Neurogenic Differentiation Potential of Human Nasal Mucosa Obtained from the Middle and Inferior Turbinates

(Potensi Pembezaan Neurogen Mukosa Hidung Manusia Diperoleh daripada Turbinat Tengah dan Inferior)

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ABSTRACT

Olfactory bulb and nasal mucosa are one of the sources for neural stem cell, including the superior and middle turbinates (MT). The middle and inferior turbinates (IT) provides the largest area of nasal mucosa which is technically easier to harvest the stem cell for future transplantation. The ability of nasal respiratory epithelial cells (RECs) and nasal fibroblasts (NFS) from both middle and inferior turbinates to differentiate into neural lineage (NL) cells were compared in this study. Six redundant human MT and IT from post-sinus surgery were digested and cultured. The RECs and NFS were separated and induced with neurotrophic factors of forskolin, human basic fibroblast growth factor (bFGF), platelet-derived growth factor-AA (PDGF-AA) and heregulin-β1-EGF-domain. Based on immunocytochemistry and quantitative PCR, the NL induced NFS of MT expressed GFAP, Nestin and P75 receptor. NL induced RECS from MT and IT expressed GFAP and Nestin but did not express the P75 receptor protein. Regarding the control, the non-induced RECS and fibroblasts expressed Nestin only. This study demonstrated that nasal mucosa cells from both IT and MT have the potential to differentiate into neural lineage cells even though the fibroblasts of MT are superior in term of quality. Hopefully, these tissues will provide better donor area with less morbidity for autologous or allograft transplantation in future neural regenerative medicine.

Keywords: Nasal epithelial cells; nasal mucosa; neural induction; neural lineage; turbinate

INTRODUCTION

Human nasal mucosa plays a vital role in humidifying and filtering the air that we breathe in by producing mucus that trap pathogens and other unwanted particles. Nasal mucosa and innate immune system served as the first line of defence to protect the host against pathogen and infection (Lane 2009). The epithelial cells and fibroblast of nasal mucosa release various pro-inflammatory chemokines and cytokines, such as interleukin (IL) - 4, IL-6, IL - 8, IL-1 β, transforming growth factor (TGF)-β1, monocyte chemoattractant protein-4 (MCP-4) and C-C motif chemokine-11 (CCL11), in response to the assault by the inhaled pathogens and toxins to modulate the innate and acquired immune responses (Ball et al. 2016; Dong Chang et al. 2018; Jae et al. 2019).

Since the development of tissue engineering and stem cell therapy, nasal mucosa has become an important source of cells for airway epithelium and tracheal reconstruction. Respiratory epithelial cells (RECs), nasal fibroblasts (NFS) and stem cells have been successfully isolated from nasal mucosa, especially from nasal turbinates (Hauser et al. 2011; Mohd Heikal et al. 2014; Nur Adelina et al. 2007;
Recurrent cell anticancer proteasome inhibitors (Ruszymah et al. 2011). The RECs has been reported to display stem cell properties through the expression of stem cell markers such as Frizzled-9, bone marrow stromal cell antigen-1, CD73, CD90 and CD105, even after serial passaging for up to passage 4. RECs has been successfuly used to develop an autologous tissue-engineered trachea that can be transplanted into sheep. It was found that the transplanted cells promoted natural regeneration of the tracheal epithelium with minimal fibrosis (Mohd Heikal et al. 2010; Ruszymah et al. 2014). Nasal mucosa in the olfactory area is well known to contain olfactory ensheathing cells (OECs). OECs are specialised cells that support the neurogenesis and rejuvination of the olfactory receptor neurons (Au & Roskams 2002). OECs were also evidenced to support the remyelination process of demyelinated axons (Kato et al. 2000). Several studies, including clinical trials, were done using OECs to treat nerve damage and diseases ranging from spinal cord injury, peripheral nerve injury to Alzheimer’s disease (Bing Chang et al. 2012; Lima et al. 2010; Lokanathan et al. 2014; Tan et al. 2013). Furthermore, human neural crest-derived stem cells have been isolated from inferior turbinates (Hauser et al. 2011). These cells were reported to be multipotent and able to improve the functional outcome of rats with neurological and psychiatric diseases (Müller et al. 2015). A study by Goldstein et al. (2013) showed that mesenchymal-like stem cells (MSC) isolated from lamina propria of nasal turbinates and septum have the capability to form neurosphere and expressed CD90, CD105, STRO-1, and nestin. They are able to transform into neuronal-like cells under differentiation conditions. To the best of our knowledge, there is no current study focusing on the quality and quantity of neural cells elements in the different part of human turbinates. Additionally, the neuronal differentiation potential of human nasal respiratory mucosa cells from the nasal turbinate has never been explored previously.

