morphology, immunocytochemistry and real-time gene expression analyses.

MATERIALS AND METHODS

The study protocol complied with the Fortaleza (Brazil) Revision of the Helsinki Declaration and was approved by the Ethical Committee of the School of Dental Medicine, University of Belgrade. Immature, impacted third lower molars were extracted from teenage patients at the

Clinic for Oral Surgery (Figure 1), School of Dental Medicine, University of Belgrade, Serbia after signing the informed consents by patient´s parents.

Preparation of graphene dispersion

For the fabrication of graphene dispersion (GD) the method of liquid phase exfoliation (LPE) has been used as a promising route for high-quality and large-scale production of 2D materials. We followed the protocol described in earlier papers (29, 30). An initial concentration of graphite powder (Sigma Aldrich-332461) in N-Methyl-2-pyrrolidone (NMP) (Sigma Aldrich-328634) was 18 mg/ml. The mixture was sonicated in a low power sonic bath (Bransonic CPXH Ultrasonic 8 Cleaning Bath) for 14 hours. In order to prevent re-aggregation and reduce the amount of unexfoliated graphite, the solution was centrifuged for 60 min at 3000 rpm. The next step was decantation that was carried out by pipetting off the top half of the dispersion. In the end, the concentration of the grey liquid consisting of graphene sheets dispersed in solution, was 320 μg/ml. The Lambert–Beer law was applied to UV/VIS absorption spectra to calculate graphene concentration by estimating the absorbance at 660 nm. The cell's length was 1 cm and the extinction coefficient of graphene (α = 24.60 mL mg^{-1} m^{-1}), in NMP solutions was taken from literature (31). Absorption spectra were measured using the UV/VIS Spectrophotometer (Perkin-Elmer Lambda 4B).

Preparation of water-soluble SWCNT functionalized with poly-m-aminobenzene sulphonic acid

The functionalization of SWCNTs, to render their water solubility, was done as described previously (10) (also See Supplemental Information for further details). In brief, commercially available purified SWCNT-COOH material (P3-SWCNT, Carbon Solutions, Inc., Riverside, CA) was reacted with oxalyl chloride in order to make an acyl chloride intermediate (SWCNT-

COCl). P3-SWNT material (1 g) was dispersed in 1 L of dry DMF by ultrasonication for 2 h and high-shear mixing for 1 h to give a homogeneous suspension. Oxalyl chloride (20 mL) was added drop-wise to the SWCNT-COOH solution at 0°C under argon. The reaction mixture was stirred at 0°C for 2 h, at room temperature for 2 h, and heated overnight at 70°C to remove the excess oxalyl chloride (boiling point 63°C). The functionalization of the resulting SWCNT-COCl intermediate was done by the addition of poly-m-aminobenzene sulphonic acid (PABS, 5 g) to form the corresponding graft copolymer (SWCNT-PABS), by allowing the components to interreact at 120^{o}C for 5 days. Afterwards, the mixture was filtered through a membrane (pore size 0.22 μm), repeatedly rinsed with 95% ethanol and then with distilled water to remove any excess of PABS. The resulting final product (SWCNT-PABS) was collected on a membrane, dried under vacuum overnight, and then reconstituted in distilled water in 2.0 mg/mL stock solutions. SWCNT-PABS has a composition of 35 weight percent (wt%) SWCNTs and 65 wt% PABS.

Isolation and cultivation of stem cells from apical papilla (SCAP)

Atraumatically extracted teeth were transferred to the laboratory in Dulbecco's Modified Eagle Medium (DMEM) containing 20% of Mesenchymal Stem Cells qualified Fetal Bovine Serum (MSC-FBS) and 1% of antibiotic-antimycotic solution (Thermo Fisher Scientific, USA). The samples were processed within 30 minutes after the extraction. Stem cells from apical papilla were isolated according to growth explant method previously described by Kerkis et Caplan, under sterile conditions(32). Briefly, teeth were extensively rinsed with Dulbecco's Phosphate Buffered Saline (DPBS, Thermo Fisher Scientific, USA) and apical papilla was gently scrapped from the root apex using sterile surgical blade. Soft tissue was minced into small pieces, and transferred into T-25 flasks containing cell culture growth medium (DMEM supplemented with

10% MSC-FBS and 1% antibiotic-antimycotic solution). Cells were then cultured under standard conditions (37 °C, 95% air-5% CO_2 atmosphere, 95% humidity) and the culture medium was changed every 2-3 days. Subconfluent, cells were detached from the plastic surface of tissue flask with recombinant cell-dissociation enzyme (TrypLE™ Express Enzyme, Thermo Fisher Scientific, USA) according to manufacturer's protocol, and seeded into new flasks. All subsequent experiments were performed with cells from the fourth (P4) and the fifth (P5) passage.

