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Dental Pulp Stem Cells Stimulate Neuronal Differentiation of PC-12 cells

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Sultan N generated the figures, table and wrote the manuscript; Grawish ME, Amin LE, Zaher AR and Scheven BA contributed to writing of the manuscript, provided intellectual contribution to the content, and made critical revisions; all authors reviewed and approved the final version of the manuscript.

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Abstract

Introduction:

Dental pulp stem cells (DPSC) secrete neurotrophic factors which may play an important therapeutic role in neural development, maintenance and repair. PC-12 is a well-established model to study neuronal cell differentiation and function. In this study the role of DPSC-derived factors on PC-12 survival, growth, migration and differentiation was investigated.

Materials and methods:

Conditioned medium (CM) was collected from 72h serum-free DPSC cultures (DPSC-CM), which were established from extracted rat incisors. PC-12 cells were treated with NGF, DPSC-CM or co-cultured with DPSC using Transwell inserts. After 8 days, the number of surviving cells with neurite outgrowths and the length of neurites were measured by image analysis. Transwell migration inserts were used to assess PC-12 cell migration under the influence of DPSC-CM. Immunocytochemical staining was used to evaluate the expression of neuronal markers NeuN, MAP-2 and cytoskeletal marker βIII-tubulin. Gene expression levels of axonal growth-associated protein 43 (GAP-43) and synaptic protein Synapsin-I, NeuN, MAP-2 and βIII-tubulin were analysed by qRT-PCR. DPSC-CM was analysed for the neurotrophic factors (NGF, BDNF, NT-3, and GDNF) by specific ELISAs. Specific neutralising antibodies against the neurotrophic factors were used to study their role in DPSC-CM effects on PC-12 survival and neurite outgrowth length.

Results:

Compared with the serum-free PC-12 controls where most of the culture was undergoing cell death, DPSC-CM significantly promoted cell survival and induced the neurite outgrowth confirmed by NeuN, MAP-2 and βIII-tubulin immunostaining. DPSC-CM was significantly more effective in stimulating PC-12 neurite outgrowths than live DPSC/PC-12 co-cultures over the time studied. The morphology of induced PC-12 cells in DPSC-CM was similar to NGF positive controls; however, DPSC-CM stimulation of cell survival was significantly higher than what was seen in NGF-treated cultures. DPSC-CM also had a chemoattractant potential as shown by an increased migration of PC-12 cells across Transwell membranes after 24h treatment. The number of surviving PC-12 cells treated with DPSC-CM was markedly reduced by the addition of anti-GDNF, whilst PC-12 neurite outgrowth was significantly attenuated by anti-NGF, anti-GDNF and anti-BDNF antibodies. Furthermore, neutralisation of GDNF and NGF resulted in significant downregulation of NeuN, MAP-2, βIII-tubulin and GAP-43 gene expression.

Conclusion:

The findings of the present study demonstrated that DPSC were able to promote PC-12 survival and differentiation. DPSC-derived NGF, BDNF and GDNF were involved in the stimulatory action on neurite outgrowth, whereas GDNF also had a significant role in promoting PC-12 survival. DPSC-derived factors may be harnessed as a cell-free therapy for peripheral nerve repair.

Keywords: Dental pulp stem cells (DPSC); Nerve growth factor (NGF); Brain-derived neurotrophic factor (BDNF); Glial cell line-derived neurotrophic factor (GDNF); Neurotrophin-3 (NT-3).

Introduction

Peripheral nerve injury may occur as a result of acute compression, trauma, iatrogenic during surgical procedures, diabetes and other health conditions and may end with the loss of sensory function, motor function, or both [1]. Regenerative capacity of the injured adult nervous system is limited due to an insufficient pool of precursor cells. Nerve regeneration is also constrained by inhibitory factors and barrier tissues in the injured microenvironment [2]. Schwann cells (SCs) transplants reported to provide a promising therapeutic strategy for the treatment of peripheral nerve injuries. SCs are peripheral glial cells responsible for clearing out debris from the site of injury. Moreover, they release growth factors to stimulate myelination and axonal regeneration [3].

Both primary SCs [4] and genetically modified SCs [5] enhance nerve regeneration in animal models; however, SCs' sourcing and availability are limited and the only method to obtain primary cells is by sacrificing a healthy nerve. Stem cell-based strategies in combination with novel technologies (e.g. Hydrogels) have heralded potential new therapeutic approaches for addressing nerve regeneration and repair [2]. Bone marrow-derived stem cells (BMSC) [6], adipose-derived stem cells (ADSC) [7] and dental pulp-derived stem cells (DPSC) have gained increasing interest as alternative sources for peripheral nervous system regeneration therapy [2].

In particular, DPSC have recently gathered much attention in the field of neuroscience research because of their neural crest origin and their apparent superior ability to secrete substantial levels of neurotrophic factors effective for neuronal survival and axonal regeneration [8-10].

In nerve repair as in other fields, numerous models have been established to test the efficacy of compounds pre-clinically [11]. Pheochromocytoma (PC12) cell line is a well-

known and tested neuronal cell model, originated from a tumor of adrenal gland, have been used extensively as a model to study neuronal differentiation, but never had been tested with DPSC. PC-12 cells are dependent on nerve growth factor (NGF) and respond to this neurotrophin by undergoing neural cell differentiation exhibiting a typical neuronal phenotype with neurite outgrowths [12, 13]. NGF is a neurotrophic factor crucial for the survival and maintenance of sympathetic and sensory neurons, and it binds to the high-affinity tyrosine kinase receptor, TrkA, leading to its phosphorylation and the subsequent activation of phosphatidylinositol-3-kinase (PI3K/Akt) pathways. This, in turn, facilitates the cytoskeletal rearrangements necessary for neurite outgrowth [14-16].

The primary objective of this study was to evaluate PC-12 cells as a model for studying the neurotrophic and neurogenic impact of DPSC secreted factors. The secondary one was to compare the efficiency of collected and pre-stored DPSC-CM as a cell-free therapy with live DPSC/PC-12 co-cultures as a cell-based therapy. In addition, this study explored the role of specific neurotrophins (NTFs) in the survival and neurite outgrowth of DPSC-stimulated PC-12 cells using specific neutralising antibodies.

Materials and Methods

All plastic-wares used were supplied by Thermo Fischer Scientific, UK, and most of the other materials used were supplied by Sigma Aldrich, UK, unless stated otherwise.

