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Regeneration of Neural Crest Cells from Human Postnatal Multipotent Dental Pulp Stem Cells

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Abstract

Objective: To identify discarded dental tissues as source for stem cells isolation and their differentiation into neurogenic lineage for regenerative applications. **Methods/Analysis**: Dental pulp tissues (Both impacted and erupted) were collected from 18-40 years old individuals having proper orthodontic reasons. The long-term in vitro culture of dental pulp stem cells (DPSCs) was established in controlled condition. Population doubling analysis was performed at different passages. DPSCs were further triggered into neurogenic cells under serum free retinoic acid defined supplements. Neurospheres development and neurogenic lineage differentiation was identified. **Findings:** More than 80% DPSCs were found to express putative dental stem cell marker CD90. We observed all basic biological characteristics of DPSCs during long-term cultivation up to 20 passages. The diameter distribution of cultured DPSCs was quite stable and was found to be 12-18μm in diameter. Highest numbers of neuropsheres were found to be at day 14 with ~100μm in size. Expression of β tubulin-III revealed highest differentiation (>15%) towards neuronal cells. **Novelty/Improvement**: The present study demonstrates that DPSCs could be a potential option to make repository of neurogenic cells for regenerative applications.

Keywords: Neural Crest Cells, Neural Lineage Cells, Neurospheres, DPSCs, PDT

1. Introduction

Cranial neural crest cells are specialized cells with neural origin. These cells have central role in the development and sustainability of mammalian tooth. They give rise to most of the dental cells/tissues and periodontium (surrounding tissue which holds teeth in position). Neural crest cells have capacity to differentiate into very specialized cell types to renew mature dental structures. Dental crest cells have their own repository of stem cells which helps to repair damages occurring throughout the life[1]. However, during severe injury/damage, the resident stem cells are not sufficient to repair the damage. The alternative approach is either activation of resident endogenous stem cells or supply of exogenous stem cells to repair the damage[2].

Although various strategies have been explored to activate endogenous stem cells however, the successful approach has not been demonstrated. Further studies are warranted for clinical applications. Site specific exogenous supply of stem cells from embryonic as well as adult stem cells do have potential which is still under exploration. In addition, due to ethical, legal, lack of expertise and oncogenic potential, their clinical utility is limited. Hence, particular interest has shown to use adult stem cells which have got potential to regenerate such organs/tissues. Mesenchymal Stem Cells (MSCs) from adult sources have been proved to have great potential in various kinds of tissues/organ regeneration[3]. They lack tumor inducing factor, allergic reactions and most importantly are hypo immunogenic in nature. Hence, major research has been focused to utilize MSCs in preclinical and clinical models.

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Various sources of MSCs have been described to be used in different disease/pathological conditions due to high proliferation and differentiation potential[4]. MSCs have great plasticity to produce a wide variety of mature cells including neuronal lineage cells[5]. Defective nervous system development or degeneration of nervous tissues leads to loss and/or functional abnormality leading to defect in central nervous system (CNS) as well as Peripheral Nervous System (PNS) tissues. Currently, there is no effective treatment for many devastating diseases and conditions that involve a destruction of nerve tissues. Nervous tissue regeneration constitutes a big social demand to develop better therapeutic strategies to restore normal function. It is very difficult to manage neurodegeneration as it poses a high expenditure and quality of life is compromised. In spite of the wide research in neural restoration strategies, nerve tissue represents inherent complications for effective structural and functional regeneration. MSCs has been considered a good potential candidate for autologous as well as allogenic transplantation[6]. Dental source of tissues have gained immense popularity to isolate MSCs due to similar genetic constituents and further to generate functional neuronal cells for their future applications in pre-clinical and clinical settings[7]. Therefore, in this study, we aimed to utilize dental tissues as prominent source to isolate MSCs population and developing the appropriate strategies to generate neuronal cells for their further applications.

There is a great hope from researchers and clinicians that such stem cell sources can have great application to regenerate dental neural crest tissues/cells as well as other to manage/or actively participate to treat several neurodegenerative conditions. However, before it reaches to the clinics, few necessary criteria's should be fulfilled such as: 1. Establishment of proper isolation techniques, 2. Well defined phenotypic and molecular markers, 3. Proper enrichment, proliferation and differentiation techniques.

The present study has explored the presence of multipotent stem cells in dental pulp tissues having highly proliferative capacity and potential sources for autologous reparative and reconstructive medicine. The study has also emphasized that human Dental Pulp Stem Cells (hDPSCs) are capable of differentiating into mesenchymal-derived neurological lineage cells. Furthermore, we also hypothesized that hDPSCs cultures include cells with the label-retaining and sphere-forming abilities; traits attributed to multipotent stem cells, and may provide evidence that these may be multipotent neural crest stem cells. Further analysis of these cells offer impending regarding for enormous supply of stem/precursor cells to apply in tissue regeneration which may possibly offer a breakthrough by combining with several other cellular resources with pharmacological compounds to regenerate dental as well as damaged nervous system.

2. Materials and Methods

Dental pulp tissues were collected from 18-40 years old individuals as per the standard guidelines after taking ethical approval from Institutional Ethics Committee of Deccan College of Medical Sciences, Hyderabad, India. Normal impacted human third molar teeth were collected (n = 15) from Dental Clinics. Before extraction of the teeth, each subject was screened for systemic diseases, health history was recorded and oral questioning was performed.

2.1 Inclusion Criteria

Healthy individuals (both males and females) with age group between 18-40 years were included in the study. Both impacted and erupted wisdom teeth were extracted having proper orthodontic reasons.

2.2 Exclusion Criteria

Subjects below 18 and above 40 years of age group were excluded from the study. Subjects having any systemic disease were also excluded from the study. Badly carious teeth, teeth with amelogenesis imperfecta, odontogenesis imperfecta or other related syndromes affecting quality of dentin or pulp, teeth with extensive restorations and root canal treated teeth were excluded from the study.