Cell differentiation capacity

In order to confirm multipotency of SCAP, cells from the fourth passage (P4) were subjected to osteogenic, adipogenic and chondrogenic differentiation. Osteogenic potential of SCAP was determined after 28 days of cell culturing in osteogenic medium (StemPro™ Osteogenesis Differentiation Kit, Thermo Fisher Scientific, USA) according to manufacturers' recommendations. Cells were seeded in six-well plates at density of 5×10^3 cells/cm^2 and culturing medium was changed every 2-3 days. Adipogenic stimulation also lasted 28 days in appropriate media (StemPro™ Adipogenesis Differentiation Kit, Thermo Fisher Scientific, USA) and seeding density of 1×10^4 cells/cm^2 on the six well plates. For the chondrogenesis, cells were seeded in a form of micromass at total number of 1.5×10^6 and grown on six-well plates in commercially available media (StemPro™ Chondrogenesis Differentiation Kit, Thermo Fisher Scientific, USA) for 21 days. Cells cultured in standard growth medium were used as a negative control. Differentiation into the three lineages was assessed by histological staining. Prior to staining procedures, cells were fixed with 4% paraformaldehyde (PFA) for 30 minutes at room temperature and after that washed twice with DPBS. Alizarin Red S staining was used for determination of calcification nodule formations of the extracellular matrix in cultures grown in

osteogenic medium. Two percent Alizarin Red S (Centrohem, Serbia) solution, at pH 4.2, was added to the wells. After 30 minutes of incubation, dye was removed and cells were rinsed twice with distilled water. Oil Red O staining was used for visualizing intracellular lipid vacuoles upon adipogenic differentiation. Cells were incubated for 15 minutes in 0.5% Oil Red O solution (Sigma Aldrich, Germany) and the excess of dye was removed by gentle rinsing with DPBS. Chondrogenesis was confirmed by 0.1% solution Safranin O (Centrohem, Serbia) positive staining. Alizarin Red S, Oil Red O and Safranin O stained areas were observed under inverted microscope (Primovert, Zeiss, Germany), and photographed.

Flow cytometry and immunophenotyping

Flow cytometry analyses were performed in order to assess the expression of specific mesenchymal markers in SCAP from the 5th passage (P5). The markers used for these analyses were: fluorescein-isothiocyanate (FITC) labeled mouse monoclonal antibodies against CD90, CD105, CD34 and phycoerythrin (PE) labeled mouse monoclonal antibodies against CD73 and CD45 (all antibodies were purchased from Exbio, Czech Republic). Cells were harvested with TrypLE™ Express solution, washed with DPBS supplemented with 10% FBS, and finally counted on automated cell counter (Countess™, Invitrogen, USA). One million of cells were resuspended in 1 ml of 10% FBS solution in DPBS, and incubated with adequate antibodies for 45 minutes in the refrigerator. After incubation, cells were fixed with 4% paraformaldehyde for 20 minutes and finally rinsed twice with DPBS. Cells were analyzed on tabletop flow cytometer (Partec, Munster, Germany) and results were processed by software (Sysmex Partec, Goerlitz, Germany).

Neurodifferentiation

For 24 hours cells were grown in standard culture medium. After that, over the next 4 hours SCAP were incubated in neural preinduction medium, DMEM with 100 mM beta-mercaptoethanol. Differentiation of SCAP was continued in neural induction medium containing recombinant human basic fibroblast growth factor (bFGF, Thermo Fisher Scientific, USA), neural growth factor (NGF, Thermo Fisher Scientific, USA), and B27 supplement (Thermo Fisher Scientific, USA) in DMEM, according to previously reported protocol(33). To evaluate potential stimulatory effects of carbon nanomaterials on neural differentiation of SCAP, four protocols were applied: neural induction medium without nanomaterials (protocol A); neural induction medium and 10 µl (of 2 mg/mL) of SWCNT-PABS (protocol B); neural induction medium and 10 µl (of 18 mg/ml) of GD prepared solution (protocol C); neural induction medium supplemented with 10 µl SWCNT-PABS and 10 µl GD (protocol D), changed with freshly made medium every 2-3 days.

Light microscopy

Cell morphology was monitored under inverted microscope (Primovert, Zeiss, Germany) and photographed. Between days 5 and 7 of neurogenic culture, the cells showed a transition from fibroblast-like to neuron-like cell bodies with long processes, suggesting that the stem cells differentiated into neurons/neuron-like cells. At that point they were subjected to qPCR and immunocytochemistry analysis.