Isolation, culture and characterization of DPSC

DPSC were isolated from 4-6w old male Wister-Hann rat incisors via enzymatic digestion as described previously (University of Aston, Pharmaceutical Sciences Animal House, Birmingham, UK) [17,18]. Isolated cells were cultured in alpha-minimum essential medium (α-MEM, Biosera, UK) supplemented with 100 µg/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10% fetal bovine serum (FBS) and

incubated at 37 ^{o}C with 5% CO_2 environment. At 70-80% confluence, the cells were sub-cultured using 0.25% trypsin-EDTA for 10 min at 37 ^{o}C and passaged to new culture flasks or used in experiments. DPSC from 2nd to 5th passages were used in this study.

At 3rd passage, DPSC were characterized by semi-quantitative polymerase chain reaction (sqRT-PCR) using mesenchymal stem cell markers; CD90, CD105, CD29, CD14 and CD45, neuronal marker; Nestin and pluripotent markers; Nanog and SOX2. Briefly, total RNA was prepared from confluent DPSC cultures by using the RNeasy Mini Kit (Qiagen, UK) consisting of RLT lysis buffer and washing buffers (RW1 and RPE). Extracted RNA was used to generate cDNA using a Tetro cDNA synthesis kit (Bioline, UK). The cDNA was amplified for sqRT-PCR by using REDTaq ready reaction mix. The intensity was normalized to expression level of the endogenous reference gene GAPDH. The sequences of the Rattus novergicus primers are illustrated in **Table 1**.

Collection of DPSC-CM

DPSC at 70–80% confluence were used for preparation and collection of CM: The growth medium was removed, cells were washed twice with PBS, and the culture medium was replaced with serum-free α-MEM. After 72h incubation, the culture medium was collected and centrifuged for 5 min at 1500 rcf, 4 ^{o}C. The supernatants were collected and centrifuged for another 3 min at 3000 rcf, 4 ^{o}C. The resulting supernatants (denoted as DPSC-CM) were filtered through a 0.22-µm filter unit (Merck, Millipore, Ireland) and stored in at -80 ^{o}C for further experiments [19].

Quantification of secreted neurotrophic factors

Total protein content was quantified by bicinchoninic acid assay (BCA) Kit (The Thermo Scientific™ Pierce™ BCA). Enzyme-linked immunosorbent assays (ELISAs) were performed to determine the concentration of NGF and BDNF (R&D systems, Biotechne, UK), GDNF (Boster Picokine™, CA, USA, EK0935), NT-3 (Fine Test, MD, USA, ER0055), and ciliary neurotrophic factor (CNTF, Thermo Scientific, UK, ERCNTF).

PC-12 Cell Culture

PC-12 pheochromocytoma cells obtained from the American Type Culture Collection (ATCC) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% horse serum (HS), 5% Fetal bovine serum (FBS), and penicillin/streptomycin. Before each experiment, PC-12 cells were plated on a poly-L-lysine coated plates (Corning Biocoat, UK) or coverslips (Electron microscopy science, UK). PC12 cells were maintained at 37°C in a 95% humidified incubator with 5% CO_2 before the experiments. For differentiation experiments, the cells were seeded at low density ($5x10^3$ cells/cm^2) in RPMI-1640 medium supplemented with 10% HS, 5% FBS and penicillin/streptomycin. After 24h of plating, the medium was replaced with serum-free RPMI-1640 medium containing either; 50 ng/ml NGF, 50% DPSC-CM or co-cultured with DPSC which had been seeded previously on Transwell culture inserts. Cells treated with serum-free culture medium alone were used as controls. Culture medium was replaced every 2–3 days. After 8 days of incubation, morphometric analysis was done using ImageJ software for average neurite length and number of cells bearing neurites.

Co-culture experiments

DPSC were seeded at 10^5 cells/ 200µl into polyester Transwell insert of 24-well plates with 1 µm pore size (Merck, Millipore, UK). DPSC were seeded on top of the membrane in complete growth medium with 10% FBS. After 24h, the culture medium was replaced with serum-free α-MEM and inserts were placed over the lower chamber containing the neuronal cell cultures and incubated for 8 days.

Assessment of PC-12 survival using live and dead cell assay

To distinguish between live and dead cells, cultures were incubated with calcein-AM and ethidium homodimer-1(EthD-1) according to the manufacturer's instructions (Live/dead assay; Invitrogen, Thermo fisher scientific, USA). The fluorescence in experimental and control cell samples were measured using excitation and emission filters (calcein=494/517 nm & EthD-1=528/617).

Assessment of PC-12 proliferation and differentiation using immunocytochemistry

Cultures were fixed with 4% paraformaldehyde (Alfa Aesar, MA, USA) for 10 min and permeabilised with 0.2% Triton X-100 for 10 min at room temperature. After blocking unspecific binding sites with 3% BSA (Sigma) and 10% goat serum at room temperature for 1h, cells were incubated overnight at 4 ^{o}C with primary antibodies against; polyclonal ant rabbit Ki-67(cat # ab 66155 at a dilution 1:1000 Abcam, Cambridge, UK), cytoskeletal marker polyclonal anti-mouse βIII-tubulin (cat# ab7751 at a 1:1000 dilution, Abcam, Cambridge, UK), mature neuronal markers; polyclonal anti-rabbit NeuN (cat# ab128886 at a 1:1000 dilution, Abcam), monoclonal anti-mouse MAP-2 (cat# ab11267 at a 1:500 dilution, Abcam). Immune reactivity was visualized with secondary conjugate Alexa flour 488 (cat# ab150113 & ab6717 at a 1:200 dilution, Abcam) and Alexa flour 555 (cat# ab150078 & ab6786 at a 1:200 dilution, Abcam) for

1h at room temperature. DAPI was used as a nuclear counterstaining. Images were captured using laser confocal microscopy (ZEISS LSM 700).

Neurite outgrowth assessments via Image analysis of neurites

Analysis was based on imaging of cells using phase-contrast microscopy followed by manual tracing of neurite length and counting the number of cells bearing neurites using ImageJ software [20]. The data was obtained from three independent experiments with 10-15 replicates for each group/experiment. Analysis was obtained by combining two fields from a single well and expressed as the average cell number and neurite length in μm.

Real time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was extracted from PC-12 cultures by using the RNeasy Mini Kit (Qiagen, UK) consisting of RLT lysis buffer and washing buffers (RW1 and RPE). Extracted RNA was converted to cDNA using Tetro cDNA synthesis kit (Bioline, UK). The cDNA template was used in real time PCR reaction by using SYBER green reagents (Roche Diagnostics, USA). The specific forward and reverse primers are shown in **Table 2**. Expression levels were obtained from Cp values for each sample by employing the Fit Points method as computed by the Light Cycler 480 software (Roche Diagnostics, USA) using HPRT1 as housekeeper control gene.

