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Differentiation capacity of dental pulp stem cell into inner ear hair cell using an in vitro assay: a preliminary step toward treating sensorineural hearing loss

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Abstract

Purpose Sensorineural hearing loss (SNHL) is commonly caused by the death or dysfunction of cochlear cell types as a result of their lack of regenerative capacity. However, regenerative medicine, such as stem cell therapy, has become a promising tool to cure many diseases, including hearing loss. In this study, we determined whether DPSCs could differentiate into cochlear hair cell in vitro.

Methods DPSCs derived from human third molar dental pulp were induced into NSCs using a medium containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) for 7 days, and then into cochlear hair cell using a medium containing EGF and IGF-1 for the next 14 days. We used the neuroepithelial protein marker nestin and cochlear hair cell marker myosin VIIa as the markers for cells differentiation. Cells expressing the positive markers under the microscope were confirmed to have differentiated into cochlear hair cell.

Results DPSCs were successfully induced to differentiate into NSCs, with mean 24% nestin-positive cells. We found that DPSC-derived NSCs have a great capacity in differentiating into inner ear hair cell-like cells with an average of 81% cells presenting myosin VIIa. Thus, DPSCs have high potential to serve as a good resource for SNHL treatment.

Conclusion We found the high potential of DPSCs to differentiate into NSC. The ability of DPSCs in differentiating into neural lineage cell made them a good candidate for regenerative therapy in neural diseases, such as SNHL

Keywords Dental pulp stem cells · Sensorineural hearing loss · Cochlear hair cell

Introduction

In mammals, the production of cochlear hair cells mostly occurs during embryogenesis, and the cells mature soon after birth. Unlike what happens in nonmammals, the cochlear hair cells of mammals cannot regenerate postnatally, and if a disruption occurs after birth, then permanent hearing loss called sensory neural hearing loss (SNHL) may occur. Other pathologies resulting in SNHL involve auditory nerve degeneration of the cochlear sensory epithelium. Cochlear implants are the only clinical treatment for people with profound SNHL. These devices deliver auditory signals through

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surviving spiral ganglion nerves (SGNs); thus, the number of SGNs is essential for the efficacy of implants [1, 2].

Regenerative medicine, such as stem cell therapy, has become a promising field for curing many diseases, including hearing loss. Several studies have reported on the cochlear transplantation of various stem cell sources, such as embryonic stem cells, mesenchymal stem cells and neural stem cells, which can survive and replace damaged hair cells and SGNs. The capability of stem cells to self-renew and differentiate into various cell types, including cochlear hair cells and SGNs, may play a key role in developing therapeutic strategies for SNHL [3–5]. Numerous efforts have been made in recent years to generate cochlear hair cells from various sources of stem cells in vitro to provide insights into the factors that contribute to their differentiation ability [6, 7]. Among these studies, Qin et al. and Ying et al. applied different sources of mesenchymal stem cells, bone marrow and adipose tissue and achieved satisfactory results. Others have examined the differentiation potential of embryonic stem cells, olfactory precursor cells and neural stem cells (NSCs) to generate inner ear hair cells [8, 9].

Studies conducted in regenerative medicine for cochlear damage mostly use neural lineage potential stem cells such as mesenchymal stem cells, because inner ear hair cells were derived from neurosensory progenitors. The ability of NSCs to differentiate into functional cells in the cochlea has been widely reported. NSCs have the ability to differentiate into SGNs, hair cells, and other neuronal type cells in the cochlea; thus, NSCs could replace damaged cells to reverse SNHL [4]. Among other neural lineage potential stem cells, dental pulp stem cells (DPSCs) have the greatest potential to differentiate into a large array of tissues, including NSCs, due to their close embryonic origin. Moreover, the extraction method of DPSCs is less invasive [5, 7, 10]. DPSCs are identified as MSCs with the same differentiation ability, and they present MSC surface markers, such as CD105, CD 73 and CD90 and lack expression for negative marker such as CD34, CD45 and HLA-DR [11, 12].