RNA isolation and gene expression analyses by real time PCR (qPCR)

For the gene expression analyses 1.25×10^5 cells were seeded in T-25 tissue culture flask. After neurogenic stimulation, total RNA was extracted using guanidinium thiocyanate-phenol-

chloroform extraction procedure with commercially available reagent (TRIzol™ Reagent, Thermo Fisher Scientific, USA) according to recommendations. RNA purity and concentration were assessed using microvolume spectrophotometer (BioSpec Nano, Shimadzu, Japan). One microgram of total RNA was reversely transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA) and oligo $(dT)_{18}$ primers. cDNA was subsequently used for Real-Time Polymerase Chain Reaction (qPCR) analyses on a Line Gene-K Fluorescence Real-time PCR Detection System (BIOER Technology, Shanghai, China). PCR reaction mix (25µl) contained 12.5 µl of real-time PCR master mix (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Fisher Scientific, USA), 1 µl of forward and reverse primer (final concentration 200 nM), 2 µl of cDNA and PCR-grade water. Each run had initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C, 15 s), annealing (55°C, 30 s) and elongation (72°C, 30 s). Data acquisition was performed in each elongation step. Specificity of PCR products was checked by melting curve analyses and the relative gene expression level was assessed using the comparative $2^{\Delta\Delta Ct}$ method (34). All reactions were carried out in duplicate. The relative expression levels of mRNA for neural markers for each sample were calculated as the ratio between the expression of the gene of interest and the expression of the selected endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). List of primers used in qPCR are given in Table 1.

Immunocytochemistry

For the immunocytochemical analyses cells were seeded onto 25 mm diameter round glass coverslips at density of 5×10^3 cells/cm^2 and subjected to neurodifferentiation protocol as described above. On the 7th day of neural induction, cells were rinsed three times in DPBS, fixed with 4% PFA solution for 20 minutes, rinsed three times with DPBS and incubated at room

temperature for 45 minutes in blocking and permeabilization buffer (10% Bovine Serum Albumin and 0.1% Triton X-100 in DPBS). For immunofluorescent detection of neuronal and glial cell marker expression, cells were incubated with following primary antibodies: rabbit anti-β III-tubulin (B3T, 1:400, Cell Signaling, USA), rabbit anti-neuronal nuclei (NeuN, 1:250, Millipore, Germany) and mouse anti-glial fibrillary acidic protein (GFAP, 1:400, Millipore, Germany). Primary antibodies were incubated at 4°C overnight and subsequently washed three times with DPBS. Cell samples were incubated with secondary antibodies - donkey anti-mouse Alexa Fluor 488 (1:200, Invitrogen, USA), donkey anti-rabbit Alexa Flour 555 (1:200, Invitrogen, USA) and donkey anti-rabbit Alexa Flour 657 (1:200, Invitrogen, USA) for 2 hours in dark at room temperature. Cells were washed three times in DPBS and stained with 4-, 6-diamidino- 2-phenylindole (1:4000, DAPI, Molecular Probes, USA) for 10 minutes in dark at room temperature. After washing in DPBS cell samples were mounted with Mowiol medium (Sigma Aldrich) on microscope slides. Immunofluorescence microscopy images were obtained by confocal laser-scanning microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Ar 488 and HeNe 543 and 633 laser lines. Micrographs were analyzed using Fiji-Image J software (NIH, USA).

RESULTS

Osteogenic, chondrogenic and adipogenic differentiation of stem cells from apical papilla demonstrated their multipotency

Osteogenic, adipogenic and chondrogenic differentiation of SCAP were verified by appropriate histological stains. Positive staining of calcification nodule formations with Alizarin Red S was

indicative of osteogenic differentiation, accumulation of Oil Red O in intracellular lipid vacuoles of adipogenic differentiation, whereas Safranin O binding to proteoglycans was a proof of chondrogenic differentiation of SCAP (Figure 2). Cells of the control group remained unstained.

Immunophenotyping revealed expression of mesenchymal stem cell markers

Flow-cytometry analyses were performed on P5 (fifth passage) stem cell from apical papilla. Flow-cytometry revealed the expression of mesenchymal stem cell markers CD73, CD90 and CD105 (99%, 91.3% and 96%, respectively), and the absence of hematopoietic markers CD34 (0.34%) and CD45 (0.01%) (Figure 3).