2.3 Isolation of Dental Pulp Stem Cells (DPSCs)

Dental pulp tissue from freshly extracted human third molar was removed after surgical dissection. Pulp tissues were kept in Dulbecco's Modified Eagles Medium-F12 (DMEM-12) containing 100 units/ml penicillin, 100µg/ml streptomycin and 1µg/ml amphotericin-B to avoid contamination. Pulp tissues were washed twice with

DMEM-12 with antibiotics, minced into 1-2mm^2 pieces and subjected for one step Collagenase digestion. Briefly, minced dental pulp tissues were incubated at 37˚C and 5% CO_2 in DMEM-12 (Gibco) containing antibiotics (SIGMA) with 0.03% collagenase (SIGMA) for 30-40 min. After incubation tube was removed from the incubator and digestion reaction was stopped by adding 1/4th of the volume of fresh DMEM-12. Cells were filtered through sterile 40µm cell strainer (BD Biosciences).

2.4 Establishment of DPSCs Culture from Extracted Teeth

Dental pulp derived cells were cultured at an initial concentration of one tooth digest per T-25 flask in DMEM-F12 with 10% FCS, 2mM glutamine, 100 units/ml penicillin, 100µg/ml streptomycin and 1µg/ml amphotericin-B. Medium was changed every after 2nd day. The culture was monitored every day and any contaminated flasks were removed immediately and recorded properly.

2.5 Sub-Culture and Population Doubling Analysis for DPSCs

Trypsinization of in vitro cultured DPSCs was performed after 21days of initial culture using 0.25% Trypsin-EDTA (SIGMA). Briefly, all the cell culture medium was removed from the cell culture dish aseptically without disturbing the cell layer. Cells were washed with 5ml of 1X PBS to remove dead cells and remaining growth medium. Added 1ml of 0.25% Trypsin-EDTA solution to the T-25 flask swirled gently and incubated at 37°C for 3min, cells detachment was confirmed by observing the flasks under microscope. All the cells were washed out from the flask surface by pipetting the fresh 5ml culture medium all over the surface. Cell clumps were dissociated by gentle pipetting. Cells were assessed for their viability, counted and further cultured in T-25 flasks at 1:3 split ratio up to 20 passages.

2.6 Cell Viability Assays

Viability of isolated DPCs was assessed by 0.2% Trypan blue exclusion and Fluorescien Di-Acetate (FDA) assays. Cell viability, counting and enumeration was carried out using a hemocytometer.

2.7 Trypan Blue Exclusion Assay

Single cell suspension was prepared after collagenase digestion of DPCs and diluted with 1X PBS to 1:100 (dilution factor = 100). In diluted cell suspension 0.2% trypan blue solution was added and incubated at room temperature for 3-5 mins. One of the counting chambers of hemocytometer was loaded with 10µl of cells mixed in trypan blue and covered with glass cover slip. Cells were counted in all counting chambers. Non-viable cells (stained in deep blue) as well as viable cells (unstained) were counted separately. Percentage of viable cells and concentration was determined using following formula:

Percentage cell viability = (No. of viable cells/No. of total cells) X 100

Concentration of viable cell (Cells/ml) = Average no. of viable cells per square X Dilution factor X 10^4

(Note: Dilution factor = Total volume/Volume of cells taken)

2.8 FDA Cell Viability Assay

1.0 X 10^3 cells were taken in a 1.5 µl micro centrifuge tube and mixed with 5µl of FDA (1µg/ml). Cells were incubated at RT for 5min. Twenty microlitre of cells stained with FDA were mounted on a glass slide and covered with glass cover slips. Cells were finally observed under an inverted fluorescent microscope and analyzed using Axiovert Version 4.2 software (Carl Zeiss, Germany).

2.9 Cell Proliferation Assessment

The proliferation of DPCs was assessed at each passage by measuring mitochondrial reductase activity. Briefly, 100µl (1 X 10^3 cells/ml) cells were seeded in triplicates into 96 well cell culture plates and cultured for 20 passages. In each passage cells were cultured for 21 days and assessed MTT reduction at day 1, day 7, day 14 and day 21. Briefly; 30µl of MTT (1mg/ml) was added in each sample at each time points. Samples were mixed with MTT and incubated for 4 hours at 37˚C in CO_2 incubator at 5% CO_2 and 100% humidity. After 4h of incubation, 50µl of Isopropanol was added in each well to solubilize the Formazan product. Optical Density of cells was determined in a microplate reader at 570nm. Readings were taken at regular intervals of time at day 1, day 7, Day 14 and day 21.

2.10 Flow Cytometry Analysis

DPCs at passage 2 were cultured at 50–60% confluency and further subjected to immunophenotypic analysis using mesenchymal specific antibodies (CD90 as positive marker and CD34 and CD45 as negative markers). Flow cytometry analysis was done using FACS Calibur flow cytometer (BD Bioscience). The number (percentage) of positive cells was determined using CellQuest software (BD Bioscience) by comparison with the corresponding control. Briefly; cells were harvested and washed twice in cold 1X PBS by centrifuging at 200x g for 7mins. The cell surface marker (CD90-FITC, CD45-FITC and CD34-FITC) was performed following the staining procedure indicated by the antibody manufacturer (R&D System, India). 5×10^5 washed cells were resuspended in 0.5 ml of cold 4% paraformaldehyde fixative and incubated at 18˚C-24˚C for 10mins. Cells were vortexed intermittently in order to maintain a single cell suspension. Following fixation, cells were washed twice in 1X PBS by centrifuging at 200 x g for 7mins. Cell pellet was harvested from each tube and resuspended in 2ml of SAP buffer (only for intra-cellular staining). Cells were centrifuged at 200x g for 7mins. Cell supernatant was decanted, ensuring that approximately 200µl of SAP buffer remains into the tube. Cells were resuspended in the remaining SAP buffer and added 10µl of antibody conjugate. For surface staining cells were directly stained with antibody conjugate. Cells were washed twice using 2 ml of 1X PBS buffer each time and centrifuged at 200x g for 7mins. Finally resuspended the cells in each tube with 200-400µl of 1X PBS for flow cytometric analysis.