Transwell migration assay

Tissue culture inserts (Greiner Bio-one, Thin-cert 8 μm pore size) were loaded with PC-12 cells in serum-free RPMI-1640. The bottom chamber contained either; DPSC-CM or 10% FBS, 0% FBS which served as a positive and negative controls, respectively. After 24h, the cultures were stained with calcein-AM fluorescent stain. The number of calcein-stained migrated cells was counted with ImageJ software and

the fluorescent intensity of the migrated cells was measured using a Tecan fluorescent plate reader.

Neutralisation experiments

Neutralisation experiments using specific blocking antibodies were conducted to determine which neurotrophic factors were involved in mediating PC-12 cell survival and neurite outgrowth. PC-12 were cultured with 50% DPSC-CM in the presence of anti-NGF antibody (0.25µg/ml; MAB556 R&D systems), anti-GDNF antibody (1µg/ml; AF212 R&D systems), anti-BDNF antibody (2µg/ml; AF1494 R&D systems), anti-NT-3 antibody (2µg/ml; AF1404 R&D systems) or in the presence of mixture of all antibodies using the concentrations stated earlier.

At the beginning of each experiment, cells were washed twice with PBS and all experiments were performed in absence of serum. The cultures were maintained under the same conditions as previous experiments for 8 days, followed by quantification of neurite outgrowth and the number of survived neurons.

Statistical analysis

The difference between mean values was determined by one-way analysis of variance (ANOVA) with Tukey post hoc test using SPSS, except for the qRT-PCR experiments which were analysed using the non-parametric Kruskal-Wallis and Mann-Whitney tests. The statistical significance was set at $p<0.05$.

Results

DPSC characterization using sqRT-PCR

DPSC exhibited adhesion capacity to plastic; spindle shaped morphology, and 80% confluence reached in approximately 3-4 days. DPSC have revealed positive expression for CD90, CD29 and CD105 markers; and negative expression for CD14

and CD45. Nanog and SOX2 expression was positive, expected for a mesenchymal stem cell profile. **(Fig. 1)**

DPSC promote PC-12 survival and proliferation

Preliminary experiments tested different DPSC-CM concentrations on PC-12 cell viability using the MTT assay. The results indicated that DPSC-CM promoted cell viability which appeared optimal at 50% concentration, while higher concentrations led to a significant reduction in cell viability (data are not shown). Consequently, 50% DPSC-CM was used in subsequent experiments.

Live/dead cell assay confirmed that DPSC-CM significantly increased the number of viable PC-12 cells in comparison with serum-free and NGF treated cultures. **(Fig. 2)**. In the serum-free control group, more than 60% of the culture was showing cell death while only 20% of cell death was detected in DPSC-CM indicating that DPSC cultures promoted PC-12 cell protection. Interestingly, there was no statistically significant difference in live cell percentage between CM treated group and DPSC/PC-12 co-cultures. **(Fig. 2A)**

Fig. 2B presents the results of all tested culture media on the expression of cell proliferation marker Ki-67. PC-12 cells treated with DPSC-CM displayed 25% Ki-67 immunopositivity while serum-free and NGF treated cultures displayed 5% and 10% Ki-67 staining, respectively. There was no significant difference in PC-12 cell proliferation between DPSC-CM and co-culture treated cultures.

DPSC stimulate PC-12 neuronal differentiation

Morphometric analysis of PC-12 cells under serum-free control condition revealed that the number of cells per field was greatly reduced with no neurite extensions compared with DPSC-CM treated culture that revealed a significantly high number of cells with extensive neurite outgrowths. **(Fig. 3)**

βIII-tubulin/NeuN and MAP-2 immunocytochemistry was used to further highlight and outline the difference in the neurite lengths between the treated groups. The immunostaining revealed that the serum-free control group were devoid of any neurite extensions, whilst DPSC-CM showed extensive neurite outgrowth stained with the mature neuronal marker MAP-2 and cytoskeletal marker βIII-tubulin. These immunostainings appeared more prominent than that seen in the co-culture treated group but appeared to be very similar to NGF treated cultures.

qRT-PCR analysis indicated that DPSC-derived factors upregulated neuronal markers in the PC-12 cultures. NeuN and βIII-tubulin were highly expressed in DPSC-CM and NGF treated culture; however, MAP-2 expression was not significantly altered in the treated groups and this is unlike the immunocytochemical protein expression of the same marker as the molecular information tends to be available only as a snapshot at the end point.

Synapsin-I is an indicator of neuronal synapse formation and GAP-43 is an important marker for neurite sprouting and regeneration after nerve injury. Our data indicated that Synapsin-I and GAP-43 were significantly upregulated after DPSC-CM and NGF treatments, even more than observed in DPSC/PC-12 co-cultures. **(Fig. 4)**

DPSC-CM stimulate PC-12 cell migration

Transwell migration assay was performed to evaluate the chemoattractive potential of DPSC-CM on PC-12 cells. Calcein-AM was used as marker to stain the migrated cells after 24h of exposure to DPSC-CM, 10% FBS and 0% FBS. PC-12 migration was significantly enhanced by DPSC-CM while exposure to serum-free culture had no significant effect on cell migration. **(Fig. 5)**

Secreted neurotrophic factors in DPSC-CM

The total protein concentration in the pooled collected serum-free DPSC-CM was 1800 µg/ml. Neurotrophic factors NGF, BDNF, GDNF and NT-3 were detected in the DPSC-CM as assessed by ELISA. NGF and NT-3 were predominately detected (**191 pg/ ml** and **161 pg/ ml**, respectively) and BDNF and GDNF amounted to **85.5 pg/ml** and **90 pg/ml**, respectively. Only a trace amounts of CNTF could be detected.

Evaluating the role of neurotrophic factors in PC-12 neuronal differentiation

Neutralising antibodies were used to block the action of specific neurotrophic factors. Neutralisation experiments were performed on PC-12 cells incubated under the same conditions as previous experiments.

The results from image analysis of NeuN-positive cells indicated that blocking NGF did not significantly affect the number of surviving neurons in DPSC-CM treated cultures, however, neutralisation of GDNF did significantly reduce the number of surviving PC-12 cells. Further, using a mixture of neutralising antibodies (Ab mix) did not result in further reduction in the number of neuronal cells suggesting that GDNF was a major contributing factor to protect PC-12 neuronal cell culture.