In this study, the neurogenic differentiation of human nasal mucosa cells obtained from the middle and inferior turbinates was evaluated. The present study aimed to compare the neurodifferentiation potential between the respiratory epithelial cells (RECs) and nasal fibroblasts (NFs) obtained from different parts of the nasal turbinate. Neuronal induction of human nasal turbinate cells has the potential application in the spiral ganglion neurons or cochlear nerve repair, by either directly replacing the neurons or via paracrine activation of the endogenous cells to do so (Bas et al. 2013; Mingliang et al. 2018). Currently, sensorineural hearing loss is on an increasing trend and affects an estimated five to 20 per 100,000 persons per year (Lin et al. 2012; Rabinowitz et al. 2006). Loss of receptor hair cells or spiral ganglion neurons, due to viral infection, vascular impairment, immune-mediated mechanisms, and inner ear and central nervous system abnormalities, were the cause of sensorineural hearing impairment (Lin et al. 2012). Repairing or restoring the damaged inner ear tissue remains a major challenge. Cochlear implantation surgery is the mainstay of treatment to restore input for those with profound sensorineural hearing loss that is not benefiting from conventional hearing-aid amplification. However, intact spiral ganglion neurons and cochlear nerve are required for cochlear implantation. Other treatment strategies to replace the defect of the nerve or loss of the spiral ganglion neurons are therefore needed and cell therapy can be an option to replace the damaged spiral ganglion neurons (Bas et al. 2013).

**Materials and Methods**

**Isolation and Culture of Human Respiratory Nasal Epithelial Cells (RECs) and Nasal Fibroblasts (NFs)**

Individual consents were obtained prior to the procedure with approval from the Institutional Ethics Committee (KPJ Healthcare University College: KPJUC/ORL/EC/2014/02). Human tissue samples derived from the lateral lamellar of the middle turbinate and the inferior portion of inferior nasal turbinate were obtained from six patients undergoing endoscopic sinus surgery and turbinoplasty. The redundant samples were taken from the donors with the age between 15-60 years.

The turbinate was cleaned by washing it several times with Dulbecco’s Phosphate-Buffered Saline (DPBS) and was minced into small pieces. It was then digested using 0.6% collagenase type I solutions (Worthington, USA) for 1-2 h in an incubator shaker at 37°C. Digested tissue containing NFs and RECs was centrifuged and the pellet was resuspended in co-culture medium (Bronchial Epithelial Cell Growth Medium (BEGM; Lonza, USA), Defined Keratinocyte Serum-Free Medium (DKSF; Gibco, USA), F-12, and Dulbecco’s Modified Eagle’s Medium (DMEM; Biowest, USA) with the 1:1:2 ratio, supplemented with 5% fetal bovine serum (FBS) (Gibco, USA)) and seeded into a six-well plate. The cells were cultured at 37°C in 5% CO₂ incubator with culture medium being replaced every two to three days. Once the cells reached 80-90% confluency, in about five to seven days, NFs were differentially trypsinised using 0.05% Trypsin-EDTA (Lonza, USA) to separate them from RECs. The remaining RECs were supplemented with DKSFM and passaged up to two times. Meanwhile, NFs were cultured in F-12 and DMEM with 1:1 ratio, supplemented with 10% FBS. Once the cell reached 80-90% confluency, they were washed with DPBS and trypsinised using TrypLESelect (Gibco, USA) and counted with haemocytometer before being used for neurogenic induction.

**Neurogenic Induction of Human Nasal Turbinates**

The neurogenic induction was adapted from Nur Hidayah et al. (2012). The cells were incubated in Alpha-Minimum Essential Medium (α-MEM) containing 1 mM β-mercaptoethanol (βME) without serum for 24 h. The culture medium was replaced with differentiation medium consisting of α-MEM containing 10% FBS and 35 ng/mL all-trans-retinoic acid (ATRA). Three days later,
cells were transferred to α-MEM containing 10% FBS and trophic factors (5 μM forskolin, 10 ng/mL recombinant human basic fibroblast growth factor (bFGF), 5 ng/mL platelet-derived growth factor-AA (PDGF-AA) and 200 ng/mL heregulin-β1-EGF-domain) and cultured for an additional four to five days before they were ready for in vitro evaluation. Cell morphology was observed and recorded using an inverted microscope. Total cell count and cell viability (induced and non-induced cells) were calculated by counting the cell after trypsinization using haemocytometer.