2.11 Triggering DPSCs into Neurogenic Cells

DPSCs at 2nd and 3rd passage were cultured in serum free human neural proliferation medium (Stem Cell Technologies, Canada) supplemented with 20 µg/ml epidermal growth factor (EGF, Millipore), 10 µg/ml basic fibroblast growth factor (bFGF, Millipore) and 1X antibiotic solution (Sigma). Cultures were incubated in a humidified incubator at 37°C and 5% CO_2. Fresh medium and growth factors were supplemented every after 3rd day and cultures were maintained for 21 days in suspension.

2.12 Assessment of Neurospheres Development Characteristics

A peculiar characteristic of neural stem cells for developing into neurospheres was assessed for DPSCs directed towards the neural phenotype using neurospheres growth characteristics. Average number of neuropsheres formed at day 1, 7, 14 and 21 was calculated and plotted against the culture period (days).

2.13 Immunocytochemical (ICC) Staining of Neurospheres Derived Cells

Dual ICC staining of neurospheres derived cells at day 21 was performed using Nestin-PE (1:100, R& D System, India) and NCAM-FITC (1:50, R& D System, India) antibodies to determine the neural cell characteristic and pluripotent nature of directed cells. DAPI (4, 6-diamidino-2-phenylindole) was used as counter dye for staining the cell nuclei. All the fluorescence images of cells were captured and documented with inverted fluorescence microscope using Axiovert software (Carl Zeiss, Germany).

2.14 Lineage Differentiation

Neural lineage differentiation ability of cells from neurospheres (derived from DPSCs) was evaluated by culturing them on fibronectin coated cover slips. The cells were allowed to adhere for 24h in Neural proliferation medium and then induced with retinoic acid in human neuronal differentiation medium (Stem Cell Technologies, Canada). During neuronal differentiation mitogens such as EGF and bFGF were withdrawn. The cells were allowed to differentiate for 21 days. Fresh medium was replenished every after 3rd day.

2.15 Quantitative Analysis of Gene Expression using RT-qPCR

Total RNA was isolated from 2nd passage of cultured cells using GITC method and further subjected to RNA quantification and cDNA synthesis for PCR analysis. Briefly; 1 X 10^6 cells were taken from differentiated cells at day 7, 14 and 21 and used for total RNA extraction. Conversion of RNA into cDNA was performed according to the method of Gubler and Hoffmann. Briefly, 16µl of RNA (1µg) and 2µl of Oligo dT was mixed in a PCR tube and incubated for 10mins at 65°C in a Thermal cycler (BIO-RAD). The

mixture was snap cooled for 2min in ice. Master-mix of RT buffer (5X) of 8µl, 1µl dNTPs (10mM), 0.5µl RTase and dH2O. A total of 12.5µl was prepared per reaction and distributed in all tubes. All tubes were incubated in a thermal cycler 1hour at 42°C and 10mins at 72°C. cDNA was quantified using nano-drop reading and further confirmed on 0.8% agarose gel using ethidium bromide staining under ultra-voilet light. All the primer sequences used in the study were obtained from Primer Bank. The annealing temperature of all the primers were standardized using conventional PCR and further subjected to SYBR Green-based quantitative Real-Time PCR for relative gene expression analysis for Nestin, NCAM, CD90, CD105, CD73, CD45, CD34, β-tubulin-III, GFAP, O4, OCN, DSPP and ALP. GAPDH was used as endogenous control to normalize the test samples.

3. Statistical Analysis

The data in present study were expressed as Mean±SD. One way ANOVA was used with post hoc Students t-test for comparing different groups and calculating the significant values. RT–qPCR efficiency was calculated with Step One real-time software (Version 2.2, Applied Biosystem). All mRNA transcripts were analyzed in triplicates. The expression for each mRNA transcript was compared with controls of respective group. Relative fold change for all transcripts was calculated by normalizing them against GAPDH expression according to the Livak method for relative quantification[8]. Fold change values for all transcripts was further validated by using Pfaffl method of relative quantification[9]. Regression value (R^2) ≥0.99 was considered significant with 100% PCR efficiency. p value <0.05 was considered statistically significant. R programming was used for statistical computing and graphical representation of fold difference values of different mRNA transcripts analyzed by RT-q PCR. The data were compiled and run on a UNIX platform during R programming using online link (https://discover.nci.nih.gov/cimminer/cimMinerUpdate.do).

4. Results

A total of 15 dental pulp tissues were used for conducting the study. We were able to extract erupted and impacted third molar dental tissues with the help of Luer's forceps or extirpation needle under sterile conditions. 8-20 million cells (12.2±3.62) from each tissue were isolated (Table 1).