Neuron like morphology was demonstrated by light microscopy

Cell morphology was observed every day, and photographed on the seventh day. Protocol A cells presented short cell projections and poor neural like morphology, similarly to cells grown under protocol B conditions. Cells in protocol C cultures had long slender projections with triangular cell bodies. Under protocol D conditions, cells also achieved a good neuron like morphology (Figure 4).

Neural markers MAP2, ngn-2, β III-tubulin, NF-M and NeuN were expressed in SCAP after neuroinduction

Pilot experiments performed in different time points (data not shown) suggested that the 7th day of neuroinduction was optimal for expression analysis. The relative expression of five neural markers (microtubule-associated protein 2 (MAP2), neurofilament medium (NF-M), neurogenin-2 (ngn-2), β III-tubulin and glial fibrillary acidic protein (GFAP)). is given in Figure 5. The predominant markers in all cell cultures were β III-tubulin and NF-M, but their ratios were different. In the presence of SWCNT-PABS the expression of NF-M was higher than the

expression of β III-tubulin while in the presence of GD it was the opposite. In cultures without nanomaterials as well as in cultures with both nanomaterials a balanced expression of NF-M and β III-tubulin was found. Cells grown in neural induction medium only, showed higher expression of MAP2 and GFAP compared to other protocols.

The markers were expressed at their proper (expected) cellular localization. As registered under light microscopy, a fine neuron-like morphology was observed in cells cultivated under protocol D, i.e. in the presence of both nanomaterials (Fig. 6A), and a robust immunoreactivity for markers β III-tubulin and NeuN was recorded (Fig. 6B and 6C, respectively). A similar morphology was obtained under protocol C although the expression of β III-tubulin was higher in cells cultivated with GD only, than with the two nanomaterials, a finding in agreement with real-time analysis (Fig.7). The expression of astroglial cell marker GFAP was observed only in cells grown in neural induction medium without nanomaterials (data not shown).

DISCUSSION

Since they originate from neural crest, dental tissue SCs have been extensively studied as a possible approach for replacing lost cells in CNS diseases or injuries (9). MSCs derived from apical papilla are becoming an increasingly attractive stem cell source because they belong to a developing, easily accessible tissue, which would be otherwise discarded as biological waste, with cells possessing high proliferation rate, plasticity and differentiation capacity (6).

Graphene, a crystalline allotrope of carbon with two-dimensional properties, may be synthesized and functionalized in various ways, and loaded with different molecules of interest. Graphene based nanomaterials have proved to be promising tools in different fields of nanomedicine, owing to their unique structure, chemical stability, exceptional mechanical properties, good

biocompatibility and bactericidal potential (35). These single-atomic layered sheet materials have also the ability to adsorb growth factors and exhibit unprecedented electrical properties, which is of particular importance when used as cell scaffolds (19, 36)**.** Their use as support for stem cell differentiation has lately been an area of intensive studies. It has been reported that both CNTs and graphene can stimulate neural stem cells differentiation, neuronal and oligodendrocyte growth, and the formation of active synaptic contacts in cell culture. But despite some similarities, they have different impact on cells (23). According to available literature, stem cells subjected to neural differentiation on substrates filmed with graphene exhibited better neural morphology and higher expression of neural markers, while SWCNT seemed to promote both glial and neuronal differentiation (37-39).

It must be emphasized that experiments on graphene dispersion (GD) utilization in stem cell differentiation are completely inexistent. One study only has previously shown GD biocompatibility and another has demonstrated good PC12 attachment and proliferation (27, 28). Moreover, there are no studies exploring the combined use of CNT and GD as materials that hold potential to synergistically stimulate stem cell neuro-differentiation and more specifically SCAP neuro-differentiation.

In the present study, it was demonstrated that GD may exert stimulatory effects on dental stem cell neuro-differentiation. Namely, cells cultivated with GD, compared to other protocols, exhibited the highest level of β III-tubulin, a microtubule protein expressed during neurogenesis and involved in axon guidance. The level of MAP2, a mature neuron marker, was also higher in cells grown with GD than in cells grown according to the other two protocols with nanomaterials. The level of ngn-2, a transcription factor inhibitor of glial cell development, was

low, and yet gliogenesis seemed suppressed as judged by the lack of expression of GFAP, a glial cell marker, a phenomenon that might be attributed to GD.

SCAPs grown with SWCNT-PABS showed better neural differentiation when compared to cells grown without the addition of nanomaterial, a result which is in accordance with previously reported data (39). Levels of ngn-2, β III-tubulin and NF-M, a neuronal cytoskeleton element found at high concentration in axons, were higher in cells grown with SWCNT-PABS than in the basic neuroinductive medium.