EVALUATION OF NEURONAL CELL SURFACE MARKERS EXPRESSION

Both induced and non-induced cells were evaluated by immunocytochemistry (ICC) and quantitative real-time PCR (qPCR) analysis. ICC was performed as previously described by Rabiatul et al. (2015). Briefly, the cells were probed with the following primary antibodies: polyclonal anti-S100b (mouse; 1:4,000; Becton Dickinson (BD), USA), monoclonal anti-Nerve Growth Factor (NGF) P75 receptor (mouse; 1:500; Chemicon Corp., Japan), monoclonal anti-Nestin (mouse; 1:500; BD) and monoclonal anti-Glial Fibrillary Acidic Protein (GFAP) (rabbit; 1:100; Chemicon Corp., Japan) and tagged with the secondary antibody, Alexa Fluor 488 anti-mouse IgG (goat; 1:50; Life Technologies, USA), or Alexa Fluor 488 anti-rabbit IgG (goat; 1:50; Life Technologies, USA). Nuclei were counterstained with DAPI (Life Technologies, USA). Cells were examined using a fluorescence microscope (Eclipse Ti, Nikon, Japan) immediately after the staining procedure.

For qPCR analysis, non-induced and induced RECs and NFs were trypsinised and the RNA was extracted from the cells using RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. The gene expression of Nestin as neural progenitor cell marker, S100, GFAP and P75NGF as Schwann cell markers on the RECs and NFs of both the MT and IT of the non-induced samples and induced samples were examined using the primers listed in Table 1. The qPCR was performed using iQ™ SYBR® Green Supermix (BioRad, USA) on Bio-Rad iCycler with MyiQ Optical Module thermal cycler.

Each qPCR reaction was performed with 1 μL forward primer, 1 μL reverse primer, 2 μL cDNA template, 12.5 μL of iQ SYBR green supermix (Bio-Rad, USA) and 9.5 μL Nuclease free water in 25 μL of total volume. Each reaction was done in triplicate. PCR amplifications included the following cycling conditions: one cycle at 95°C (3 min), followed by 40 cycles of denaturation at 95°C (30 s), annealing and extension at 55°C for 45 s. Finally, melt curve analyses were done by heating the PCR mixtures from 65°C to 95°C in increments of 0.5°C every 5 s with simultaneous measurements. The data were analysed using iQ5 optical system software (Bio-Rad, USA).

STATISTICAL ANALYSIS

SPSS version 16 (SPSS Inc. USA) was used for data analysis. The comparison of the mean of total cell count viability between induced and non-induced groups of RECs and NFs were assessed with Student’s paired t-test.

The qPCR data were presented as mean ± standard deviation (SD). ANOVA test was used to determine the statistical significance of qPCR data. A p-value<0.05 was considered significantly different.

RESULTS

CELLULAR MORPHOLOGY OF NON-INDUCED AND INDUCED HUMAN NASAL MUCOSA CELLS

Phase-contrast microscopic observation showed the morphological changes of human nasal mucosa cells before and after induction. Following 24 h of induction, the non-induced RECs looked relatively similar to the induced RECs where they had a prominent non-uniform polygonal shape (Figure 1). On day three of post-induction, the non-induced RECs became more confluent compared to the induced RECs and remained symmetrical, whereas the induced RECs became more asymmetrical in shape and appeared to be apoptotic. One-week post completion of induction with neurogenic induction medium, the non-induced RECs continued to appear matured and confluent. Apoptotic morphological changes were observed in the induced RECs (Figure 1).

Initial culturing of nasal fibroblast cells showed no difference (Figure 1). Proliferation was observed in non-induced NFs upon day ten post-infection with elongated cytoplasmic extension. The induced NFs showed neuronal changes, where they exhibited a longer spindle-shaped fibroblastic morphology.

VIABILITY ANALYSIS

There is no significant difference observed in the total viable cell analysis between non-induced and induced

| Target gene | Forward primer | Reverse primer |
|-------------|----------------|---------------|
| GFAP        | GTGGCCAGGTTGGAGCTTGGAT'TCT | CTGGGCCGCGCTGGATACGA |
| Nestin      | AGAGGGGAAATCTTGGAGAGG | CTTAGGGACCAGGACTCTCTA |
| S100B       | ATGCTGCACTGGAGAAGG | CTTGCTGCTTTCCTGATG |
| P75         | CCTACGGCTACTACCAGATGAG | TGGCCTGTCGGAATACG |

TABLE 1. List of primer used for qPCR analysis (5'-3')
human nasal mucosa cells. The number of viable non-induced cells was higher compared to induced cells after one week (Figure 2). However, the difference was not statistically significant ($p > 0.05$). The percentage of viable cells also showed the same trend, whereas the non-induced REC$s$ and NF$s$ had a higher percentage of viable cells compared to their induced counterparts, both for middle turbinate and inferior turbinate, however, the differences were not statistically significant (data not shown).

