A comparative study on the secretion of various cytokines by pulp stem cells at different passages and their neurogenic potential

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Aims. By measuring the extent of cytokines secreted by human dental pulp stem cells (hDPSCs) from passages 2 through 10, the optimal passage of hDPSCs was determined. This offers a potential theoretical basis for the treatment of neurological disorders.
Method. After isolation and culture of hDPSCs from human teeth, the morphological features of the cells were observed under an inverted microscope. hDPSCs were identified by their immunophenotypes and their multiple differentiation capability. Cytokine concentrations secreted in the supernatants at passages 2-10 were detected by ELISA.
Results. hDPSCs were viewed as fusiform or polygonal in shape, with a bulging cell body, homogenized cytoplasm, and a clear nucleus. Moreover, they could differentiate into neuroblasts in vitro. hDPSCs at passage 3 were positive for CD29 (91.5%), CD73 (94.8%) and CD90 (96.7%), but negative for the hematopoietic markers CD34 (0.13%). ELISA results showed that hDPSCs at passage 3 had the highest secretion levels of vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF), with the highest secretion level of Neurotrophin-3 (NT-3) being at passage 2.
Conclusion. hDPSCs have steady biological features of stem cells and exhibit optimal proliferation potential. hDPSCs at different passages have different capacities in the secretion of VEGF, BDNF, NGF, and NT-3. In conclusion cytokines secreted by hDPSCs may prove to be appropriate in the treatment of neurological diseases.

Key words: dental pulp stem cell, vascular endothelial growth factor, brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3. immunophenotype, multidirectional differentiation, cytokines

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INTRODUCTION

Trauma, iatrogenic injuries, and neurodegenerative diseases can lead to neurite degeneration, synapse loss, or even neuronal loss. This is mainly due to the limited ability of the mature nervous system to differentiate into functional neuronal and glial cells especially after nerve damage[1]. The human body can maintain the genetic stabilization of the genome in many DNA repair pathways under normal physiological conditions. However, there are often cases, where dysregulation of replication and metabolism of DNA may influence cell proliferation, differentiation, migration, and consequently, the formation of synaptic connections. The treatment of irreversible diseases of the nervous system caused by neuronal loss, necrotic and apoptotic cell deaths represents a significant and common clinical burden worldwide. Nevertheless, the development of tissue engineering in recent years has demonstrated that stem cells can serve as a source for the replacement of damaged cells, thereby offering new therapeutic strategies to repair neurological tissue defects[2-4].

Mesenchymal stem cells (MSCs) for instance have a neural differentiation potential and are present in several tissues of mesenchymal origin, such as bone marrow, teeth, and adipose tissue[5-7]. Several articles have reported that Adipose-derived stem cells (ADSCs) tend to secrete glial cell line-derived neurotrophic factor (GDNF), where its overexpression has been used to satisfactorily treat sciatic nerve injuries in rats[8].

Since dental pulp stem cells (DPSCs) originate from neural crest cells, they thereby share with neural cells similar phenotypical characteristics and gene expression patterns[9], Mead et al.[10] found that DPSCs especially, secrete more neurotrophic factors (NTF) than bone marrow-derived mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ADSCs). Where the expression of neurotrophic growth factors (NGF), brain-derived neurotrophic factor (BDNF), and nerve growth factor-3 (NT-3) from DPSCs can be up to 2–3 times higher than the BMSCs. Likewise, neuroprotective effects are also often superior to BMSCs and ADSCs. When compared to mesenchymal elements of the dental papilla, dental follicle,

and periodontal membrane from the same teeth, Ullah et al. found that DPSCs exhibited a more neurotrophic character[11]. Therefore, and due to further advantages, such as their pluripotent differentiation ability, their low risk of rejection, minimal ethical controversies, and being operative friendly, DPSCs are regarded as a promising source of stem cells for the treatment of neurological diseases[12].

Changes in cytokine levels secreted from the pulp stem cells in relation to the cell passage number varies in vitro[13,14]. Therefore, it is of great practical significance to explore the cytokine levels from different passages of dental pulp stem cells to determine the optimal passage of DPSCs that can serve as an initial theoretical basis for the treatment of neurological disorders.