The combination of the two nanomaterials resulted in a seemingly well-adjusted ratio of NF-M and β III-tubulin levels, accompanied with a robust expression of NeuN. This treatment ensured the most compelling cell morphology with clear neuron shaped body and long processes.

The predisposition of SCAPs to differentiate toward neural lineages, as well as the neuroinductive properties of GD and SWCNT, should warrant further studies of dental stem cells in conjunction with these nanomaterials, with the aim of finding a superior solution for autologous neuroregenerative therapy.

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FIGURE LEGENDS

Figure 1. (A) Patients' panoramic X ray with non-erupted developing third molar; (B) extracted third molar with apical papilla.

Figure 2. Multipotency confirmation by Alizarin Red S staining for osteodifferentiation, Safranin O staining for chondrodifferentiation and Oil Red O staining for adipodifferentiation.

Figure 3. The presence of mesenchymal markers CD73, CD90 and CD105, and the absence of hematopoietic markers CD34 and CD45 were assessed by flow-cytometry, on P5 cells.

Figure 4. Cell morphology after seven days: (A) protocol without nanomaterials; (B) protocol with SWCNT-PABS; (C) protocol with GD; (D) protocol with the combination of nanomaterials. Magnification 200×.

Figure 5. Real-time PCR analysis of neural marker expression after 7 days in cultures with neural induction medium (protocol A), neural induction medium and SWCNT-PABS (protocol B), neural induction medium and GD (protocol C), neural induction medium and SWCNT-PABS + GD (protocol D).

Figure 6. (A) The combination of SWCNT-PABS and GD resulted in a good neuron-like morphology (β III-tubulin in red, nuclei in blue-DAPI); (B) strong immunostaining of β III-tubulin (red) and (C) NeuN (turquoise).

Figure 7. (A) β III-tubulin expression was higher in cells cultivated with GD than with GD and SWCNT-PABS (B). The difference was statistically significant (C).

Table 1. Sequences of primers used for quantitative PCR

Gene	Sequence of primers (5'→3')	Accession number
NF-M	F: TGGGAAATGGCTCGTCATTT R: CTTCATGGAAACGGCCAA	NG_008388.1
ngn-2	F: CCTGGAAACCATCTCACTTCA R: TACCCAAAGCCAAGAAATGC	NM_024019.2
GFAP	F: GCCTCAAGGACGAGATGG R: TCGCCCTCTAGCAGCTTC	NM_002055.3
B III-tubulin	F: GCCAAGTTCTGGAAGTCA R: GCCTCGTTGTAGTAGACGC	NM_006086.2
MAP2	F: AACCCTTTGAGAACACGACA R: TCTTTCCGTTCATCTGCCA	NM_002374.3
GAPDH	F: TCATGACCACAGTCCATGCCATCA R: CCCTGTTGCTGTAGCCAAATTCGT	BC083511

Abbreviations: NF-M - neurofilament medium; ngn-2 – neurogenin-2; GFAP - glial fibrillary acidic protein; MAP2 - microtubule-associated protein 2; GAPDH - glyceraldehyde 3-phosphate dehydrogenase.

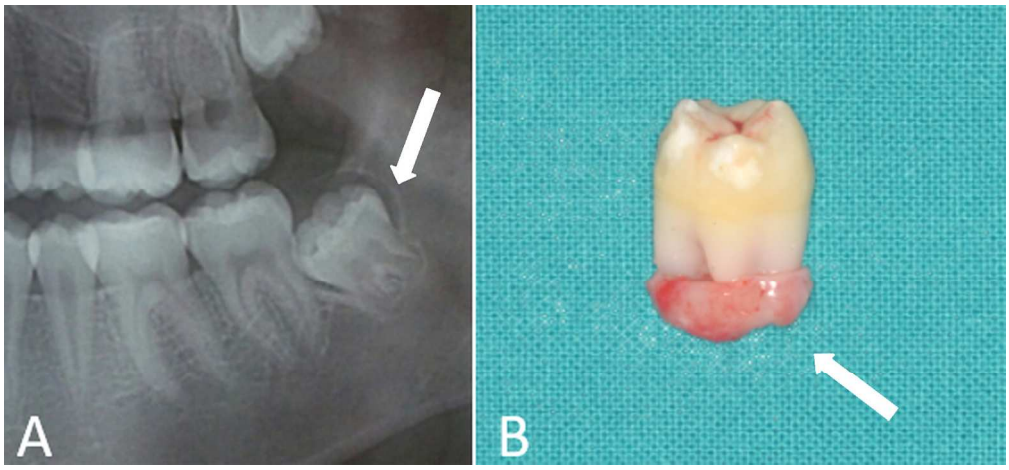
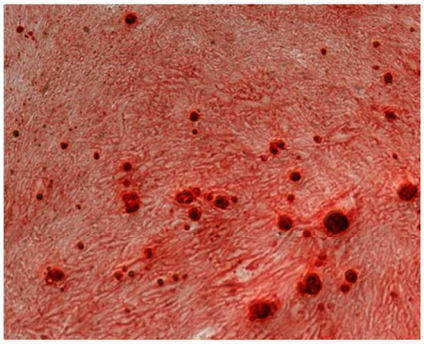