**QUANTITATIVE ANALYSIS OF NON-INDUCED AND INDUCED HUMAN NASAL MUCOSA CELLS**

The RT-PCR analysis showed statistically-significant upregulation for GFAP and Nestin genes in induced REC$s$ compared to its respective control ($p < 0.05$). In terms of S100B and P75NGFR, upregulations were observed but insignificant (Figure 3). In terms of tissue origin, the expression of GFAP and Nestin genes were significantly higher in induced REC$s$ of MT compared to that of IT ($p < 0.05$). However, no significant difference was observed in the expression of S100 and P75NGF between MT and IT. Next, comparison of NF$s$’ gene expression among MT and IT derived cells showed upregulation of all neural gene markers for the induced NF$s$ of MT and IT compared to their respective control (Figure 3). However, only upregulation of GFAP, Nestin and P75 in the NF$s$ of MT were statistically significant ($p < 0.05$). The gene upregulations in induced NF$s$ of IT were insignificant. Finally, in terms of tissue origin, the expression of GFAP, Nestin and P75NGFR were significantly superior in NF$s$ of MT ($p < 0.05$), but not significant for the expression of S100B ($p > 0.05$).

**IMMUNOCYTOCHEMISTRY ANALYSIS OF SPECIFIC MARKERS PROTEIN EXPRESSION IN REC$s$ AND NF$s**

Induced REC$s$ were positive for neuronal cell markers such as GFAP and Nestin but negative for S100B and P75NGF.
(Figure 4). However, Nestin was expressed in all the tested cell groups, regardless of the induced or non-induced cell (Figures 4 & 5). The induced NFs of MT showed positive expression for GFAP, P75NGF and Nestin while the induced NFs of IT showed positive expression for Nestin only (Figure 5).

**DISCUSSION**

Neurogenic differentiation of human nasal mucosa cells has been reported in earlier studies (Xingjia et al. 2013; Yang et al. 2017). These studies mainly focused on the isolation of olfactory epithelial-derived progenitors from rodent and human and their differentiation into neural lineage cells (Delorme et al. 2009; Meng et al. 2012; Murrell et al. 2008; Zhang et al. 2006). This study aimed to determine the quality and quantity of the REC s and NF s derived from the MT and IT, with a focus on their induction to neural lineage cells for future tissue engineering purposes. It was found that the human nasal mucosa cells from both MT and IT can undergo neurogenic differentiation, but there were differences in terms of quality and quantity of the neuro-differentiated cells.

In term of cell viability, there were no statistical differences between induced REC s of MT and induced REC s of IT as well as induced NF s in MT and IT. However, high variability was observed in the total number of cells cultured per cm$^2$ from sample to sample. This could be attributed to the size of the cultured turbinate and the number of REC colonies per plate as it is well known that prolonged culturing might compromise subsequent growth (Fulcher & Randell 2013). Due to sample variation, REC s from some samples took a longer time to achieve the desired confluency compared to others. NF s had a higher proliferative rate at all passage compared to REC s. This may be due to the ability of NF s to secrete various cytokines and growth factors to maintain its stability (Ball et al. 2016).

Generally, the presence of trophic factors in the culture medium will increase the cell proliferation rate and viability of the induced cells. Contradictorily, lower number of viable cells were observed in our induced NF and REC compared to their non-induced counterparts. This was due to the addition of BME at the pre-induction stage that reduces the number of viable cells drastically before the trophic factors were supplemented.

Morphology of the non-induced REC s and non-induced NF s only start to show more obvious differences as compared to the induced REC s and NF s after one-week post completion of induction with neurogenic induction medium. We hypothesized that the morphology results presented in this study using the induction mediums adapted from Nur Hidayah et al. (2012) may not be an optimal medium for human nasal turbinates. Further studies need to be done to ensure the appropriate induction medium specific for human nasal turbinates are used to demonstrate the ability of adult human nasal turbinates to undergo neurogenic differentiation for both middle turbinate and inferior turbinates.