MATERIALS AND METHODS

Wisdom teeth were obtained from the biological waste of teeth extracted in the outpatient clinic of the Department of Oral and Maxillofacial Surgery under approved guidelines set by the Ethics Committee of our hospital. All patients provided their informed written consent. Teeth were anonymized and immersed in Phosphate Buffered Saline (Cat. no 14190094, Gibco) followed by immediate transportation to the laboratory.

Culturing human dental pulp cells

Every tooth used in the experiment was immersed entirely in sterile DPBS containing 500U/ml penicillin streptomycin (cat. no 15140122, ThermoFisher Scientific) and 500 µg/mL amphotericin-8 (cat. no 15290026, ThermoFisher Scientific) for 15 min. Soon after this step and under aseptic conditions, the dental crown was separated from the root, exposing the pulp. To isolate DPSCs, the outgrowth methodology was employed, where the pulp tissue was chopped into small fragments, which were then cultured onto a tissue culture plate. Cells were then observed to slowly grow out of the tissue fragments in the medium (cat. no 31095029, ThermoFisher Scientific) containing 15% heat-inactivated FBS (cat. no 10082147, ThermoFisher Scientific), 1% penicillin-streptomycin (cat. no 15140122, ThermoFisher Scientific), 1% amphotericin-B (cat. no 15290026, ThermoFisher Scientific) at 37 °C with 5% CO_2 and 95% humidity.

The adherent cells were defined as passage zero. When the cells grew to 70% confluency, they were passaged at 1/5 dilution after digestion by 0.05% trypsin-EDTA (cat. no 25300054, Thermo Fisher Scientific) and then passaged for further experimentation. Media was replaced every 3 days until the cells grew to the desired confluency. The images of cell morphology were captured under an inverted microscope.

Pulp stem cells were obtained by the limited dilution method

The cells in suspension were serially diluted to 10 cells/mL with a limiting dilution assay. The diluted cell suspension was distributed at 100 µL per well in a 96-well plate (Corning Life Sciences). Wells containing no cell or more than one single cell were excluded. The wells with a single cell were marked and inspected daily microscopically to count the number of cell clones. DPSCs were then stained with antibodies against STR0-1 (cat. no 398401, ThermoFisher Scientific) with a dilution of 1:500 - a stem cell surface marker. After STR0-1 was incubated with cell samples overnight at 4 °C followed by incubation with the secondary antibody, goat anti-mouse lgG-HRP (cat. no sc2371, Santa Cruz Biotechnology) with a dilution of 1:500 for 1 h at room temperature in the dark. After being rinsed thoroughly with PBS for 5 min three times, the samples were assessed using fluorescence microscopy.

Cell growth curves

Cells were identified according to the number of passages (P), starting with passage 1 (P1). DPSCs that were passaged for 3 generations were made into cell suspensions. Suspensions with a cell concentration of 4×10^3/mL were cultured in 24-well plates for 8 days. Cells from 3 wells were counted using MTT colorimetric analysis in each group every day. Numerical values were obtained by UV spectrophotometry at 490 nm using a Nanodrop spectrophotometer (NanoDrop™ 2000 or 2000c Spectrophotometer, ThermoFisher).

Immunophenotypic analysis was performed using flow cytometry

By passaging, cells were harvested using trypsin at 0.25%. Cell suspensions of 2×10^6 cells at P3 were collected and washed twice with PBS. Surface markers of 3rd generation cells were identified by flow cytometry using antibodies against CD29 (cat. no. 11-0299-42; Thermo Fisher Scientific), CD73 (cat. no. ab239246; Abcam), CD90 (cat. no. ab139364; Abcam), CD34 (cat. no. 560941; BD Biosciences). All antibodies were used at a dilution of 1:500. The antibodies were gently mixed and incubated for 30 min at 4 °C, then identified through surface antibody: antigen labeling by the use of flow cytometry (BD).

Neural differentiation

DPSCs at a density of 2×10^4 cells/well were seeded into 24-well plates and cultured in a standard culture medium. Upon confluency, neural differentiation was started by exchanging the standard medium for neural induction medium [Neurobasal Medium (cat. no. 21103049, Gibco); fortified by the following ingredients: 1x8-27™ Supplement (50x: cat. no. 17504044, Gibco); 2 nM human heregulin (rHRG-$\beta1_{1177-244}$; provided by Dr. Steven Carrol & Dr. Jody Longo, University of South California); 0.5 nM 3-lsobutyl-1-methylxanthine (IBMX) (cat. no.17018, Sigma-Aldrich); 5 µM Forskolin (cat. no. F6886, Sigma-Aldrich); 10 ng/mL basic fibroblast growth factor (bFGF) (Cat No. F0291; Sigma Aldrich). The media was changed every 3 days and the neural differentiation period was continued for 21 days.