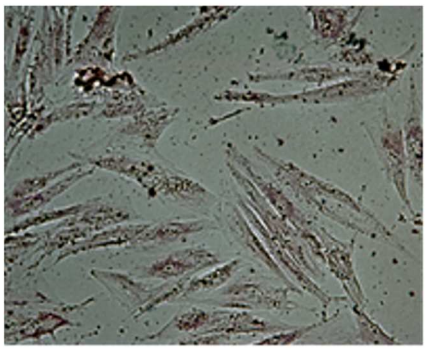
Figure 1. (A) Patients' panoramic X ray with non-erupted developing third molar; (B) extracted third molar with apical papilla.

152x69mm (300 x 300 DPI)



OSTEODIFFERENTIATION

CHONDRODIFFERENTIATION



ADIPODIFFERENTIATION

Figure 2. Multipotency confirmation by Alizarin Red S staining for osteodifferentiation, Safranin O staining for chondrodifferentiation and Oil Red O staining for adipodifferentiation.

67x182mm (300 x 300 DPI)

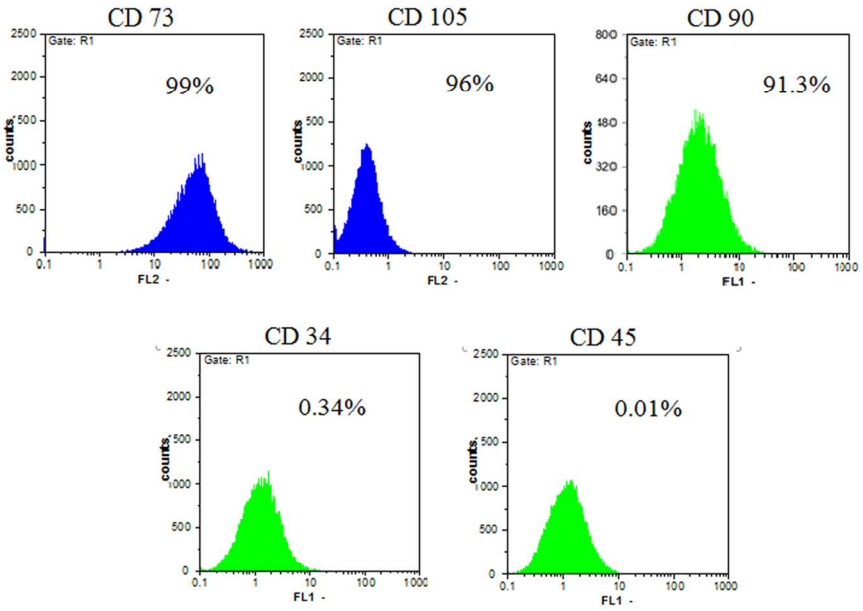


Figure 3. The presence of mesenchymal markers CD73, CD90 and CD105, and the absence of hematopoietic markers CD34 and CD45 were assessed by flow-cytometry, on P5 cells.

135x86mm (300 x 300 DPI)