Neutralisation of NGF, BDNF and GDNF respectively significantly reduced neurite outgrowth stimulated by DPSC-CM. However, blocking of NT-3 did not significantly affect neurite outgrowth in PC-12 cells. These findings suggest that NGF, BDNF and GDNF were important for neurite outgrowth promoted by DPSC-CM. Gene expression analysis using qRT-PCR indicated that neutralisation of NGF and GDNF resulted in significant downregulation of NeuN, MAP-2, GAP-43 and ßIII-tubulin underscoring the impact of these neurotrophic factors in the gene expression of neuronal markers. **(Fig. 6)**

Discussion

Neuronal differentiation is dependent on a single or collective effects of extracellular signalling neurotrophic factors which are endogenously to regulate the nervous system development, regeneration and function [21]. PC-12 cells have been widely used as a convenient model system for neurobiological and neurotoxicological studies and neuronal cell differentiation. PC-12 cells are rapidly dividing, suspended cells growing in clusters and are highly responsive to NGF; upon exposure to this neurotrophin, cell proliferation is ceased followed by a dramatic change in phenotype with the cells acquiring typical neurite extensions together with a number of properties characteristic of sympathetic neurons as chemical messenger secretions such as acetylcholine or noradrenaline [13,15,16, 22]. NGF has a well-known multifunctional role in nociceptive processing. The mediators released from inflammatory cells such as bradykinin, histamine, ATP and serotonin has been shown to be increased with NGF application. These mediators are released from ruptured cells during inflammation or injury and are able to stimulate receptors and ion channels located on the peripheral terminal of the nociceptor, leading to neuronal depolarization and sensitization that manifests as pain hypersensitivity [23].

In recent years, DPSC have been reported to demonstrate ability to secrete a variety of cytokines, growth and angiogenic factors such as vascular endothelial growth factors (VEGF), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF) which have a beneficial effect on various diseases [24-26]. in particular, DPSC are increasingly gaining attention in the field of stem cell research and therapy for neurodegenerative disease or nerve injuries because of their neural crest origin and significant neurogenic and neurotrophic properties [27]. In accordance with Mead 2017 [8-10], who illustrated that neurotrophic and neuroprotective effects of DPSC were

attributed to the plethora of secreted factors through paracrine mediated mechanism, DPSC were shown here to be able to induce PC-12 differentiation and survival. The neuroprotective effect, i.e cell survival action, of DPSC-CM was much more prominent as compared to NGF treatment. However, the neurotrophic effect of DPSC-CM on neurite outgrowths was very similar to that obtained following NGF treatment underlining the importance of NGF for PC-12 differentiation. The neuroprotective advantages of CM over NGF treatment could be due to the presence of other soluble factors within CM like GDNF, BDNF, NT-3, and CNTF which have been proven to promote neuronal cell survival in vitro [28-30]. This was in the line with a study done by Palmores et al. 2018, who used adipose stem cells conditioned medium (ADSC-CM) and the SH-SY5Y neuronal cells and concluded that BDNF, an exogenous neurotrophic growth factor, was able to recover oxidized neuronal cells, but the effect was less pronounced than that obtained with ADSC-CM [28]. In contrast, Gervois et al. 2017, used DPSC-CM to promote neurite outgrowth in SH-SY5Y cell line and concluded that exogenous BDNF was able to induce significantly longer neurites in SH-SY5Y than DPSC-CM [31].

The present study using the PC-12 cell model for the first time with DPSC, suggested that DPSC were able to secrete supportive neurotrophic factors of greater neuroprotective action than the widely used PC-12 inducer, NGF. This was further confirmed by our finding that neutralising antibodies against NGF, GDNF and BDNF, significantly attenuated the CM-mediated neurite outgrowth and produced a significant loss of morphological features associated with differentiation as assessed by MAP-2 immunostaining images. Indeed, GDNF and Abs mixture inhibition resulted in significant reduction in the number of survived cells and this was not markedly observed with NGF and BDNF inhibition. These novel results underline that GDNF

was critical for PC-12 cell survival, whereas NGF, GDNF and BDNF detected in DPSC-CM were essential for neurite outgrowth. This was in accordance with De Vicente et al. 2002, who confirmed the cytoprotective role of GDNF in the knocked-out mice and concluded that GDNF inhibits cell death induced by serum deprivation [32].

Blocking of NT-3 did not result in any observed influence on DPSC-CM stimulated PC-12 cell survival or neurite outgrowth. A study done by Willerth et al. 2009, [33] concluded that low NT-3 concentrations provided an initial starting point for neurite outgrowth but did not support complete differentiation of neuronal cell lines. This could possibly give an explanation of our finding that NT-3 concentration in DPSC-CM may not be enough to sustain PC-12 neuronal survival and complete differentiation. It would therefore be interesting to further evaluate the potential impact of different neurotrophic factors, including GDNF, NT-3 and BDNF independently or synergistically on PC-12 survival and neuronal differentiation, and their associated signalling downstream over time.

The co-culture system used in this study prevents direct cell-cell contact by using a semi-porous membrane allowing the infusion of soluble molecules from DPSC to the cultured cells. It was found that there was no remarkable difference in the neuroprotective effects of DPSC-CM and DPSC co-cultures as both promoted cell survival and prevented cell death to the same extent. This finding is in the line with Hao et al. 2014, who failed to detect any difference in cortical neuronal survival between ADSC-CM and ADSC co-cultures [34]. Another elegant study compared the capacity of ADSC-CM and ADSC-co-cultures to recover SH-SY5Y cell viability and concluded that both had the same capacity to recover cell viability [28]. However, here we demonstrated that DPSC-CM promoted longer neurite outgrowths from PC-12 cells than that observed in DPSC/PC-12 co-cultures. The neurotrophic advantages of

CM over co-cultures on PC-12 neurite outgrowth length could be attributed to the rapid and concerted action of the neurotrophic factors detected in DPSC-CM, but in the co-culture group, the time studied probably was not sufficient for the live cells to induce full terminal differentiation of PC-12 cells. Further studies are required to analyse the dynamics of secretome production by live DPSC over extended period of time.

DPSC-CM was also able to significantly promote PC-12 cell migration highlighting the chemoattractive potential of DPSC which may be important for the attraction of endogenous stem cells to the injury site to promote nerve regeneration. These findings are consistent with other studies describing stimulation of endothelial [35, 36] and neuronal cell migration by DPSC in vitro [31].

