Int. J. Morphol.,
35(2):413-419, 2017.

Olfactory Epithelium as an Infinitive Source of Neural Stem Cells for Derivation of Inner Ear Hair Cells

El Epitelio Olfatorio como Fuente Infinita de Células Madre Neurales para la Derivación de los Vestibulocitos del Oído Interno

Tahere Bahmani [1] ; Samira Gholami[1]; Ali Mostafaie[2]; Ebrahim Karimi[3]; Zohreh Hoseinkhani[1]; Taha Azad[4]; Hamid Mahdizadeh[5,6] & Kamran Mansouri[1]

BAHMANI, T. ; GHOLAMI, S.; MOSTAFAIE, A.; KARIMI, E.; HOSEINKHANI, Z.; AZAD, T.; MAHDIZADEH, H. & MANSOURI, K. Olfactory epithelium as an infinitive source of neural stem cells for derivation of inner ear hair cells. *Int. J. Morphol., 35(2)*: 413-419, 2017.

SUMMARY: Surgical techniques for treatment of sensory neural hearing loss (SNHL) have unpredictable outcomes and in recent years cell therapy investigated for treatment of SNHL. Olfactory epithelium proceed neurogenesis during life time and provide an easily accessible source of neural stem cells. So the aim of this study was isolating neural stem cells from olfactory epithelium of rat and differentiation of these cells into hair cells of inner ear *in vitro*. The epithelium tissue of olfactory mucosa of rats were removed and digested by collagenase H. The digested tissue was cultured in flasks in suspension forms to create spheres. Spheres were passaged and from passage 2 spheres selected for differentiation. At this stage cells of spheres isolated from each other and placed in flask containing defined differentiation medium. Cells at this stage cultured in adhesive form. Immunohistochemistry and RT-PCR were used for neural stem cells and hair cells identification. Spheres formed from olfactory epithelium culture and immunohistochemistry revealed that cells of spheres from passage one and two expressed the neural stem cells markers. After culture of isolated cells in differentiation medium, the morphology of cells begun to change. The cells presented neural cells projections and after 10 days the projections elongated more and interact to each other in multi layers. RT-PCR and immunohistochemistry revealed that differentiated cells expressed hair cells specific genes. In this study we showed that neural stem cells of olfactory epithelium can differentiate into hair cells of inner ear and therefore can be used for treatment of SNHL.

KEY WORDS: Olfactory mucosa; Neural stem cells; Hair cells; Differentiation; Rat.

INTRODUCCIÓN

Sensory neural hearing loss (SNHL) is one of the most disabilities in the world that mainly results from dysfunction of mechanosensory hair cells of the cochlea (Okano & Kelley, 2012). Previous researches revealed that the mammalians in contrast with non-mammalians lack the capacity of regeneration of hair cells of inner ear (Ryals & Rubel, 1988). Therefore, nowadays the surgical techniques such as cochlear implantation and amplification techniques such as a hearing aid are used for treatment of SNHL still the outcomes of these techniques are unpredictable (Limb, 2012). It is more efficient to treat SNHL by biological methods rather than surgical or amplification techniques (Patel *et al*., 2004). Therefore recently, attentions have turned to the regeneration of hair cells by cell therapy specifically stem cell therapy (Koehler *et al*., 2013). Although recent studies revealed that there are evidences of the existence tissue specific stem cells of inner ear in mammalians (Lopez *et al*., 2004; White *et al*., 2006; Oshima *et al*., 2007). These studies also showed that the regeneration capacity of inner ear specific stem cells is very low and age-dependent. Therefore, it is critical to find proper sources of stem cells for regenerating the hair cells of inner ear. Embryonic stem cells have legal and immunologic problems for clinical usage. Induced

[1] Medical Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran.
[2] Department of Immunology, Kermanshah University of Medical Sciences, Kermanshah, Iran.
[3] Department of Otorhinolaryngology, Tehran University of Medical Sciences, Tehran, Iran.
[4] Department of Biochemistry, Tarbiat Modarres University, Tehran, Iran.
[5] Student Research Committee, Babol University of Medical Sciences, Babol, Iran.
[6] Cellular and Molecular Research Medicine, Babol University of Medical Sciences, Babol, Iran.

pluripotent stem cells (iPS cells) also have some problem for clinical usage because of viral vectors using to create them. Mesenchymal stem cells have a low potential for neuronal differentiation, so it seems that a patient's specific neural stem cells are the most proper stem cells for treatment of SNHL (Okano & Kelley).