The viability of isolated cells was found to be approximately 95% (95.69±1.96). The plating efficiency of DPCs Figure 1(a) isolated from third molar tissue was approximately 70% (72.18±4.26) (Table 2). Almost all the cells

Table 1. Details of tooth donors and number of cells obtained from the dental pulp of each donor tooth (Age: 28±6.78)

Patient No.	Gender	Age (Year)	Extracted Teeth	No. of Cells (Millions)
DN/01	M	19	M3 Impacted	10
DN/02	M	20	M3 Impacted	8
DN/03	M	25	M3 Impacted	15
DN/04	F	20	M3 Erupted	16
DN/05	M	28	M3 Erupted	20
DN/06	F	32	M3 Erupted	11
DN/07	F	38	M3 Impacted	8
DN/08	F	39	M3 Impacted	12
DN/09	F	38	M3 Erupted	11
DN/10	F	31	M3 Erupted	16
DN/11	M	22	M3 Erupted	15
DN/12	M	25	M3 Impacted	10
DN/13	M	29	M3 Impacted	8
DN/14	F	31	M3 Impacted	9
DN/15	M	23	M3 Erupted	14

were stained positive for FDA showing higher percentage of cell viability and suitable for the culture and further experimental purposes (Figure 1(b)).

Table 2. Percentage cell viability and plating efficiency of cells isolated from third molar dental pulp tissue of each donor

Patient No.	Cell viability (%)	Plating efficiency (%)
DN/01	96.03	73.5
DN/02	93.41	68.1
DN/03	93.01	79.71
DN/04	94.56	74.23
DN/05	95.78	72.13
DN/06	98.23	76.12
DN/07	99.46	70.23
DN/08	98.12	69.01
DN/09	97.26	68.52
DN/10	93.02	66.23
DN/11	94.01	67.12
DN/12	95.06	69.21
DN/13	96.17	77.23
DN/14	95.13	78.18
DN/15	96.12	73.28

4.1 Cellular Morphology

The primary cultures established for DPCs on plastic surfaces showed adherence after 24h and appeared as small colonies after 2-3 days. Whereas, in continuous culture morphological changes were observed from spherical phenotype to spindle shaped. The number of spindle shaped cells was found to be enhanced after day 7 to day 21 (Figure 1(c)-(j)). Figure 1(c) shows spherical cells during initial culture which was later changed to spindle shaped at/after day 7. Cells were found to be >70% confluent at day 21 and ready for the further passages.

4.2 Immunophenotypic Characterization of DPSCs

To confirm the mesenchymal stromal phenotype of in vitro cultured cells 2nd passage cells were subjected to flow cytometry for expression analysis of positive and negative markers. Flow cytometry analysis revealed that >80% DPSCs express putative dental stem cell marker CD90 and were negative for hematopoietic markers (CD34 and CD45) (Figure 2). The absence of expression for CD34 and CD45 suggests the lack of cells from the hematopoietic and leucocytic lineages.

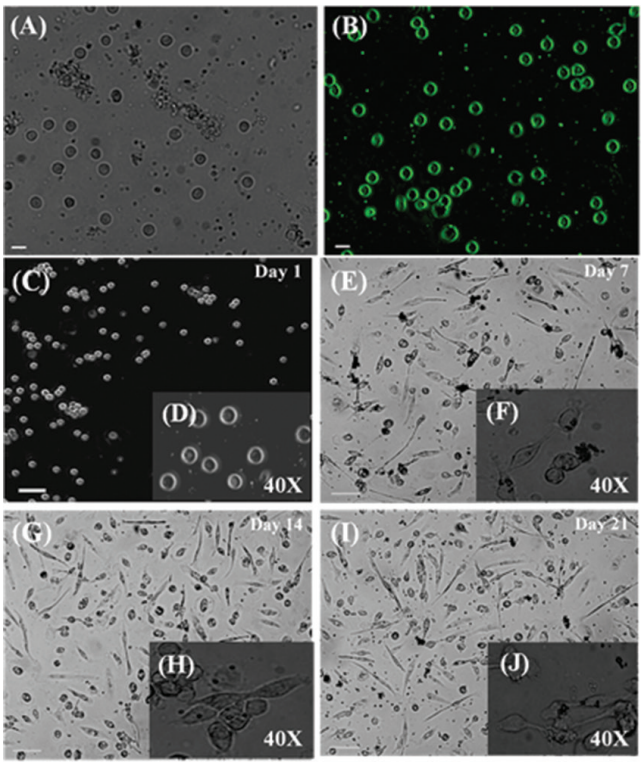

Figure 1. (a) Plating density of DPSCs. (b) FDA stained DPSCs. Morphology of in vitro cultured DPSCs at (c-d) day 1 (e-f) day 7 (g-h) day 14 and (i-j) day 21. Figures a,b,c,e,g, and i represent 10X magnification images of cells whereas d,f,h and j represent 40X magnified cell images at different time points as described above. (Scale bar: 50μm).

4.3 Cumulated Population Doublings (CPD) and Diameter Distribution

We observed all basic biological characteristics of in vitro cultured DPSCs during long-term cultivation up to 20 passages. Initial 5 doubling time was found to be approximately 20h whereas from 6-15 doubling the time was increased up to 40h and further was consistent till 20 passages (Figure 3(a)).

The diameter distribution of cultured DPSCs was quite stable and was found to be 12-18μm in diameter (Figure 3(b)). The distribution of cell diameter was not related with the morphological differences at later stage of culture.