In this study, under the influence of DPSC-CM a dramatic upregulation of neuronal markers NeuN, MAP-2, ßIII-tubulin in addition to Synapsin-I and GAP-43 was detected. GAP-43, an important partner in neuronal differentiation and Synapsin-I, an indicator of synapse transmission, were particularly expressed in growing neurons coinciding with the beginning of neurite outgrowth [21]. These data suggest that soluble secreted factors from DPSC were able to stimulate relevant neural-associated gene expression in PC-12 cells, increasing the expression of neuronal markers and axonal regeneration underlining the significant impact of CM on PC-12 neuronal differentiation and synapse formation. The enhanced expression of MAP-2, NeuN and GAP-43 under the influence of DPSC-CM was significantly reduced upon NGF and GDNF inhibition suggesting that neurotrophic factors NGF and GDNF in the CM mediated PC-12 neuronal differentiation and axon formation.

Summary and Conclusions

To the best of our knowledge, this is the first study addressing the paracrine effects of DPSC in the well-established neuronal PC-12 cell model. Thus, this study demonstrated that CM derived from DPSC profoundly enhanced the viability, proliferation, migration, and differentiation of PC-12 cells in vitro. DPSC-derived GDNF proved in particularly to be critical for PC-12 viability and survival. DPSC-CM appeared more effective than DPSC/PC-12 co-cultures in promoting PC-12 survival and differentiation underscoring that the live cells co-cultures had a delayed lag time in producing effective amounts of trophic factors. These new findings highlight the useful application of the PC-12 cell line for exploring the role of specific signalling factors present in DPSC/MSC secretomes.

Ethics declaration

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

Data Availability

The datasets used and analysed in this study are available from the corresponding author on reasonable request.

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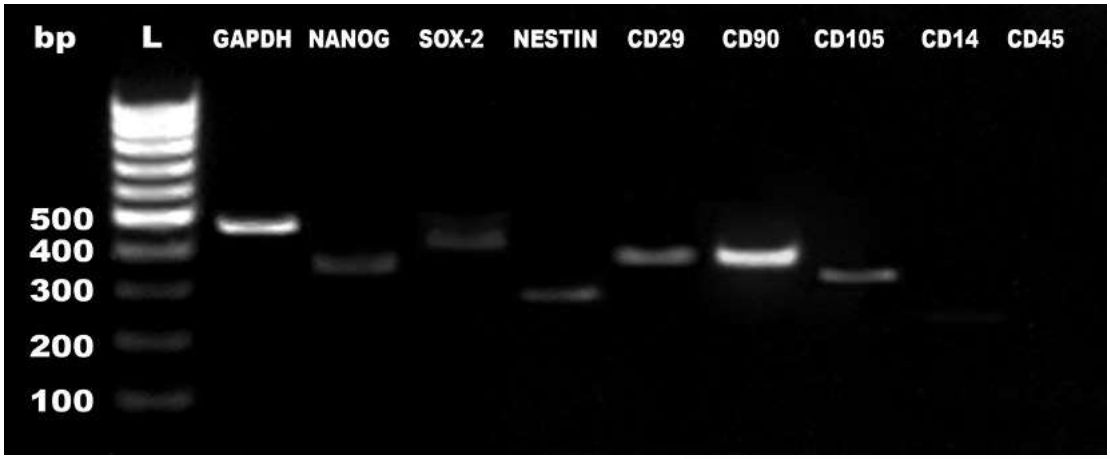

Figure 1: **DPSC characterization by sqRT-PCR**. Gene expression profile of Nanog, Sox2, Nestin, CD29, CD90, CD105, CD14 and CD45 in DPSC in relation to the expression of a housekeeping gene, GAPDH. L: ladder, bp: primer band size.