To stain the neural differentiation results, β-tubulin and MAP-2 were employed. First, the induction media was removed and cells were washed with PBS. Cells were

then fixed in 4% paraformaldehyde (Cat No. 30525894, Santa Cruz Biotechnology) (pH7.4) at room temperature for 10 min, followed by a triple rinse using ice-cold PBS. The fixation procedure was then completed by adding 60% isopropanol (Cat No. 19516, Merck) for 5 min. Subsequently, the cells were permeabilized by incubating them in 0.15% Triton X-100 (Cat No. X100, Sigma-Aldrich) for 10 minutes, and then washed 3 times with PBS for a duration of 5 min each time. Specimens were then incubated for 30 min with serum blocking solution, which is composed of PBS containing 1% bovine serum albumin (BSA) (Cat No. A7906, Sigma- Aldrich), 22.52 mg/mL glycine (Cat No. G8898, Sigma-Aldrich) and 0.1% Tween 20 (Cat No. P1379, Sigma-Aldrich), to suppress the nonspecific binding of the antibodies. Afterward, cells were labeled with primary antibodies overnight at 4 °C: β-tubulin (Cat No. H0417, Santa Cruz Biotechnology) and MAP-2 (Cat No. 3173528, Merck) at a dilution of 1:500. For immunofluorescence staining, cells were stained with the following fluorochrome-conjugated secondary antibodies, Alexa Fluor 488 goat anti-mouse (cat. no A11029, ThermoFisher Scientific) and Alexa Fluor 633 goat anti-mouse (cat. no A21050, ThermoFisher Scientific) respectively, both of which were diluted to 2 µg/mL in PBS with 1.5% normal blocking serum for 1 h at room temperature in the dark. The nuclei were then stained using 4'-6-diamidino-2-phenylindole (DAPI) (cat. no D9542, Sigma-Aldrich). Following triple PBS washes for 5 min each time in the dark, coverslips were mounted on glass slides with a permanent mounting solution (cat. no S3023, Dako). Finally, stained cells were examined under a fluorescence microscope.

ELISA analysis of conditioned media (passages 2-10)

The DPSCs were seeded into T25 culture flasks at a cell density of 1×10^6 and were cultured in DMEM with 10% FBS until 70–80% confluency is reached. The culture medium was then discarded, and the cells were washed with PBS 2 times, followed by the addition of 1.6 mL fresh media to the cells for a further 24-hour incubation period. Conditioned media and cell lysates were then collected. The levels of VEGF, BDNF, GDNF, and NT-3 were determined using enzyme-linked immunosorbent assay (ELISA) kits according to the protocols of the manufacturer (BDNF and NGF from Promega; VEGF and NT-3 from R&D Systems).

To test BDNF and NT-3 levels: 50 µL of standards and samples were added to the respective coated reaction cups and incubated at room temperature for 2 h. For BDNF, the binding solution was added without washing the plate, whereas, with NT-3, binding solution was only added after the plates were washed. Plates were then incubated at room temperature for 1 h and 1.75 h respectively. After washing the plates, respective substrates were added at room temperature for 30 min, followed by termination solution and absorbance measurement at 450 nm within 30 min.

To test VEGF and NGF levels: 200 µL of standards and samples were added to the coated reaction cup and incubated at room temperature for 2 h. After washing the plates, VEGF and NGF binding solutions were added and incubated at room temperature for 2 and 1.75 h respectively. After washing the plates, respective substrates were added and incubated at room temperature for 30 min, then the termination solution was added and absorbance was measured at 450 nm within 30 min.

Statistical analysis

Statistical analysis was performed by a statistical package and expressed as mean±standard deviation (SD). The distinction of the proportion of cytokines was processed via the student's t-test for statistical calculation. $P<0.05$ was considered statistically significant. Graphs were prepared with Prism version 8.0 (Graph Pad Software).