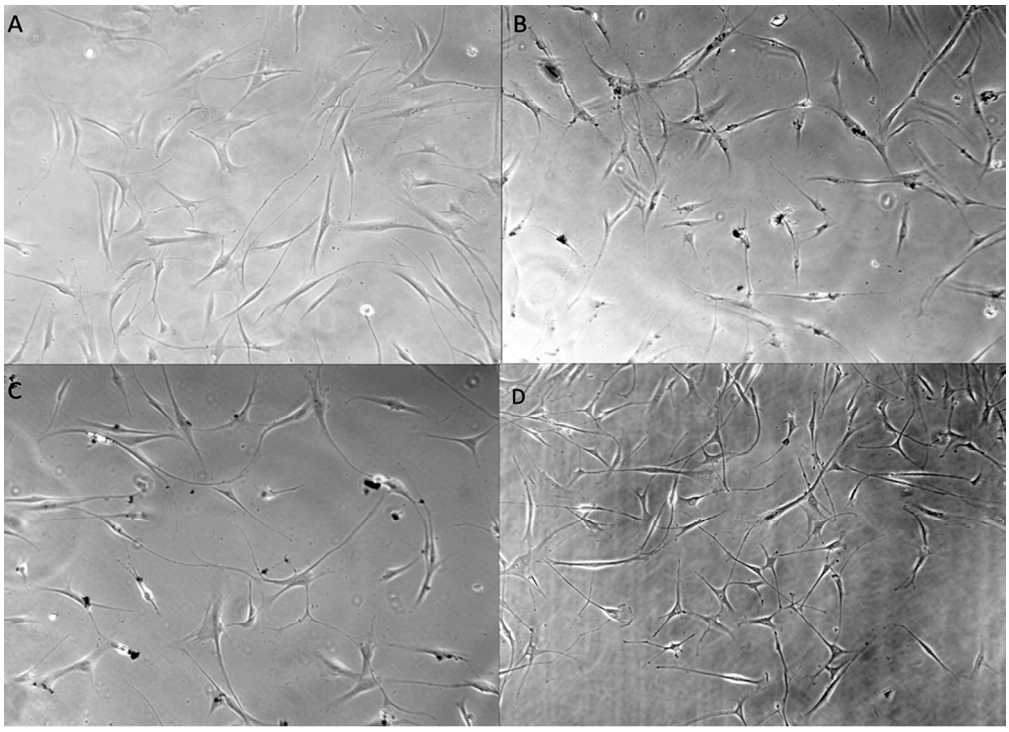


Figure 4. Cell morphology after seven days: (A) protocol without nanomaterials; (B) protocol with SWCNT-PABS; (C) protocol with GD; (D) protocol with the combination of nanomaterials. Magnification 200×.

127x91mm (300 x 300 DPI)

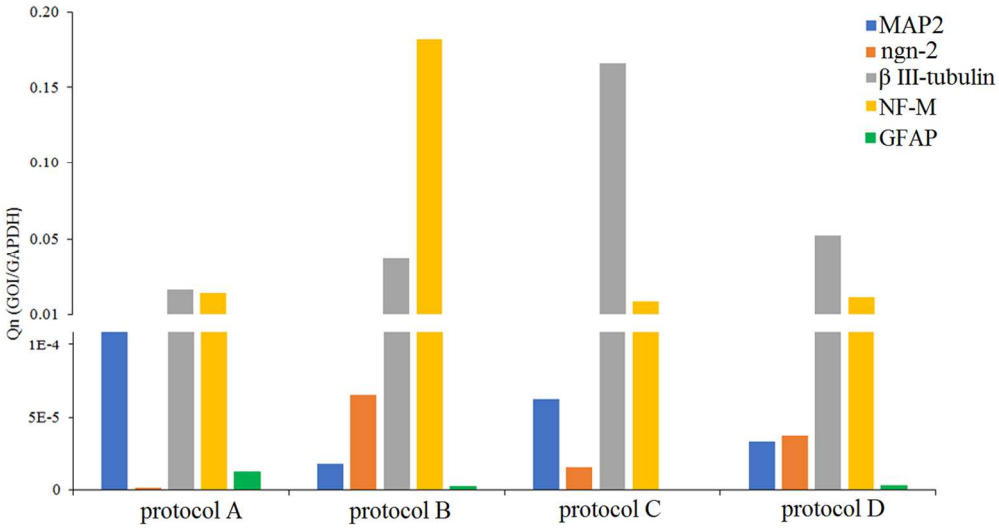


Figure 5. Real-time PCR analysis of neural marker expression after 7 days in cultures with neural induction medium (protocol A), neural induction medium and SWCNT-PABS (protocol B), neural induction medium and GD (protocol C), neural induction medium and SWCNT-PABS + GD (protocol D).

135x73mm (300 x 300 DPI)