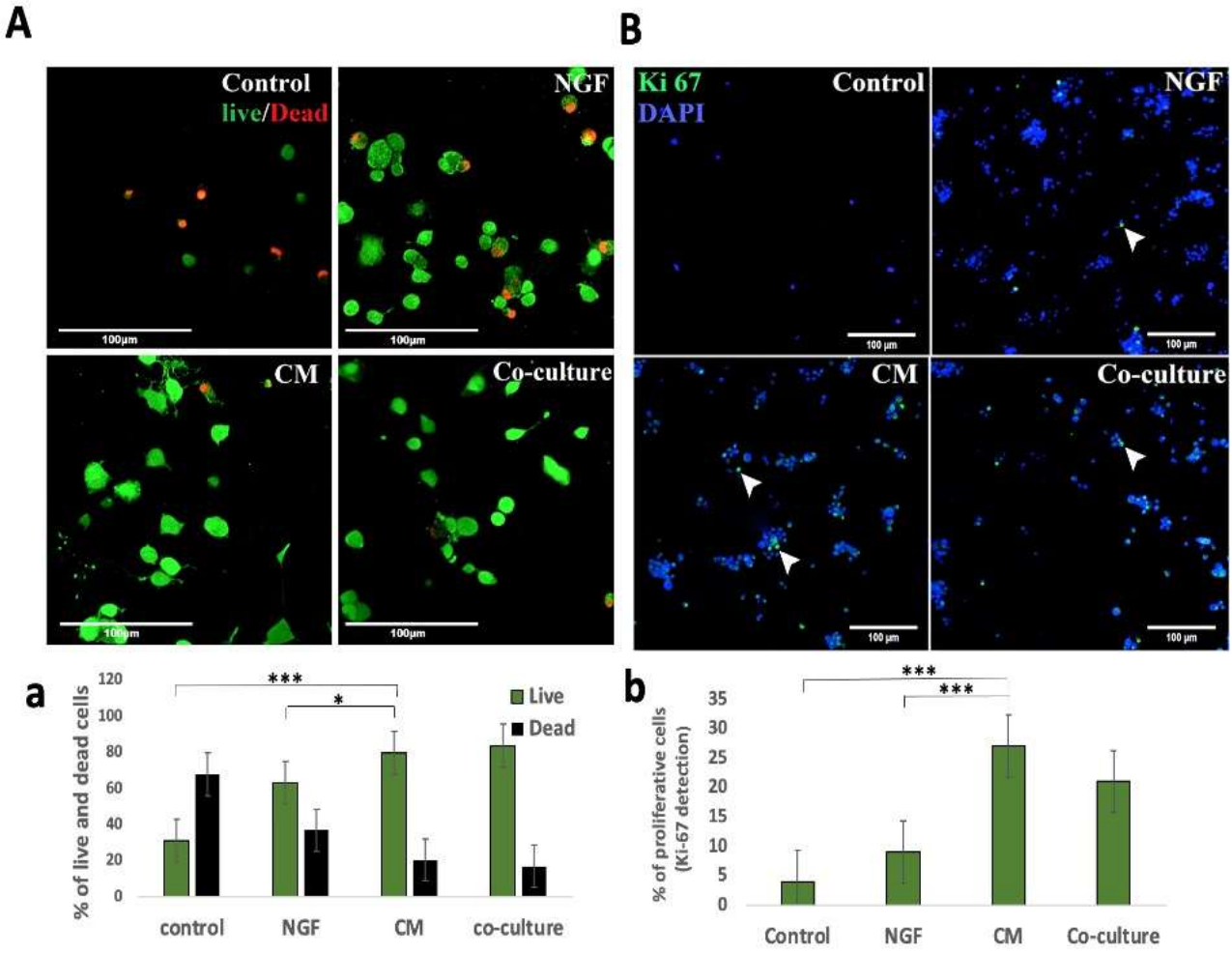

Figure 2: DPSC mediate PC-12 survival and proliferation. PC-12 cells were cultured in; serum-free RPMI-1640, 50ng/ml NGF, 50% DPSC-CM or DPSC co-cultured with PC-12 cells for 8 days. (**A**) Live/dead assay laser confocal images and (**a**) quantitative assay results obtained from Tecan fluorescence plate reader. (**B**) Ki-67 immunostaining images and (**b**) counting the number of Ki-67 positive cells using ImageJ software. Data are presented as mean ± SEM, from 3 independent experiments with 10 replicates for each group/experiment. * $p<0.05$, *** $p<0.001$. Scale bar is 100µm.

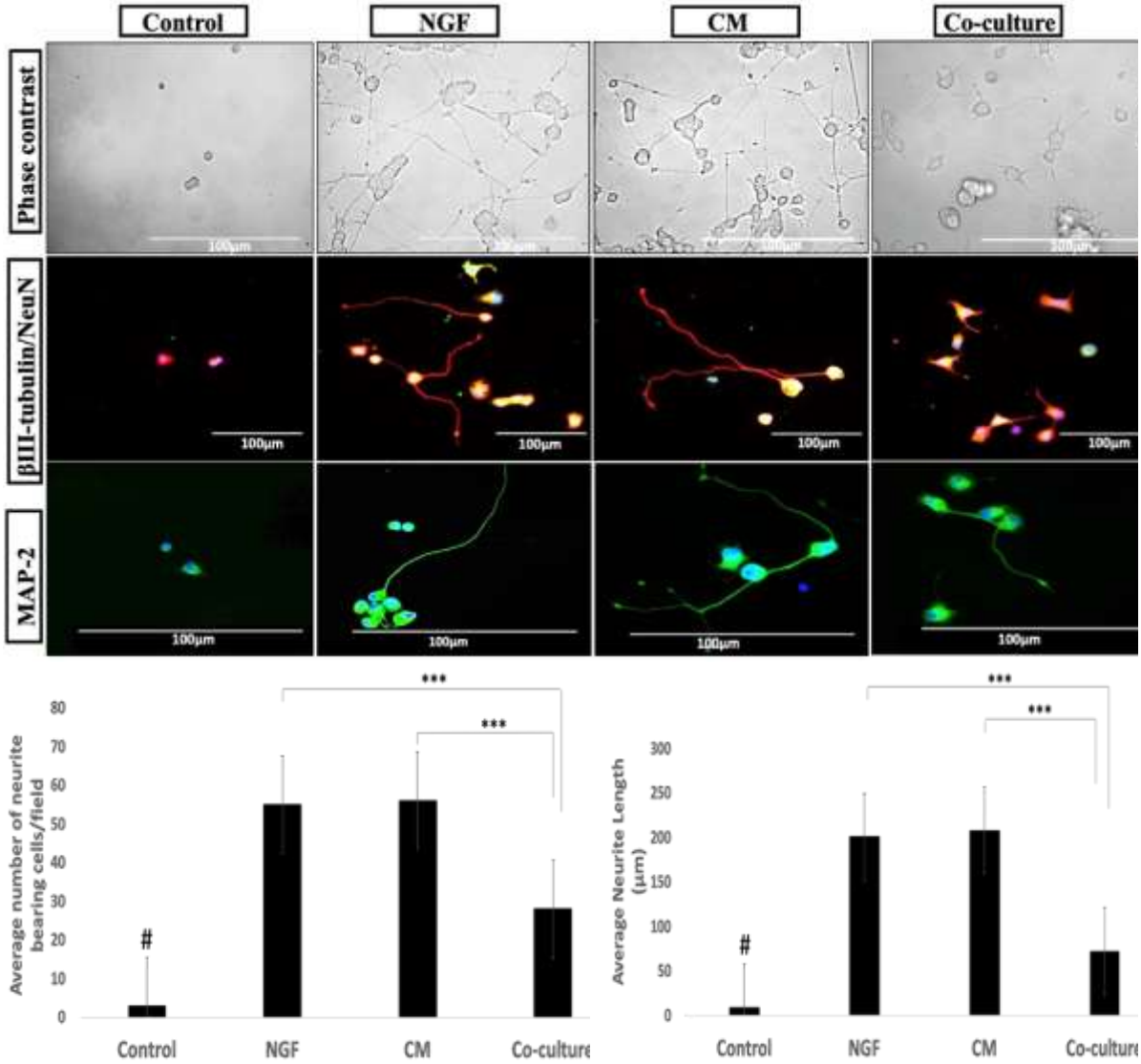

Figure 3: DPSC mediated induction of PC-12 differentiation. Phase-contrast microscopic images of PC-12 cells cultured on poly-L-lysine coated plates for 8 days in; serum-free RPMI 1640, 50 ng/ml NGF, 50% DPSC-CM or DPSC/PC-12 co-cultures. DPSC-CM and NGF prominently induced outgrowth of neurites from PC-12 cells. Cytoskeletal marker βIII-tubulin (red) and mature neuronal marker MAP-2 (green) were used to outline the differences in the neurite length between different treated groups. DAPI was used as a counterstain for nuclei. Bar charts quantitative analysis of the average neurite length and the average number of neurites bearing cells/field using ImageJ analysis. Data are presented as mean ± SEM, from 3 independent experiments with 15 replicates for each group/experiment. *** $p<0.001$, # means significant to all groups. Scale bar is 100µm.