The potential of DPSCs has begun to attract the attention of researchers since the use of these cells was first reported by Gronthos et al. [13]. To date, DPSCs have been reported to differentiate into various cell lines, such as odontoblasts, osteoblasts, adipocytes, smooth and striated muscle cells, cartilage cells, endothelial cells, and nerve cells, either in vitro or in vivo. Thus, DPSCs may represent a promising source for stem cell therapy in neurological disorders due to their neural characteristics and ease of extraction. In a study conducted by Karaoz et al. [14], DPSCs showed a better ability to differentiate into nerve and epithelial cells than bone marrow mesenchymal stem cells (BMMSCs); however, the ability of DPSCs to differentiate into specialized cell type, such as cochlear hair cells, was not investigated. Regarding this matter, we sought to determine whether DPSCs can be induced to transform into cochlear hair cells, one of the sensory epithelial cells in the cochlea. In this study, we evaluated the neural differentiation potential of DPSCs using basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) for 7 days, followed by induction to cochlear hair cells. The expression of neuron cell-specific cell markers in DPSCs cultured in neuronal induction media and cochlear hair cell-specific markers after hair cell induction was assessed by immunocytochemistry. The morphological changes were also observed.

Materials and methods

DPSCs were obtained from extracted partially impacted third molars and transported in microcentrifuge tubes with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (FBS) (Gibco). The experiment was approved by the Health Research Ethical Committee of Medical Faculty of Universitas Sumatera Utara, Indonesia (no: 310/TGL/KEPK FK USU-RSUP HAM/2019).

Isolated cells should present MSC markers to demonstrate that they are DPSCs. We tested the expression of some specific cell surface markers and the pluripotent differentiation potential of the cells. The isolated cells were analyzed by flow cytometry. The markers evaluated in this study using monoclonal antibodies against CD90 (FITC), CD105 (PerCP-Cy5-5), CD73 (APC, all from BD Biosciences, USA). We also examined CD45/CD34/CD11b/CD19/HLA-DR negative Lin antibodies (PE, BD Biosciences, USA).

NSC induction and in vitro differentiation

During NSC induction, passage 3 MSCs were trypsinized, and then approximately 2×10^4 cells were transferred to 24-well plates. The cells were incubated overnight in MEM with 10% FBS at 37 °C in a 5% CO_2 atmosphere. Half of the medium was changed after 24 h. When cells became confluent, the medium was changed to 1:1 DMEM/F12 media containing N2/B27 (Gibco, Carlsbad, CA, USA), 20 ng/mL EGF, and 10 ng/mL bFGF (PeproTech, Inc., Rocky Hill, NJ, USA) for 7 days. After 7 days, the medium was discarded and the cells were washed using phosphate-buffered saline (PBS). Then, the cells were subjected to an immunocytochemical assay for characteristics specific for neural progenitors using anti-nestin antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The induced cells were examined, and the cells expressing nestin were counted [6].

Cochlear hair cell induction

To induce DPSC-derived NSCs into inner ear hair cells, NSCs obtained from the neural induction of DPSCs for 7 days were cultured on DMEM/F12 1:1 media containing supplements of N2/B27, 20 ng/mL EGF, and 50 ng/mL IGF-1 for 2 weeks. After 2 weeks of induction, the cells underwent immunocytochemical examination for characteristics specific to hair cells using anti-myosin VIIa antibodies. Cells expressing myosin VIIa were counted.

Control group I

For the control group, DPSCs were cultured in conventional culture conditions containing DMEM and 10% FBS for 7 days and subjected to immunocytochemical examinations using anti-nestin antibodies. Cells expressing nestin were counted.

Control group II

For the control group, DPSC-derived NSCs were cultured in conventional culture conditions containing DMEM and 10% FBS for 2 weeks and were subjected to immunocytochemical examinations using anti-myosin VIIa antibodies. Cells expressing myosin VIIa were counted.

Immunohistochemistry

In the early stages of immunocytochemical examination, the cells must be fixed and stabilized. The culture media were drained and removed. Then, the cells were gently washed with PBS, added to 1 mL of 4% formaldehyde in PBS, and incubated for 15 min. The fixation fluid was discarded, and the cells were washed again using PBS. The fixation and stabilization procedure was the same for nestin and myosin VIIa examination.