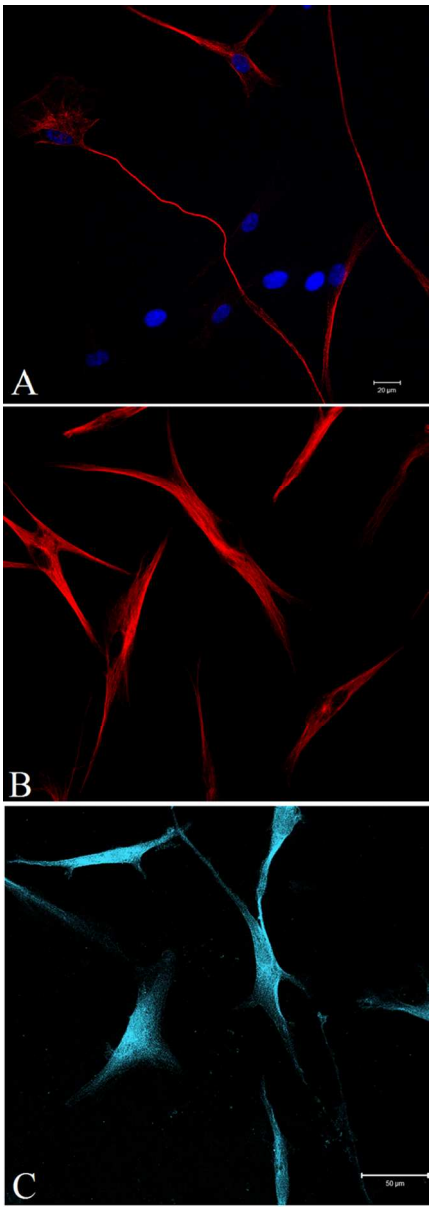


Figure 6. (A) The combination of SWCNT-PABS and GD resulted in a good neuron-like morphology (β III-tubulin in red, nuclei in blue-DAPI); (B) strong immunostaining of β III-tubulin (red) and (C) NeuN (turquoise).

53x152mm (300 x 300 DPI)

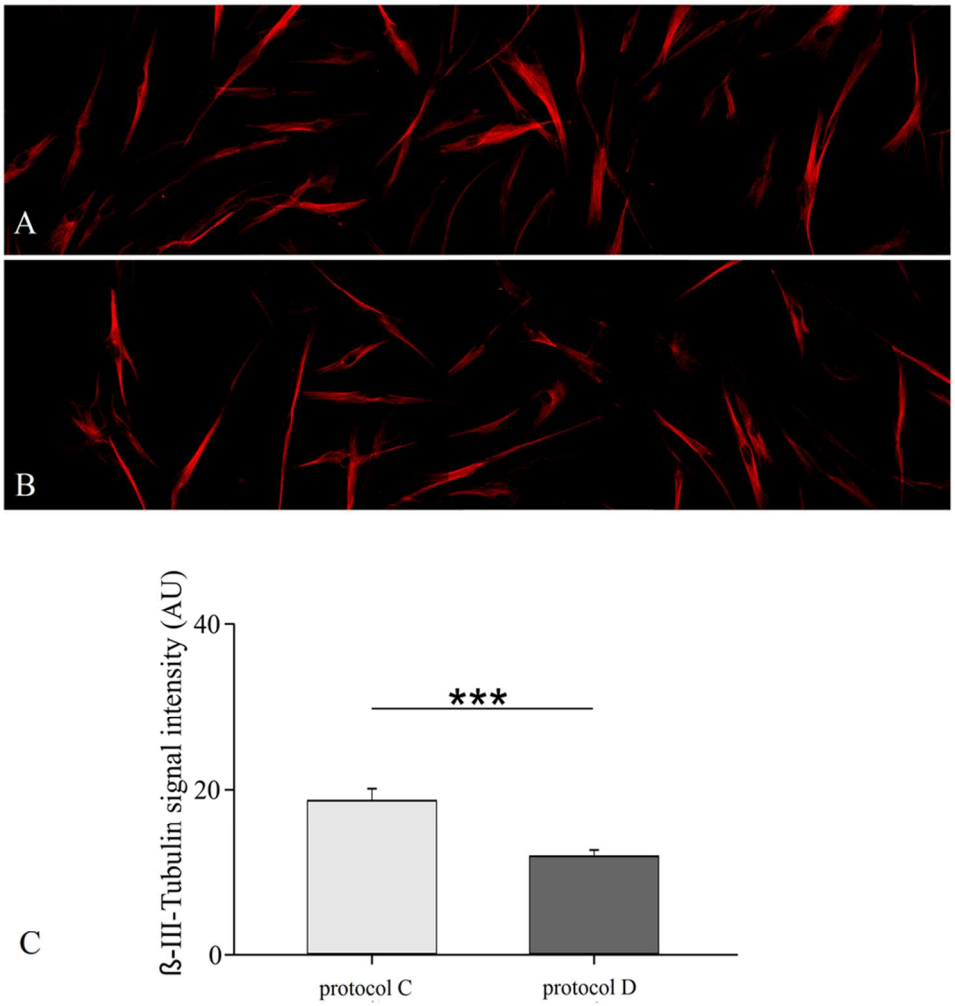


Figure 7. (A) β III-tubulin expression was higher in cells cultivated with GD than with GD and SWCNT-PABS (B). The difference was statistically significant (C).

101x108mm (300 x 300 DPI)