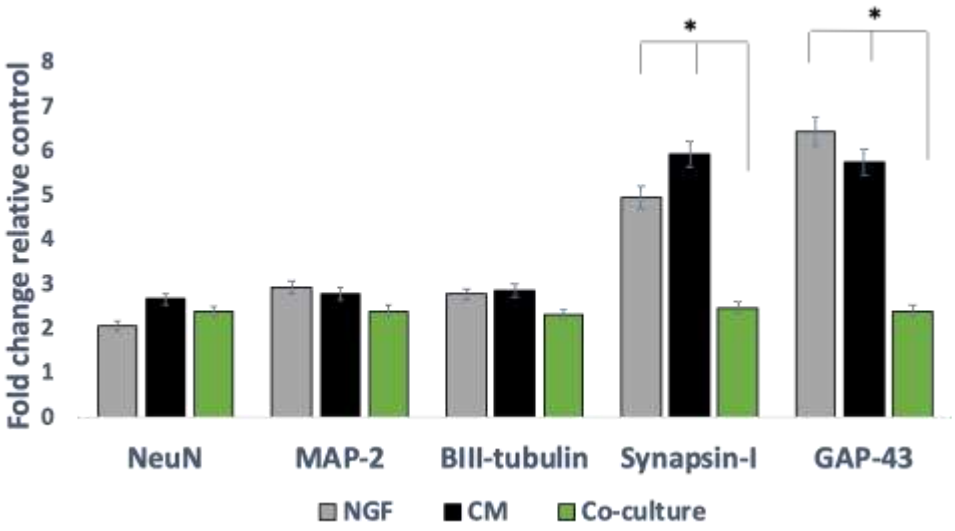

Figure 4: Gene expression analysis of PC-12 cells after 8 days of culture with 50 ng/ml NGF, 50% DPSC-CM and DPSC/PC-12 co-cultures. The fold change in the expression was relative to HPRT1 housekeeping control gene. Data are presented as mean ± SEM (n=3). * $p<0.05$.

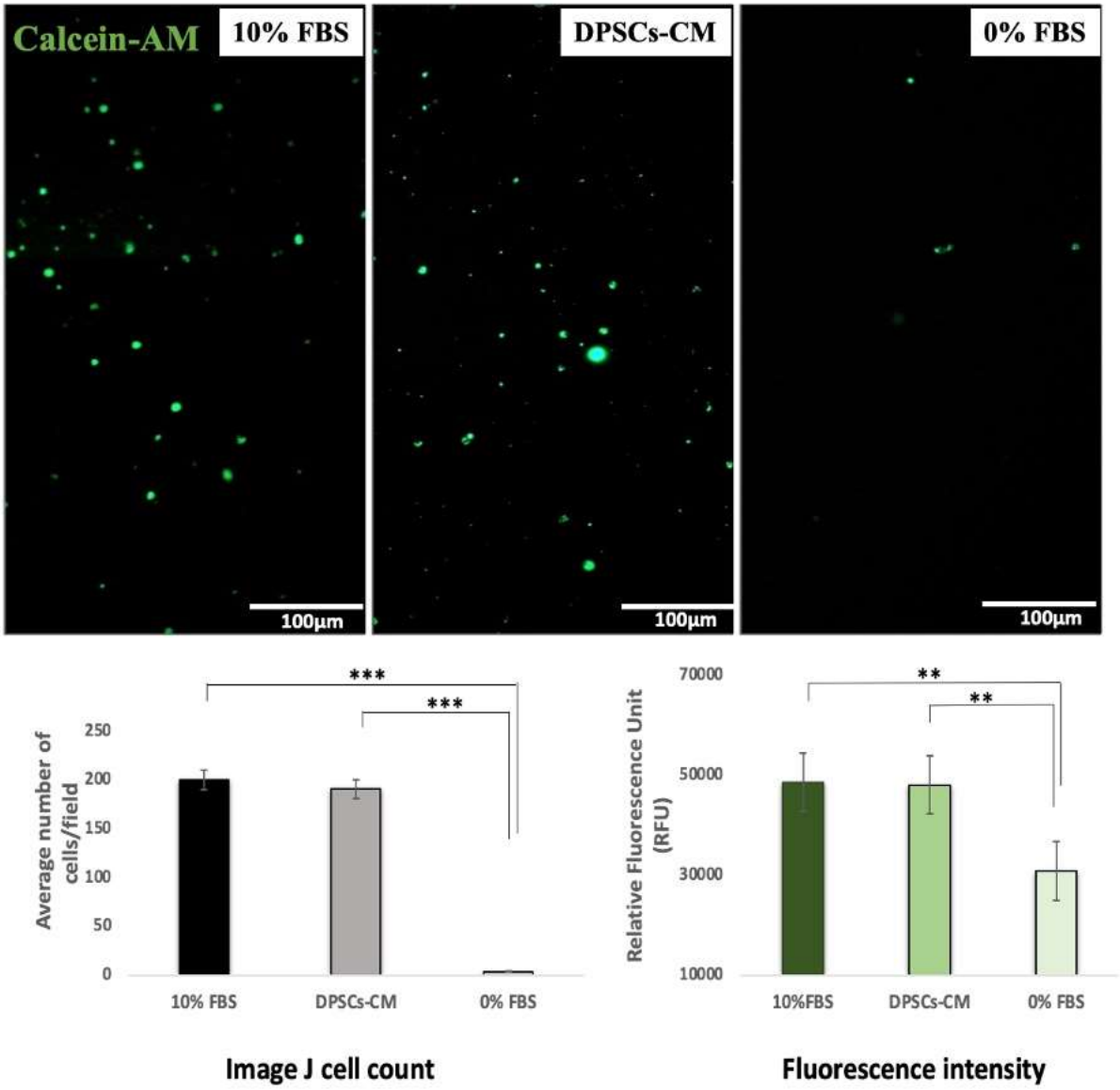

Figure 5: DPSC-CM promotes PC-12 cell migration. Calcein-AM staining of migrated cells after 24h exposure to 10% FBS, 0% FBS and DPSC-CM revealed that CM had a significant chemoattractant effect on PC-12 cell line migration. Data are presented as mean ± SEM, from 3 independent experiments with 10 replicates for each group/experiment. ** p<0.01, ***p<0.001.