For nestin examination, the cells were incubated overnight at 4 °C with anti-nestin antibody at 1:100 dilutions. After washing with PBS 3 times, anti-mouse IgG HRP-linked secondary antibody was used for 2 h. Then, the cells bound with the antibody were visualized using a DAB substrate kit (Cambridge, MA, USA) for 15 min, documented, and counted using a microscope.

For myosin VIIa examination, the cells were incubated overnight at 4 °C with anti-myosin VIIa antibody at a 1:50 dilution. Then, the cells were washed 3 times using PBS, followed by the administration of anti-mouse IgG HRP-linked secondary antibody for 2 h. Then, the cells were visualized using a DAB substrate kit for 15 min, documented, and counted using a microscope.

Statistical analysis

All results are reported in terms of the mean ± standard deviation of the percentage of positive cells for specific markers. Statistical analyses were performed with the Wilcoxon test, and the two experimental groups were compared using SPSS (Statistical Package for the Social Science).

Results

Biological properties of DPSCs

DPSCs were obtained from extracted partially impacted third molars and transported to the laboratory in microcentrifuge tubes with complete medium. The tissue was cultured for 21 days, and the media were changed every 3–4 days. CD90, CD105, and CD 73 were highly expressed based on a RT-PCR analysis (> 98%). In contrast, negative lineage marker (CD45, CD34, CD11b, CD19, and HLA-DR) were minimally expressed (0.4%) (Fig. 1).

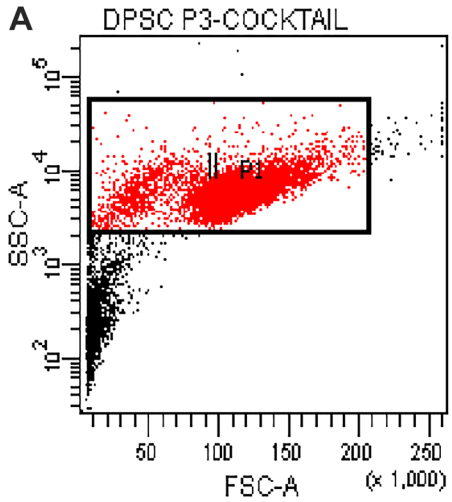
Potential of DPSCs to differentiate into NSCs

In this study, DPSCs that were not induced by NSCs could not display features showing appearance of nestin (an NSC marker) (Fig. 2b); whereas after the induction of NSCs, the mean nestin percentage was as high as 24% ± 7.80% (Fig. 2a).

In this study, the Wilcoxon test was performed to analyze the difference in nestin expression, and nestin expression was significantly increased in DPSCs induced by EGF and bFGF compared to DPSCs that were not induced ($p = 0.028$). This finding suggests that DPSCs have the potential to differentiate into NSCs (Table 1). We also observed that the morphology of DPSCs changed from a spindle-shaped appearance (Fig. 2b) to a neuron-like shape after 7 days of neuronal induction (Fig. 2a). This result shows that induction using EGF and bFGF successfully differentiated DPSCs into NSCs.

The Wilcoxon rank sum test showed that the expression of myosin in cochlear hair cells was significantly different before and after induction (Table 2; $p = 0.028$). After cochlear hair cell induction, morphological changes were observed consisting of neuron-like turning hemispherical-shaped, which showed the induction process to form cochlear hair cells (Fig. 2c).