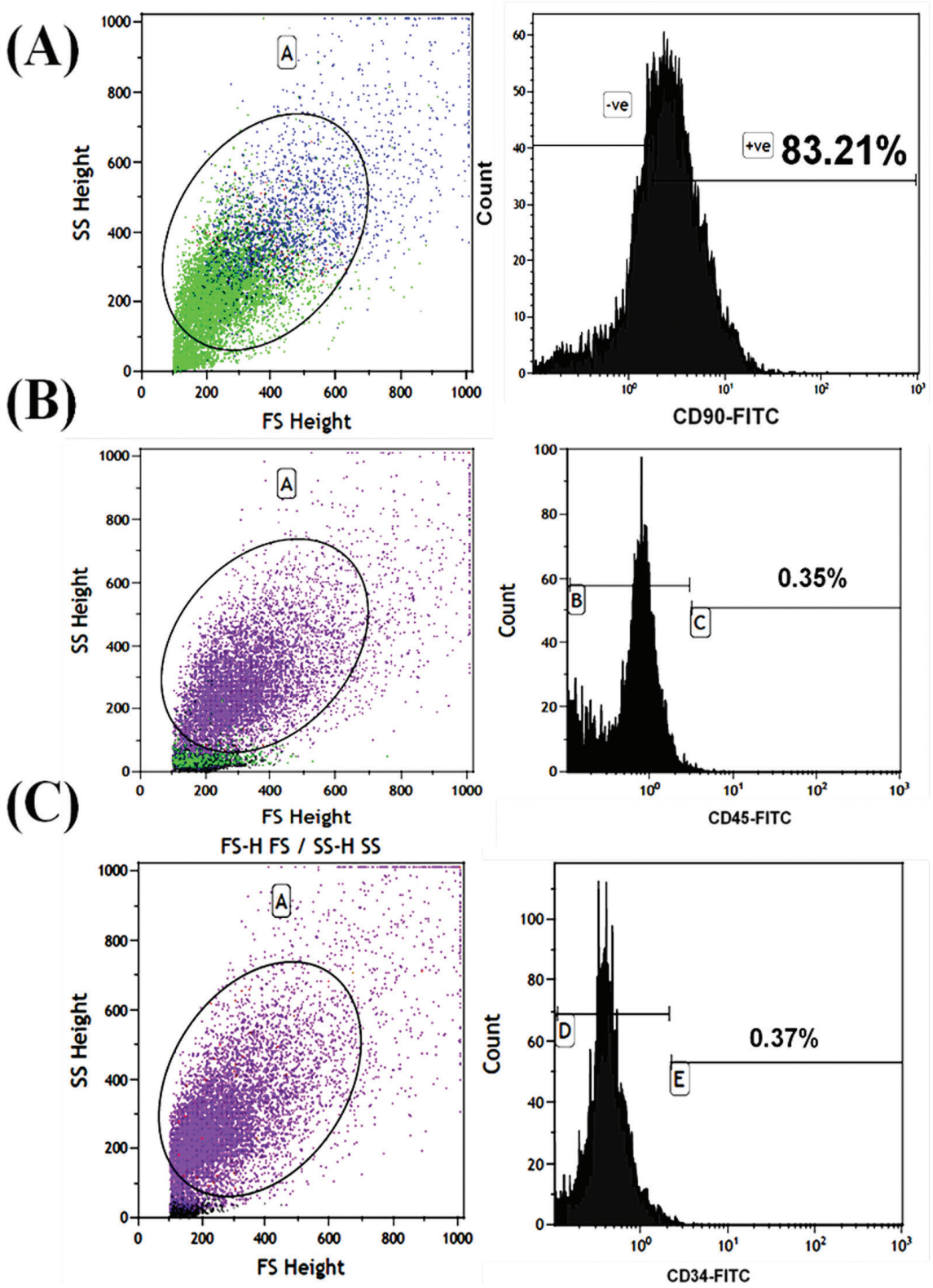

Figure 2. Flow cytometry showing immunophenotypic analysis of DPSCs cultured on plastic plates at 2nd passage (day 21). DPSCs were tested against human antigens such as hematopoietic markers (CD34 and CD45) and MSCs specific marker (CD90). (A-C) Dot plot and histographs for CD90, CD45 and CD34 respectively showed percentage of cells expressing specific antigens.