All the induced and non-induced samples of REC s and NF s in our study demonstrated upregulation of all the tested markers at the gene level. Xingjia et al. (2013) showed that positive Nestin expression was seen in NF s of MT, but the expression was not tested in the OECs from the human MT. Another study by Hauser et al. (2011) also
reported on the positive expression of Nestin marker in respiratory epithelial cell and neural crest-derived stem cell derived from adult human inferior turbinate. A recent study demonstrated that early passage of fibroblasts does contain cells that express Nestin but not Vimentin, a common specific marker for fibroblast. Thus, they hypothesised that these group of cells might be the multipotent stem cells of the human skin (Fang et al. 2017). Nestin expression was seen in both induced and non-induced samples in this study. Such findings were not surprising as it is present in neuroepithelial cells that may exist in both induced and non-induced proliferating nasal mucosa tissues. We believe that Nestin was not downregulated due to insufficient differentiation into a more matured stage, as supported by the lack of S100B expression in induced samples. S100 is known to indicate a late developmental stage whereby the GFAP-expressing cells lose their neural stem cell potential (Raponi et al. 2007).

GFAP marker was not detected in induced NFs of IT by ICC analysis. However, qPCR showed a notable upregulation of GFAP in induced NFs of IT compared to that of non-induced. One possible explanation could be that while qPCR can detect a minute amount of mRNA, the amount of protein translated might be insufficient for ICC detection.

Upregulation of S100B gene expression was observed in the qPCR analysis of all of the induced RECs and NFs of MT and IT, but they were not statistically significant (p>0.05). In contrast, no expression of S100B was observed in any of the samples by ICC analysis. A possible explanation is that S100B tends to be expressed in cells at a more mature stage among the GFAP-positive cells. Expression of GFAP and Nestin in our samples indicates that only a minimal portion of the cultured cells achieved maturity, which explained the low expression of S100B. A similar observation was also reported by Raponi et al. (2007) in neural stem cells.

Although upregulation of P75 gene marker was observed in all induced samples for both RECs and NFs, they were not significant (p<0.05), except for induced NFs of MT. The P75 protein was also not detected by ICC analysis in any of the groups. This suggests that the differentiation of our sample probably had achieved adequate differentiation towards Schwann cell lineage and thus were not expressing a significant amount of P75NGFR. However, Hauser et al. (2011) found that when sorted based on P75NGFR expression, both positive and negative fractions of the inferior turbinate cells were positive for Nestin expression and showed the ability to form neurospheres, despite positive fractions showed shorter population doubling.

**FIGURE 4. Immunocytochemical analysis of non-induced and induced RECs. GFAP (green), S100 (green), P75NGF (green), Nestin (green), nuclei (blue)**
time. This suggests that IT samples could still be used in neural stem cells induction even though they did not show significant P75NGFR upregulation.

The induction medium adapted from Nur Hidayah et al. (2012) protocol may not be optimal for neurogenic differentiation of the human nasal mucosa in this study. In addition, neurogenic induction medium containing β-mercapethanol can cause cells to undergo apoptosis besides acting as a pre-induction factor. Further studies need to be done to optimise the appropriate induction mediums specific for human nasal mucosa cells, which drive the neurogenic differentiation with higher efficiency for both middle turbinate and inferior turbinate.

The limitation of this study includes the variation in patient samples and also the small sample size. The variation in patient samples might cause sample bias, where the general population’s tissue may behave slightly different from those of the diseased. However, it will be challenging to obtain nasal turbinate tissues from healthy humans. The insufficient sample number may mask any significant difference with the control, and the overall result may not correlate with the larger population. Thus, larger sample size and inclusion of healthy nasal turbinate tissues may provide a better indication of the ability to differentiate into neural lineage cells. Besides that, the spontaneous expression of Schwann cell markers such as Nestin, S100 and P75 in nasal mucosa cells and other cells (Hauser et al. 2011; Khairunnisa et al. 2019) may hinder the identification of cells that differentiated into neural lineage cells. Therefore, a functional assay is needed to validate a successful neurogenic differentiation.

**CONCLUSION**

Human nasal respiratory mucosa is indeed a potential autologous cell source, which is useful for future tissue engineering applications. In this study, the induced RECs and NFs showed neuronal appearance. The induced RECs expressed the neuron cell markers, namely GFAP and Nestin, while the induced MT NFs expressed GFAP, Nestin and P75NGFR. All the samples (regardless of non-induced or induced) expressed Nestin markers. These concluded that induced RECs and NFs of MT are of superior quality than those induced from IT. However, it must be recognised...
that cells from both the MT and IT have the capability for neurogenic differentiation. Given the advantages of ease and safety in harvesting IT compared to MT, IT is still a better donor tissue candidate than the MT. It is with great hope that this study can serve as a stepping stone for future studies on tissue engineering and regeneration for the neurogenic differentiation from the human nasal mucosa.

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