DPSC-derived NSCs have the potential to differentiate into cochlear hair cell-like cells In this study, there were no stem cells presenting myosin VIIa (a cochlear hair cell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P1
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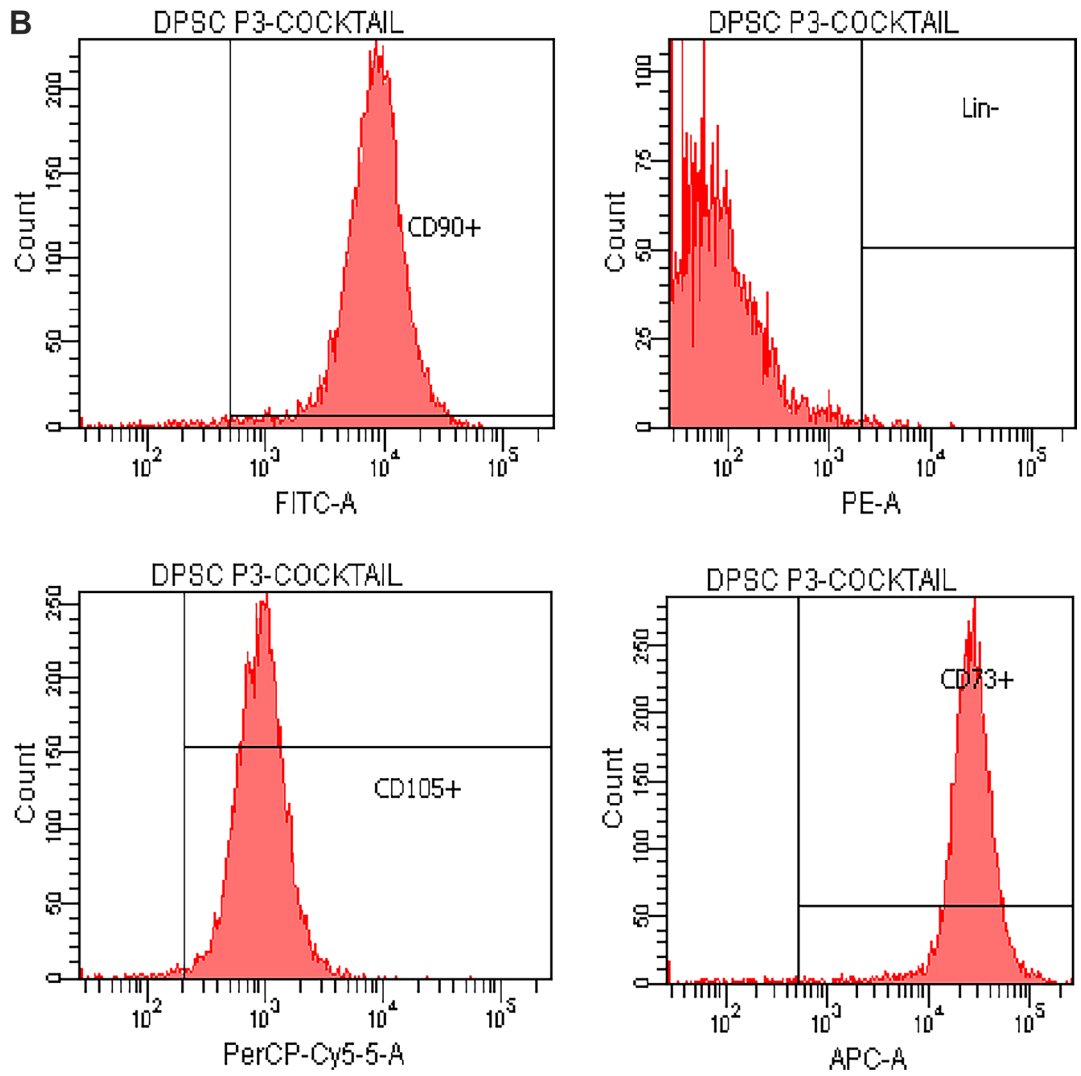
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CD90+
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Lin-
Count
PE-A
DPSC P3-COCKTAIL
CD105+
Count
PerCP-Cy5-5-A
DPSC P3-COCKTAIL
CD73+
Count
APC-A

◂**Fig. 1** MSC characteristics of DPSCs analyzed using flow cytometry. **a** The initial analysis region used forward and side scatter to determine the population of interest. **b** Flow cytometric analysis of cultured stem cells with monoclonal antibodies, indicated the high presentation of CD90 (FITC), CD105 (PerCP-Cy5-5) and CD73 (APC) whilst lacking of negative Lin antibodies (PE)

marker) in DPSC-derived NSCs that were cultured in conventional media for 2 weeks (Fig. 2d); whereas after cochlear hair cell induction for the same period, the mean percentage of myosin VIIa was 81% ± 8.65 (Fig. 2c).