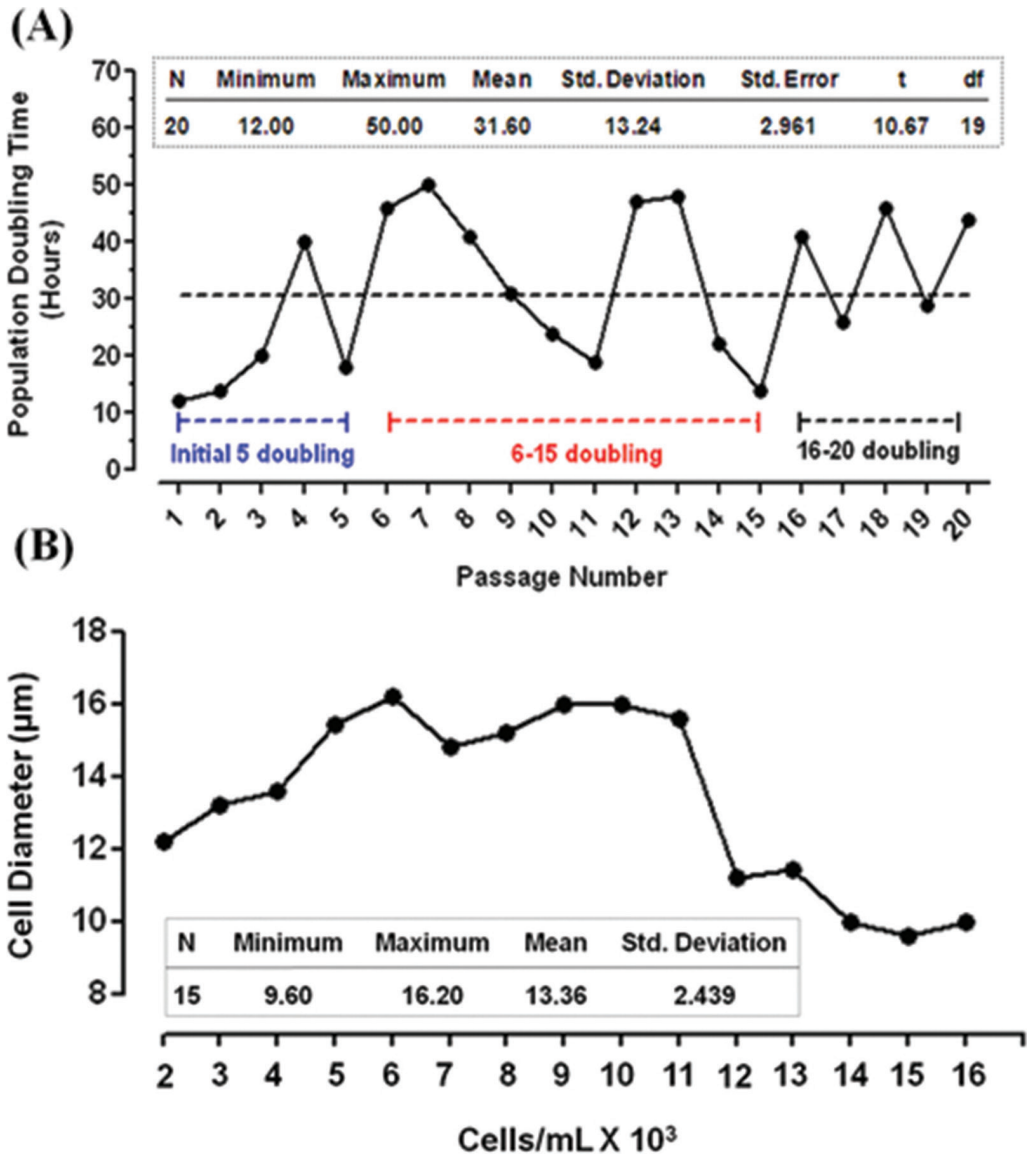

Figure 3. (a) PDT for in vitro cultured DPSCs from passage 1 to 20. The increase in PDT was observed from passage 6-20 and was found to be approximately 40-50h. (b) The diameter distribution of in vitro cultured DPSCs at passage 2 was found predominantly 12-18µm in diameter.

4.4 Neurospheres Development

Replacing the regular serum medium after 24h with serum free medium containing neural mitogenic growth factors (EGF and FGF), cell started communicating to each other and initiated to develop spheroids at day 7. These spheroids continuously increased in size up to 100µm in diameter at day 21 and termed as neurospheres (Figure 4(a)). The average number of neurospheres developed at day 7, 14 and 21 were calculated and plotted (Figure 4(b)). Highest numbers of neuropsheres were found to be at day 14 and were slightly decreased at day 21 due to increase in their size and limited division capacity.

4.5 Identification of Neural Cells in Developing Neurospheres Derived from DPSCs

To determine the presence of neural cells inside the developing neurospheres, the cells were harvested and stained with neural precursor cell specific markers such as Nestin

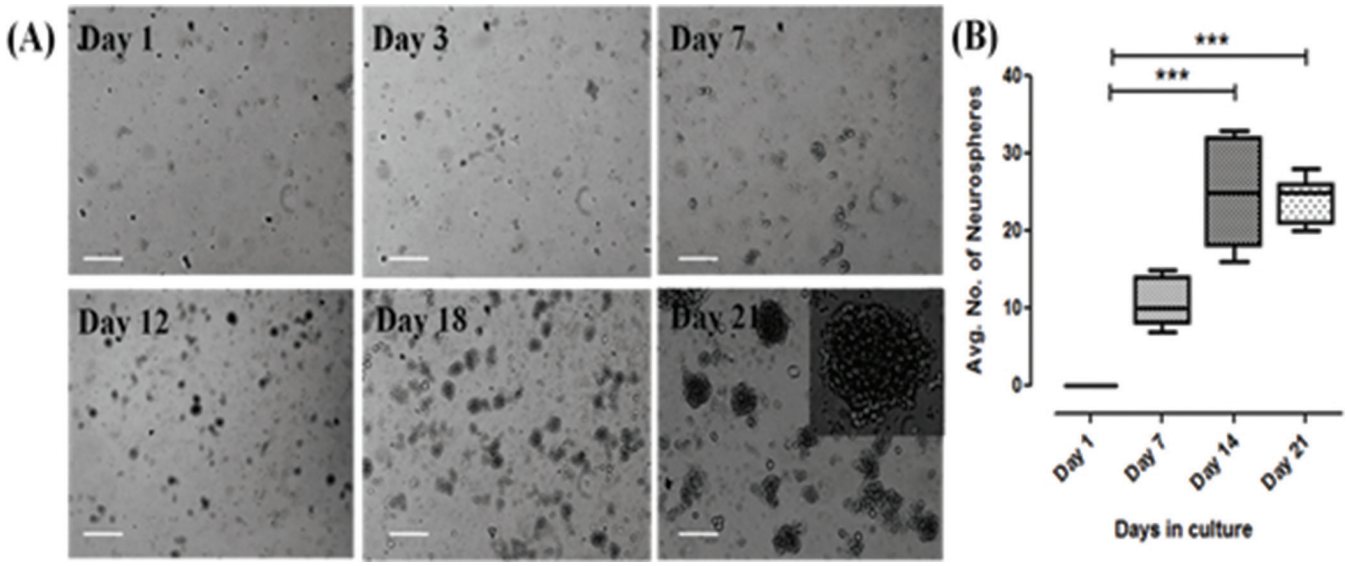

Figure 4. (a) Neurospheres development of DPSCs at different time points from day 1 to day 21. (b) Average number of neurospheres formed at day 7-21.