RESULTS

Morphological characterization of DPSCs

After 5 days of primary culture, a small number of elongated spindle-shaped cells surrounding and growing out of the tissue fragments were observed. Eight days later, cells fused together and exhibited characteristic polygonal and spindle-shaped morphology. They were seen to have clear nuclei with a huge chromatin core, together with a granular aggregation in the cytoplasm.

After cells were selected via limited dilution in 96-well plates, monoclonal cells were seen to significantly grow and appeared as elongated spindle-shaped cells with a swirling growth pattern.

Proliferation of hDPSCs

From P2-P10, cells maintained an optimal growth curve (see Fig. 1). The proliferative capacity of hDPSCs peaked at P4 ($P<0.05$).

Identification of hDPSCs

Cell surface markers were detected using flow cytometry to determine the mesenchymal origin of the hDPSCs. The following markers were positively expressed at respective percentages; CD29 at 91.5%, CD73 at 94.8%, CD90 at 96.7%, whereas the hemopoetic stem cell marker,

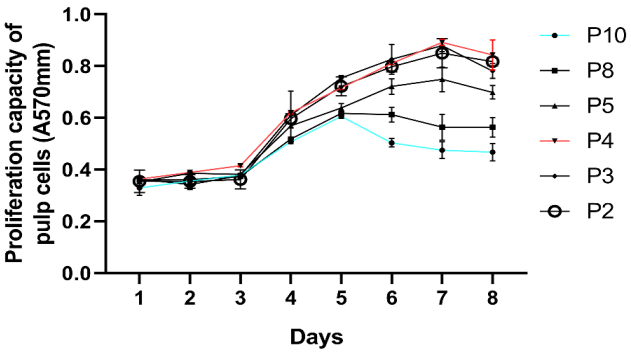

Fig. 1. Proliferation curves of human dental pulp stem cells at different passages.