Discussion

This study was the first to demonstrate the potential of DPSCs to differentiate into inner ear hair cells. A previous study found that DPSCs have great potential to differentiate into NSCs, which prompted us to investigate the potential of DPSCs to differentiate into inner ear hair cells [14]. Arthur et al. [15] also found a positive marker of nestin (BIII tubulin) and mature nerves (NF-M and NF-H) in DPSCs that were exposed to neural induction media for 3 weeks. This result shows the potential of DPSCs as a therapy for diseases caused by nerve cell damage in the human body. Zhang et al. [16] evaluated the effectiveness of DPSC therapy in mice suffering from focal cerebral ischemia and showed satisfactory results, and they reported the transdifferentiation of DPSCs into neuron-like cells and cell migration toward ischemic areas. They also found that DPSC injection can reduce the infarct size and edema in the brain. Compared to other stem cells, DPSCs have a higher potential in forming the neurosphere and in differentiating into nerve cells, which may be related to the embryonic origin of the DPSCs from the neural crest.

NSCs have high pluripotent ability. Nestin is downregulated and replaced by a protein specific for neuron cells upon differentiation as shown in studies on nestin knockout rats, which found a decrease in the number of viable and regenerating NSCs. These NSCs can later proliferate or differentiate into mature neuron cells or other cell types according to signals from the surrounding environment [17, 18]. In a study conducted by Ito et al. [19], NSCs transplanted into the cochlea of mice survived within 2–4 weeks. The study showed that some transplanted cells can adapt morphologically and replace the position of damaged hair cells. This finding suggests that NSCs can adapt to cochlear conditions and provide new hope for the treatment of cochlear damage causing hearing loss. In this study, the induction of NSCs was performed using a combination of growth factors EGF and bFGF, which is consistent with previous studies. EGF and bFGF are able to induce bone marrow-derived mesenchymal stem cells to differentiate into NSCs or neural cell progenitors [6, 20–22]. Pacey et al. [2006] published the optimal protocol required for performing NSC culture and proliferation. Stem cells are known to proliferate when exposed to certain growth factors. In this protocol, EGF and FGF are the best growth factors that can be used to culture NSCs. The optimal time period used for culture is 7 days because the biological activity of growth factors can decrease over time.

In this study, a significant difference in cells presenting myosin VIIa was found between NSCs that were induced with EGF and IGF-1 for 14 days and those that were not ($p = 0.028$). This finding shows that the growth factors EGF and IGF-1 successfully induced NSCs to differentiate into cochlear hair cell-like cells by presenting the myosin VIIa marker. Interestingly, we found that the mean percentage of cells presenting the myosin VIIa marker was high (81%). This result shows that DPSCs could be a better source of stem cells to repair the cochlea and treat hearing loss. This study is similar to that of Qin et al. [6], who successfully induced BMMSCs into NSCs and then into cochlear hair cells using media containing EGF and IGF-1. Apart from presenting the marker for myosin VIIa, this study also assessed their cell morphology and found cell growth resembling cochlear hair cells. A study from Li et al. [8] also found that media containing EGF/IGF-1 and a combination with bFGF could induce embryo stem cells (ESCs) into cochlear hair cells.

In previous studies, ESCs were shown to have a high survival rate and migrate well when implanted into the cochlea. However, mixed results have been obtained on the proper functional outcome. The possibility of tumor formation, rejection reactions or infection is higher because animal embryos are usually used. The clinical applications are also quite controversial because of the ethical problems linked to ESCs. The administration of mesenchymal stem cells from humans can minimize the reaction of graft–host disease. Mesenchymal stem cells from bone marrow also have a fairly good survival rate and can migrate to the desired sites [23].