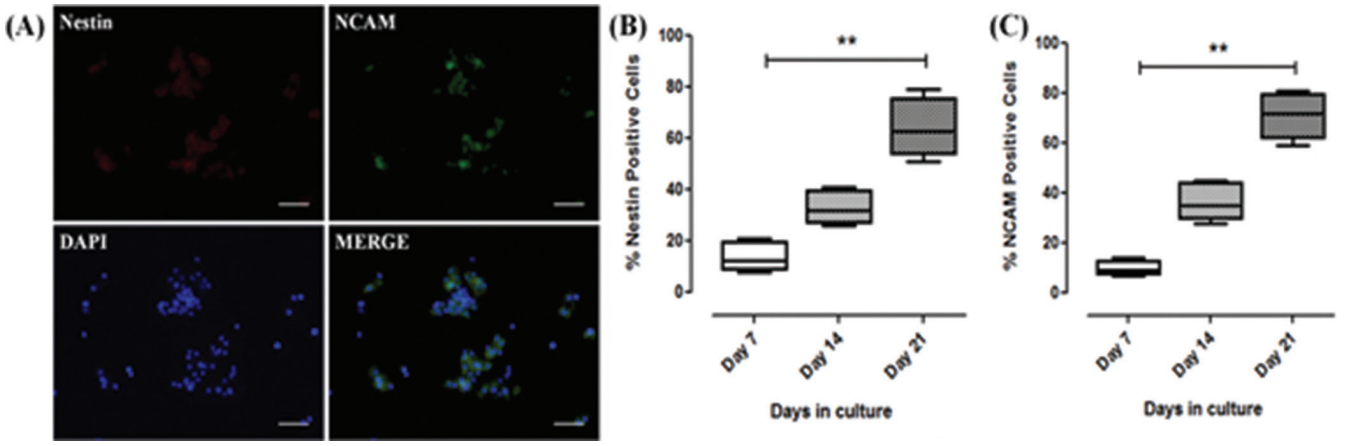

Figure 5. (a) ICC staining of cells derived from developing neurospheres of DPSCs cultured in serum free medium showing positivity for both Nestin and NCAM. Highest percentage of positive cells were found to be at day 21 for both. (b) Nestin (Day 7: 13.50±5.80, Day 14: 32.75±6.652, Day 21: 64.00±11.60) and (c) NCAM (Day 7: 10±2.915, Day 14: 36.40±7.403, Day21: 71.00±9.083).

and Neural Cell Adhesion Molecule (NCAM). The percentage positive cells for both the markers were found to be enhanced with increasing the time points during in vitro proliferation and neurospheres development from day 1 to day 21. Highest percentage of positive cells for Nestin/NCAM was found to be approximately 60-80 % (Figure 5).

4.6 Analysis of Neuronal Lineage Differentiation Ability

After culturing neurospheres derived cells on fibronectin coated coverslips in defined medium under retinoic acid induction, cells started changing their morphology to neuronal like cells. The complete neuronal differentiation was observed at day 21 post retinoic acid exposure (Figure 6(a), (b)). Well-developed neuronal network was observed with defined boundaries for axons and dendrites communicating to neighboring cells.

Quantitative gene expression analysis of differentiated cells revealed the expression of several neural stem cells and lineage markers. One of the most important characteristics of these cells was found to be their maximal differentiation towards neuronal cells as observed through the highest increase in the expression of β-tubulin III in

differentiating cells population (Figure 6(c)). The expression levels of neural precursor cells markers such as Nestin and NCAM was found to be enhanced with increasing the time during in vitro culture from approximately 4 fold to 10 fold. Whereas, the expression levels of stromal cell markers (CD90, CD105 and CD73) was reduced showing the commitment of cells towards lineage differentiation. In addition, the hematopoietic markers (CD34 and CD45), osteocytes/Osteoblasts marker (OCN) and dental marker (Dental Sialophospho Protein, DSPP) were also decreased with the time. More interestingly, early/intermediate neuronal cell marker (β-tubulin III), glial marker (Glial Fibrilliary Acidic Protein, GFAP) and oligodendrocyte marker (O4) was significantly enhanced with the time in neural differentiation medium (Figure 6(d)).