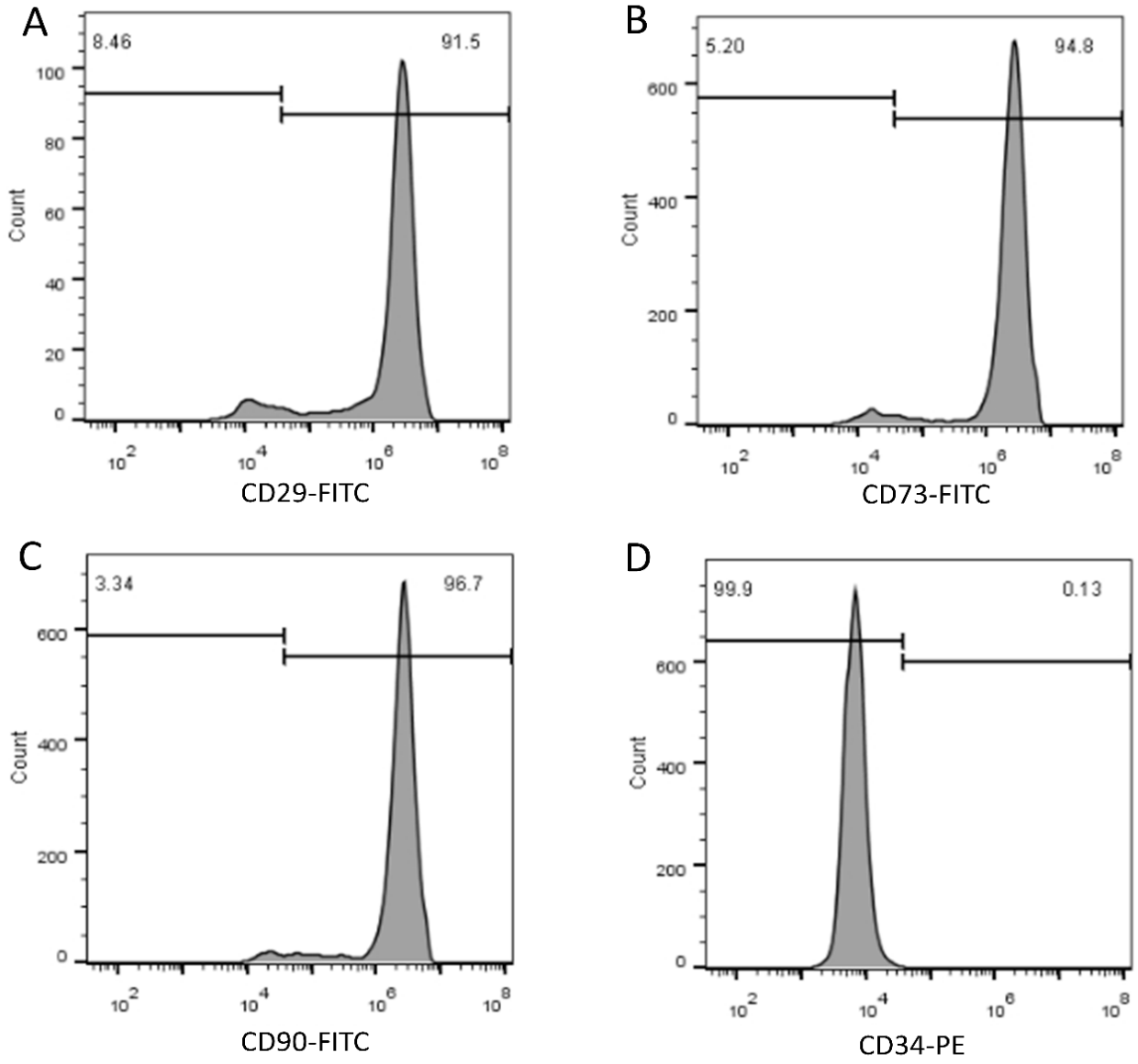

Fig. 2. Flow cytometry detection of passage 3 dental pulp stem cells.
Cells expressed mesenchymal surface markers CD29 (91.5%, A), CD73 (94.8%, B), and CD90 (96.7%, C), while hardly expressing the hematopoietic stem cell surface marker CD34 (0.13%, D).

CD34 was minimally expressed at a percentage of 0.13% (see Fig. 2).

Most hDPSCs were positive for the cell surface marker STRO-1 (in Fig. 3).

Cellular identification based on differentiation potential

Before neural differentiation induction, hDPSCs displayed a fibroblast-like appearance with an elongated and flattened body. Along the induction process, differentiating hDPSCs started to change their morphology towards neuron-like cells with multiple extensions. At the end of the differentiation period of 21 days, the expression of β-tubulin (neuronal-specific tubulin) and MAP-2 (mature neurocyte) was detected (in Fig. 3). Lack of neuronal staining in some cells with nuclei stained with DAPI denoted the specificity of the immunofluorescence staining.

Changes of hDPSCs secretion of cytokines through passages 2-10

ELISA showed that the peak secretion of VEGF, BDNF, NGF at P3 of hDPSCs. Significant differences between the VEGF, NGF, and BDNF groups (P<0.05) (see Fig. 4B,D,F). hDPSCs secretion of NT-3 peaked at P2 (P<0.05) (see Fig. 4H).

DISCUSSION

The central and peripheral nervous systems govern all activities of the body and perform important physiological functions. While it is established that damage to central nerves is irreversible, peripheral nerves do possess repair functions, their role is however limited, and therefore, severe nerve damage ultimately leads to irreversible