The main source of mesenchymal cells is the bone marrow. However, the process of extracting the patient's bone marrow is painful and invasive. MSCs can also be found in many other cell populations, such as adipose tissue, skin, cartilage, and dental pulp. DPSCs present CD105, CD73 and CD90, thus confirming their mesenchymal phenotypes, and they can differentiate with the help of growth factors, transcription factors, extracellular proteins and receptor molecules into other cells, such as nerve cells, cardiomyocytes, corneal epithelial cells and beta cells. In a recent study, DPSCs were found to be an alternative to BMMSCs, which have great potential in cardiac repair. Cardiac repair performed from the BMMSC transplantation process was found to be similar to DPSC transplantation [11, 24].

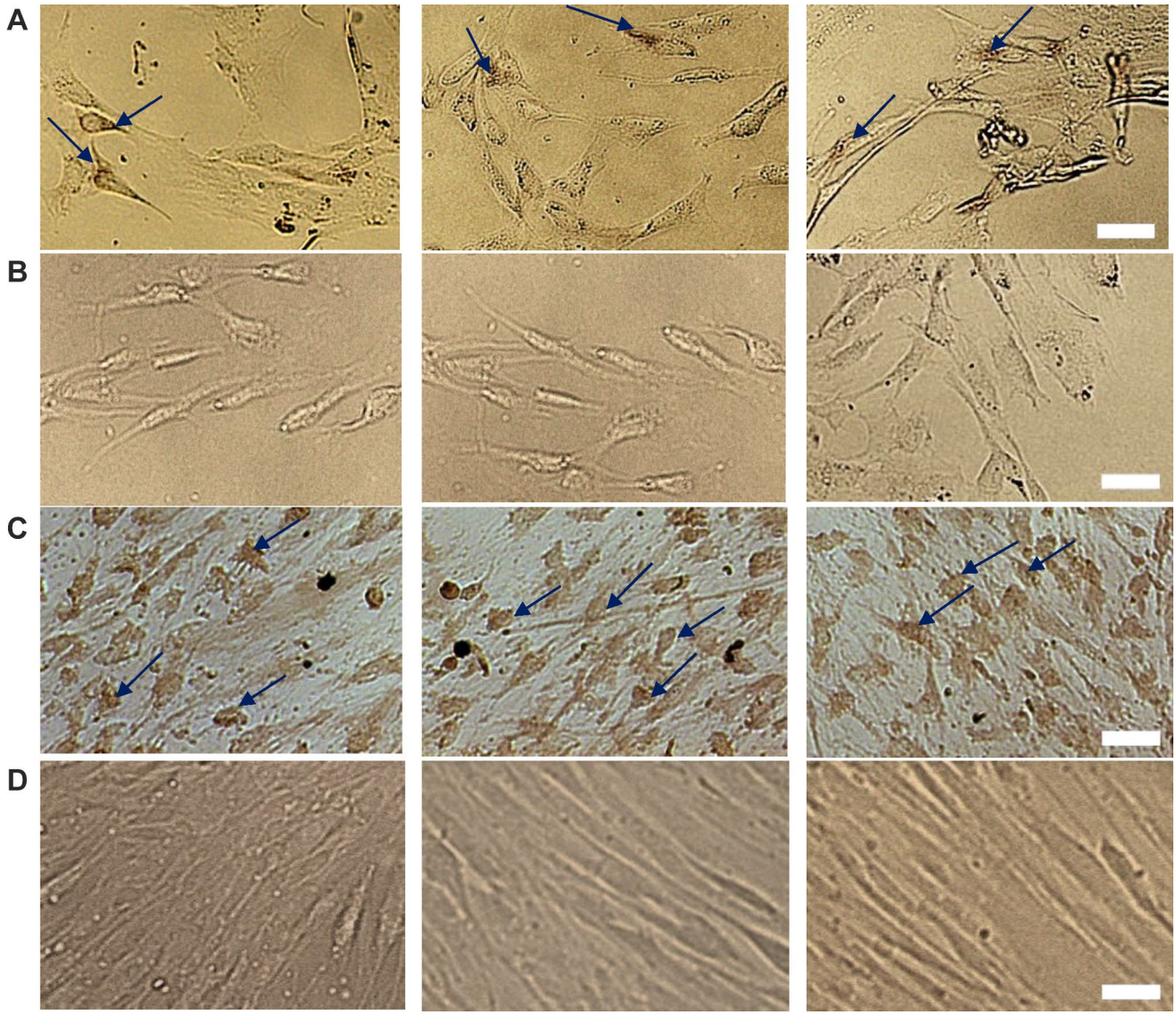

Fig. 2 Morphological and differentiation characteristics of DPSCs. **a** Nestin detection (arrow) in NSCs induced from DPSCs, and cultured cells adopted neuron-like morphology. **b** No nestin marker was detected in DPSCs in conventional culture condition for 7 days without induction. **c** Myosin VIIa was detected in the inner hair cell-like cell from DPSC-derived NSC with hair cell induction for 2 weeks (arrow). **d** No myosin VIIa was detected in DPSC-derived NSC after 2 weeks of culture in conventional media. Scale bars represent 20 µm

Table 1 Mean percentage of cell presenting nestin marker

	Nestin, mean (SD), %	*p*
Control group I	0 (0)	0.028
Neural stem cell induction	24 (7.80)	

Table 2 Mean percentage of cell presenting myosin VIIa marker

	Myosin VIIa, mean (SD), %	*p*
Control group II	0 (0)	0.028
Hair cell induction	81 (8.65)	

Apart from bone marrow, dental pulp is a good source of mesenchymal stem cells and is known to have excellent neuronal differentiation capabilities. In this study, dental pulp stem cells were also shown to differentiate into cochlear hair cells. However, a cell that can form hair cells morphologically and present cochlear hair cell markers may not necessarily function in response to mechanical stimuli and deliver stimuli to the auditory nerve. Stem cell transplantation to the cochlea has been performed by many researchers. Most of these stem cell sources have neural lineages, which may be because all hair cells, neuron cells, and nonsensory tissues associated with the cochlea come from a single embryonic