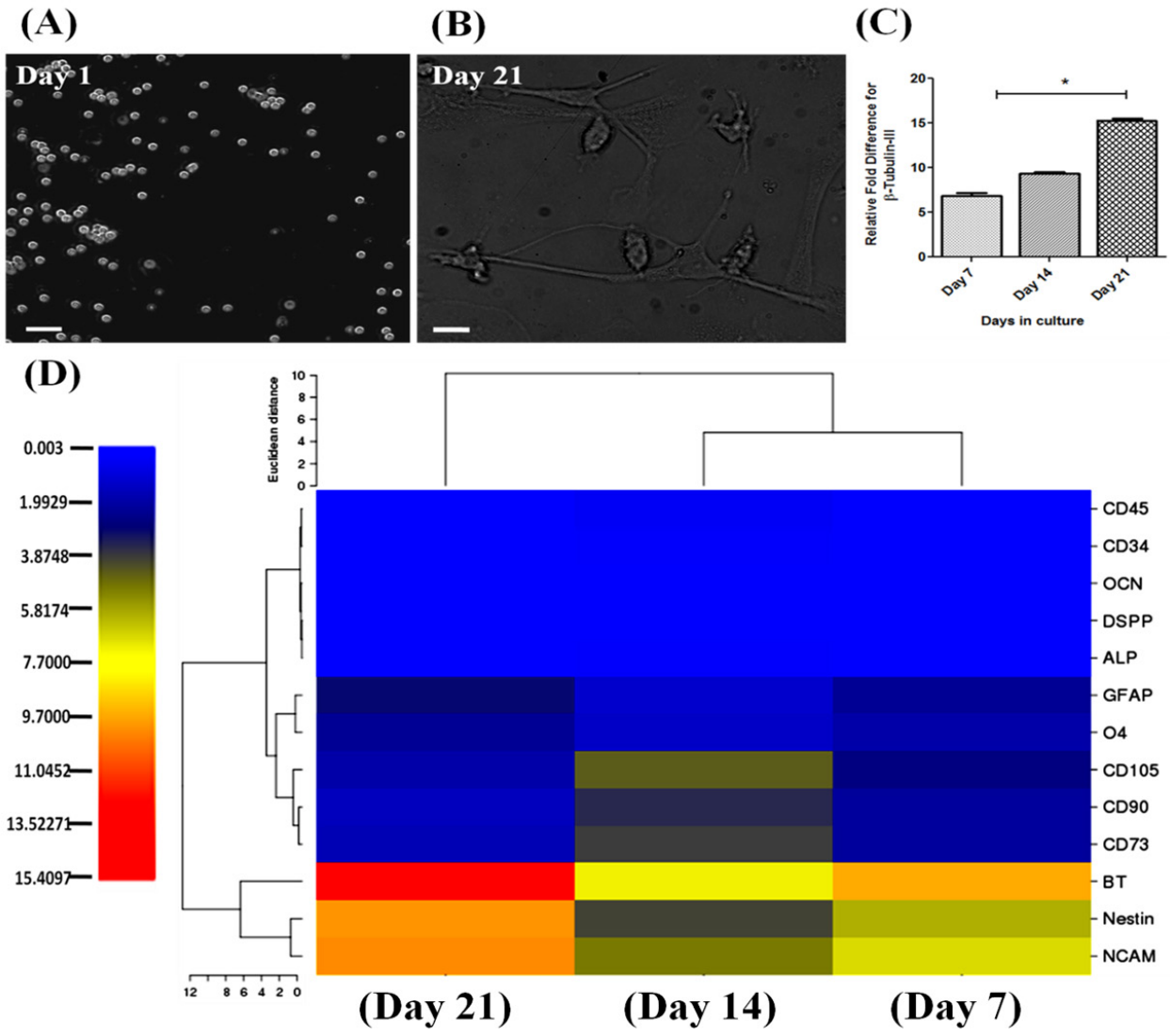

Figure 6. (a) Neurospheres derived cells seeding density and phenotype at day 1 during in vitro differentiation into neural lineage cells. (b) In vitro differentiated neuronal cells showing axons, dendrites and well developed cellular communication. (c) Relative fold values for the expression of β-tubulin III in defined neural differentiation medium at day 7, 14 and 21. (d) Heat map showing variation in relative fold values for the expression of different markers at day 7, 14 and 21. (BT: β-tubulin III, OCN: Osteocalcein, ALP: Alkaline phosphatase, DSPP: Dental siophospho protein, NCAM: Neural cell adhesion molecule).

5. Discussion

Neural tissue regeneration is a major area of interest nowadays due to immense increase in nerve related injuries/damage. There is immense burden on individuals suffering from different kinds of neurological disease which significantly affects their quality of life and presents an inherent difficulty for effective regeneration[10]. Presently, no effective therapeutic strategy exists to treat several critical debilitating neural diseases and poses a great challenge. Hence, there is huge demand for restoration and rehabilitation strategies to effectively support the damaged neuronal tissues. Moreover, elderly individuals having at major risk of different devastating diseases who may require stem cell-based therapies are more prone to experience periodontal problems[11]. Hence, these hopeless teeth might be a tremendous source for isolation of multipotent stem cells further to obtain desired neuronal cells. Dental pulp represents one of the alternatives for production of stem cells which have potential to regenerate neural crest tissues. However, various other sources have also demonstrated the potential towards neural lineage cells. Among other sources, dental pulp represents closely related source to generate desired cells for relevant applications[12]. Because DPSCs are adult pulp stem cells and are easily available, have no ethical or legal complications. They are the ideal candidates for generating large quantities of nerve cells and can be applied for cellular therapy in conditions like spinal injury, stroke, alzheimer's and other neurodegenerative diseases.

In present study, we have explored the presence of multipotent stem cells in dental pulp tissues having highly proliferative capacity and potential source for autologous reparative and reconstructive medicine. The study has also emphasized that these DPSCs capable of differentiating into neurogenic lineages which represents an alternative source for neurological regeneration. Furthermore, we demonstrated that human DPSCs cultures include cells with the label-retaining and sphere-forming abilities; traits attributed to multipotent stem cells, and may provide evidence that these may be multipotent neural crest stem cells (Figure 4 and 5). We observed all basic biological characteristics of in vitro cultured DPSCs during long-term cultivation up to 20 passages including their PDT Figure 3(a) and diameter distribution from 12-18μm Figure 3(b).

The neurospheres forming ability of DPSCs revealed a unique property having potential to produce large number of neurons which can be utilized for various neurological regeneration applications. These neurosphere derived neural crest sort of cells have multi-division potential and can be easily differentiated into neurological lineages for future neural regeneration. We also report that removal of growth mitogenic growth factors and addition of retinoic acid triggers multipotent neural cells obtained from neurospheres tend to differentiate into neural lineage cells as demonstrated in Figure 6. Further characterization of various other markers specific to osteocytes/osteoblasts, dental cells, hematopoietic lineages revealed no such commitment of these cells towards other undesired lineage cells (Figure 6(d)).

In summary, hopeless teeth provide a tremendous source for isolation of multipotent DPSCs further to obtain desired neuronal cells under controlled environment. Dental pulp tissues are easily available, have no ethical or legal complications, and hence might be one of the ideal candidates for generating large quantities of desired neuronal population which can be applied as cellular therapy in various devastating neurological problems.

6. Conclusion

The present study demonstrated that DPSCs could be a potential source which can be differentiated into neurological lineage cells under defined growth factors to enhance the neurological regenerative processes. This study may bridge the gap between the basic and clinical sciences to apply these cells as a possible replacement to regenerate peripheral/central nerve injuries.

Conflict of Interest: The authors declare that they don't have any financial conflict towards the publication of this manuscript.

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