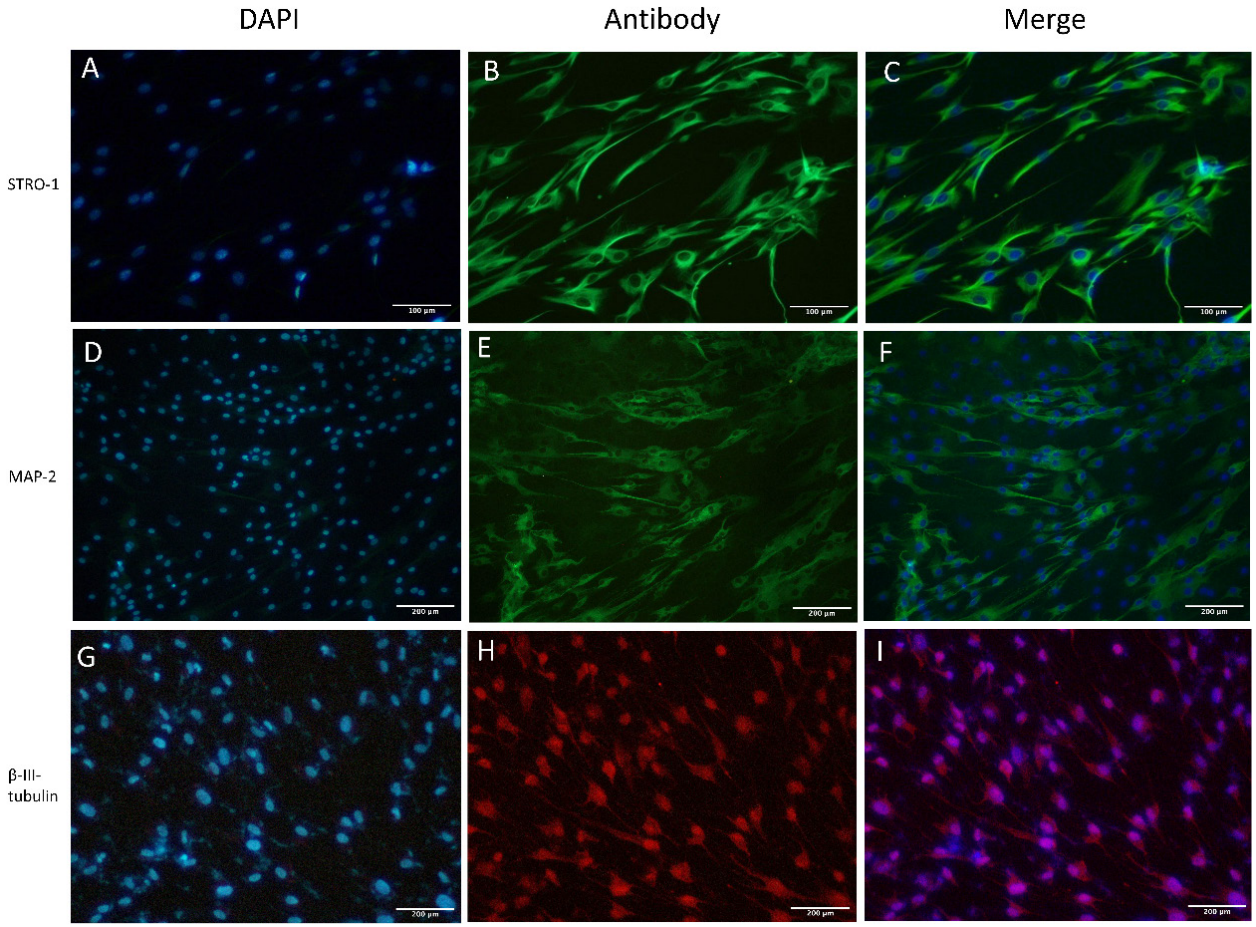

Fig. 3. Induced neural differentiation of passage 3 dental pulp stem cells *in vitro.*
Nuclei of the cells were stained with DAPI (blue) shown in Fig. (A, D, G). STRO-1 was marked by immunofluorescence (green) shown in Fig. B. hDPSCs were stained with both DAPI and STRO-1. in Fig. C. Expression of MAP-2 stained in green shown in Fig. E. Expression of β-III-tubulin stained in red shown in Fig. H.