source called the otic placode. The otic placode is the epithelial cell on the surface of the embryo adjacent to the developing neural tube [23, 25]. A recent study found that human DPSCs show better neural properties than BMMSCs. Therefore, DPSCs could be a great alternative source for providing regenerative therapy in neuronal disorders, such as SNHL [14]. Research conducted by Estrela showed that DPSCs could differentiate into active neuron-like cells and present nestin markers. This finding suggests that DPSCs constitute an alternative to BMMSCs as a good source of stem cells to be transplanted into damaged cochlea, thus providing regenerative therapy in SNHL [25, 26].

Stem cell-based therapy for cochlear damage has been extensively studied. Several candidate stem cell sources have been investigated for their potential to replace damaged hair and nerve cells in the cochlea. One of the challenges in approaching stem cells for cochlear disorders is whether the given stem cells can survive, migrate to the desired location, replace damaged cells, and work functionally. The organ of Corti is a highly organized structure, and the transplanted cells would have to be able to repopulate the epithelium without disrupting its precise architecture. To do that, the transplanted cells will need to graft into a cell layer by disrupting strong tight junctions, which will need to be reconstituted quickly to maintain the sealed nature of the scala media. Moreover, the best route for delivering stem cells into the cochlea is still under investigation. Some researchers have indicated that it occurs through the scala tympani and scala media, and some even go directly to the auditory nerve. Most researchers chose the scala tympani route and obtained good results. However, more research is still required to overcome this challenge [1, 3, 23, 27].

Conclusion

Dental pulp has indeed been widely used for the treatment of neurological disorders. In our study, we found that DPSCs have great potential to differentiate into inner ear hair cells-like cell in vitro. The ability of DPSCs to differentiate into neural lineage cells makes them good candidates for regenerative therapy in neural diseases, such as SNHL. However, whether DPSCs can survive in the cochlear environment, replace damaged cells and function properly needs further investigation.

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Declarations

Conflict of interest The authors have no conflicts of interest to declare.

Ethical approval The experiment was approved by the Health Research Ethical Committee of Medical Faculty of Universitas Sumatera Utara, Indonesia (No: 310/TGL/KEPK FK USU-RSUP HAM/2019).

Informed consent Informed consent was obtained from participant included in this study.

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