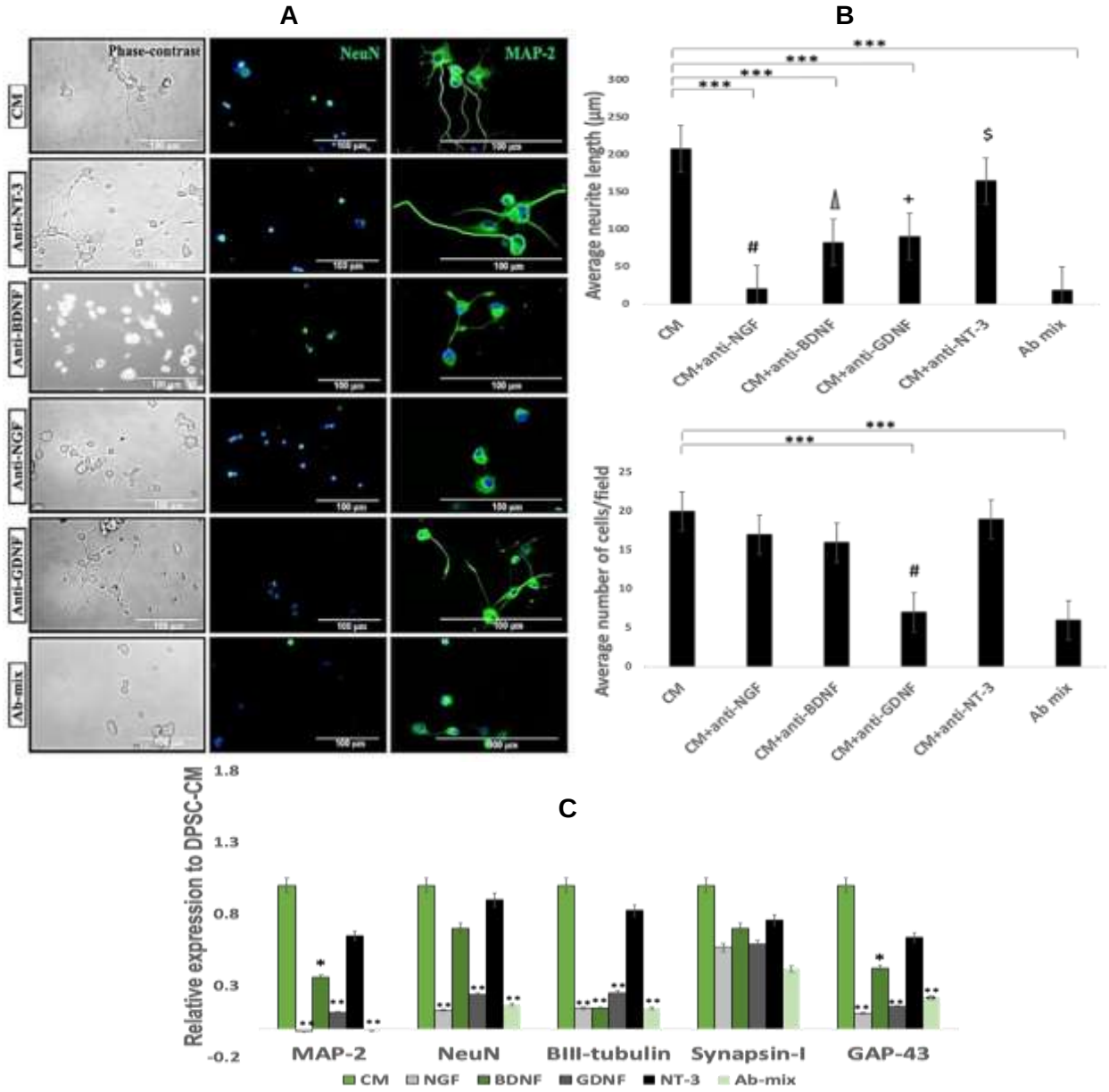

Figure 6: Inhibition studies evaluating the role of DPSC-derived neurotrophic factors after treatment with specific neutralising antibodies on PC-12 cells. (**A**) Immunolocalization of NeuN and MAP-2 showing the difference in the number of surviving neurons and the neurite length. DAPI (blue) was used as a nuclear staining. Treatment with anti-NGF antibody completely blocked neurite outgrowth evoked by DPSC-CM while the addition of Anti-GDNF antibody showed significant impact on neuronal survival. Scale bar is 100µm. (**B**) Bar charts show quantification of average neurite length and average number of surviving neurons under the effect of specific neutralising antibodies using Image J analysis. # means significance compared to all groups except Ab mix. Δ Means significance to all groups except anti-GDNF. **+** means significant to all groups except anti-BDNF. **$** means significant to all groups except CM. (**C**) qRT-PCR data showing that neutralisation of NGF and GDNF resulted in significant downregulation of MAP-2, NeuN, βIII-tubulin and GAP-43. Data are presented as mean ± SEM, from 3 independent experiments with 10 replicates for each group/experiment except for qRT-PCR with 3 replicates for each group/experiment. *p<0.05, **p<0.01, ***p<0.001.

Table 1: **Specific forward and reverse primer sequences used in sqRT-PCR.**		
Genes	**Primer sequences (5'-3')**	
	Forward	**Reverse**
CD105	F- TTCAGCTTTCTCCTCCGTGT	R-TGTGGTTGGTACTGCTGCTC
CD90	F- AGCTCTTTGATCTGCCGTGT	R- CTGCAGGCAATCCAATTTTT
CD29	F- ATCATGCAGGTTGCAGTTTG	R- CGTGGAAAACACCAGCAGT
CD14	F- GTTGGGCGAGAAAGGACTGA	R- GCTCCAGCCCAGTGAAAGAT
CD45	F- AGCTACCCCTCAAACGAAGC	R- TGTGAGTCCCTGGTGGTACA
Nestin	F- CAT TTA GAT GCT CCC CAG GA	R- AAT CCC CAT CTA CCC CAC TC
NANOG	F- TATCGTTTTGAGGGGTGAGG	R- CAGCTGGCACTGGTTTATCA
SOX2	F-TCCAGTCAAGCCCCACATC	R-TCCGAGTCACCCTTCCCA
GAPDH	F-CCCATCACCATCTTCCAGGAGC	R-CCAGTGAGCTTCCCGTTCAGC

Table 2: Specific forward and reverse primer sequences used in qRT-PCR.		
Gene	**Primer sequences (5'-3')**	
	Forward	**Reverse**
NeuN	F-CATGACCCTCTACACGCC	R-TGGAGTTGCTGGCTATCTGT
MAP-2	F-GATCAACGGAGAGCTGACCT	R-TTGGGCCTCCTTCTCTTGTT
βIII-tubulin	F-ATGAGGGAGATCGTGCACA	R-CACGACATCCAGGACTGAGT
GAP-43	F-GTTGAAAAGAATGATGAGGACCA	R-GCATCACCCTTCTTCTCGT
Synapsin-I	F-CCCAGATGGTTCGACTACAC	R-GGGTATGTTGTGCTGCTGAG
HPRT1	F-CCCAGCGTCGTGATTAGTGATG	R-TTCAGTCCTGTCCATAATCAGTC