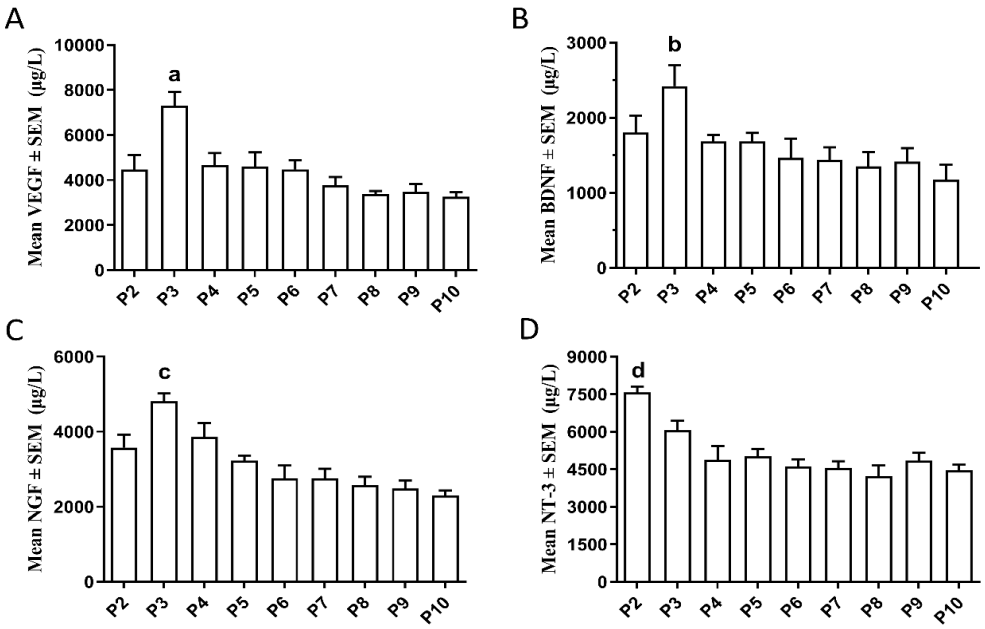

Fig. 4. Secretion of vascular endothelial growth factor, brain-derived nerve growth factor, nerve growth factor, and neurotrophin-3 from human dental pulp stem cells at different passages.
A, B, C, and D are the secretion levels of VEGF, BDNF, NGF, and NT-3 from P2-P10 DPSCs Compared with other passages [a]$P<0.05$; [b]$P<0.05$; [c]$P<0.05$.

dysfunction. Stem cells are characterized by self-renewal and multi-directional differentiation ability, and in recent years a variety of stem cells have been confirmed to have the potential to differentiate into neuronal cells, amongst which are the hDPSCs. hDPSCs possess a potent differentiation ability, as well as favorable paracrine effects, rendering them a therapeutic means for the treatment of nerve injuries.

Furthermore, hDPSCs not only express conventional neuronal markers such as nestin, glial fibrillary acidic protein, and neuron-specific nuclear protein, but they also express further markers such as nucleolin, glial fibrillary acidic protein, neuron-specific nuclear protein (NeuN), and S-100. Moreover, they secrete a variety of neurotrophic factors, such as BDNF, GDNF, and other NGFs which play important roles in neuroprosthetics[15]. BDNF and GDNF act directly on dopaminergic neurons and have strong nutritional repair effects that promote recovery of dopaminergic neurons after injury. These neurotrophic factors affect themselves and the surrounding microenvironment through autocrine and paracrine effects, creating a microenvironment conducive to cellular growth and neuronal differentiation.

It has been shown that the biological properties of MSCs up to 10 passages are stable. Yu Jinhua et al. showed that Stro1+-labeled hDPSCs obtained by immune magnetic bead sorting were highly expressed at P9 compared with P1. Nevertheless, hDPSCs at the P1 were observed to highly express the following dentin-specific genes; dentin sialophosphoprotein (DSPP), dentin sialoprotein (DSP) in addition to osteogenic-specific genes such as alkaline phosphatase (ALP) and osteopontin (OPN), suggesting that P1 hDPSCs are potent in terms of multipotential differentiation that is directed toward dentin, bone tissue and cartilage formation, whereas hDPSCs at P9, i.e. at a more advanced passage are particularly more inclined towards osteogenic differentiation. The aforementioned data denotes that the advancement of in vitro passages has a directional effect on the differentiation potential of hDPSCs.

Moreover, the results of ELISA showed that hDPSCs secreted the highest level of VEGF at P3, peaking up to 7300 μg/L. VEGF is an important regulator of physiological and/or pathological vasculogenesis and angiogenesis, and plays an important regulatory role in the proliferation, survival, migration and infiltration of vascular endothelial cells. Since hDPSCs can also promote vascular regeneration by expressing VEGF, thereby further promoting and contributing to axonal growth and Schwann cell proliferation. More specifically, VEGF-A is one of the key factors in angiogenesis, and in a study where differentiated hDPSCs were implanted into a rat model with a 15 mm sciatic nerve defect, VEGF-A was found to be responsible for enhancing angiogenesis and promoting the inward growth of the initial nerve projections[16].

Moving on to the neurogenic paracrine effect, ELISA results showed that the level BDNF secreted by the 3rd passage of hDPSCs was 2421 μg/L. BDNF is physiologically widely distributed in the central nervous system (CNS) and plays an important role in the survival, differentiation, growth and development of neurons during the development of the CNS, while also preventing neuronal death and contributing to the reversal of the pathological state of neurons. BDNF is a neurotrophic factor that promotes biological effects such as regeneration and differentiation of injured neurons, and is also necessary for the survival and normal physiological functions of mature neurons in the central and peripheral nervous system. Consequently, changes in the expression level of BDNFs are closely related to various neurological diseases, such as Depression, Epilepsy, Alzheimer's, Parkinson's and Huntington's disease[17].

The ELISA results showed that the 3rd passage of hDPSCs secreted the highest level of NT-3, which reached 7571 μg/L. NT-3 were observed to significantly increase the number of neuritis βIII-tubulin+ retinal cells and the length of neural dendrites in co-cultures in vitro. It was also observed, that retinal cell activity decreased when treated with NT-3 receptor inhibitors, demonstrating the protective effect of NT-3 on RGCs (ref.[10]). Moreover, retinal ganglion cells (RGC) are known to express a large number of neurotrophic factors, including NGF, BDNF, NT-3 and GDNF, which can bind to the RGCs receptors and thereby enhance not only the survival of RGCs, but also axonal regeneration[18]. hDPSCs may also be used as a resource in treating retinal injury because the paracrine effect of hDPSCs entails the relevant expression of the aforementioned neurotrophic factors.

ELISA results also showed that the 3rd passage of DPCSs secreted the highest level of NGF which reached 4814 μg/L. Moreover, NGF is one of the earliest discovered factors of the neurotrophin family and are known to maintain the survival of sensory neurons, promote the growth of nerve fibers, as well as increase the expression of sensory neuropeptide genes. In the retina, NGF is produced and utilized by the retinal ganglion cells, bipolar neurons, and glial cells in a local paracrine or autocrine manner. Furthermore, Colafrancesco et al.[19] found that NGF protects the retina by reducing ganglion cell apoptosis. During the development of the visual system, NGF and its receptor are both highly expressed in visual centers and can influence neuronal proliferation, survival, and selective apoptosis. Moreover, NGF activates signaling pathways by binding to tropomyosine receptor kinase (TrkA) receptors to maintain neuronal survival and differentiation. In a guinea pig model of Form-Deprivation Myopia (FDM), Davis et al.[20] used immunohistochemistry and fluorescence quantitative PCR to detect the protein and gene levels of NGF and TrkA, respectively. Results showed that the expression of NGF and TrkA proteins and nucleic acids decreased in the retina with the prolongation of shape perception deprivation time. Information regulation in retinal cells promoted differentiation to a functional retinal developmental stage. Moreover, Spalding et al[21] found that NGF and TrkA were expressed in both neuronal and non-neuronal cells in the retina of a guinea pig model of FDM. The experiments only discussed the secretion level of cytokines from the 2nd through the 10th passage of hDPSCs. Therefore, the secretion level of cytokines of subsequent passages still

needs to be clarified; and the clinical applications of hDPSCs also need further investigation in terms of indications and contraindications. In addition, further research on the exact mechanism of paracrine secretion of hDPSCs and the factors influencing paracrine function, as well as how to use this potential function of stem cells in a reasonable and effective manner, are issues that need to be resolved.

CONCLUSION

In summary, our study discussed the biological characteristics of hDPSCs and the cell passages optimal for cytokines secretion, all of which provided new ideas for the clinical translation of cellular therapy.

ABBREVIATIONS

hDPSCs: Human dental pulp stem cells; VEGF: Vascular endothelial growth factor; BDNF: Brain derived neurotrophic factor; NGF: Nerve growth factor; NT-3: Neurotrophin-3.

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Author contributions: MY: conceived the study, supervised the experiments and drafted themanuscript; ON: conceived the study, supervised the experiments and drafted the manuscript; LF: data evaluation, manuscript preparation; LC, DL: data evaluation, manuscript preparation; MG, RF: analyzed the data and revised the manuscript; RS: performed the data collection; HF: conceived the study, designed the data evaluation, manuscript preparation.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

Ethics approval and consent to participate

Administrative permissions were acquired by our team to access the data used in our research. The study protocol was approved by the Guiyang Stomatological Hospital that approved the study. Accordingly, all teeth were coded with number and all personal identification of the patients were removed. All parent or guardian of participants provided written informed consent for using their teeth which otherwise would have been discarded as waste.

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