Olfactory epithelium consists of a specific neural stem cell with neural crest origin (Suzuki *et al*., 2013) which unlike the other regions of central nervous system (CNS) proceed with neurogenesis throughout life (Beites *et al*., 2005). This capacity of regeneration candidates the stem cells of olfactory epithelium as an efficient source for treatment of neurodegenerative disorders of CNS (Evgrafov *et al*., 2011; Mackay-Sim, 2012). The neural stem cells of the olfactory epithelium were previously isolated and characterized (Calof *et al*., 2002; Hahn *et al*., 2005). These cells express neural stem cells markers, including Nestin and Vimentin and can easily be isolated by culture methods. These cells can differentiate into both neuronal and glial cells (Beites *et al*.).

Based on the data, olfactory epithelium can be used as an easily accessible source of neural stem cells with high renewal potential for treatment of SNHL by regenerating the hair cells. Based on our literature review, there is not enough evidence of successful differentiation of neural stem cells of olfactory epithelium into hair cells of inner ear. So we performed this study in order to isolate neural stem cells from the olfactory epithelium of rat at first, and then differentiate them into hair cells of inner ear *in vitro*.

MATERIAL AND METHOD

Isolation of olfactory epithelium from rat nose. Outbred Sprague adult rats were anesthetized with chloroform and then the rat head was removed under sterile conditions. The removed head was placed in Hank's balanced salt solution (HBSS) buffer (AppliChem, Germany) and transferred under a class II laminar airflow hood. Under the hood, with sterile surgical instruments, the head skin was carefully removed and the skull was precisely divided into two sagittal parts. The olfactory epithelium, which has a pale tissue, was removed as the next step and placed in a 50 ml Falcon centrifuge tube containing fresh HBSS buffer.

Primary culture of rat olfactory epithelium and isolation of neural stem cells. The epithelium sample was washed three times in 50 ml HBSS buffer, and then incubated in 2 mg/ml collagenase H (Roche, Mannheim, Germany) at 37oc for 60 minutes. Cellular suspension obtained from tissue digestion filtered and centrifuged for 5 minutes in 700g. The pellet was resuspended in 5 ml culture medium and put into 25 cm^2 culture flasks [NUNC, Denmark) and placed in a CO_2 incubator. The culture medium was DMEM (Gibco, Breda, the Netherlands) / F-12 (Gibco) mediums in a 3:1 ratio containing: B-27 minus vitamin A (Invitrogen, Carlsbad, CA, USA) 2 %, βFGF (Relia-Tech, Australia) 40 ng/ml, EGF (Biovision, CA, USA) 20 ng/ml, LIF (Sigma, St. Louis, MO, USA) 10 ng/ml, Pen/Strep (Applichem) 100 IU/ml, Amphotericin-B (Bristol-Myers Sqible, NY, USA) 1 µg/ml, and $NaHCO_3$ 2 g/li. In this stage the cells cultured in suspension form, so the flasks were coated with Poly-Hema (Sigma) before the culture. The fresh culture medium was added three times a week to flasks and the cellular spheres begun to generate after 2 days. After 10 days the cellular spheres were centrifuged, resuspended in culture medium and pipetted up and down for isolating the cells from each other. Then the isolated cells were passaged. The passage procedure was repeated every 10 days.

Identification of neural stem cells markers by immunohistochemistry. Immunohistochemistry was used for identification of neural stem cells. Both cellular spheres and single cells from passage 1 and 2 were used for immunohistochemistry study. Monoclonal anti-human Nestin antibody (R&D, Minneapolis, USA) and monoclonal anti-human Vimentin antibody (R&D) were added to the fixed cells and spheres, then secondary anti-mouse IgG antibody conjugated with FITC (produced in Medical Biology Research Center of Kermanshah University of Medical Sciences) was added in appropriate dilution. After immunohistochemistry staining the cells were evaluated under a fluorescence microscope (TS100, Nikon, Tokyo, Japan).

Differentiation of neural stem cells into hair cells. Spheres from passage 2 were selected for differentiation. The plates were centrifuged and after that the 2/3 upper medium removed and the cellular pellet resuspended in remaining 1/3 medium. The cellular suspension was pipetted up and down frequently to isolate the cells from each other. Then the differentiation medium was added to the tubes and the suspension was transferred to 25 cm^2 flasks. In this stage the culture condition was adhesive, so the flasks were coated with Poly-D-lysine (Sigma) before culture. The flasks placed in a 5 % CO_2 incubator in 37 ºC. The differentiation medium contains: DMED/F12 in a 3:1 ratio, FCS (Gibco) 1 %, B-27 serum free (Invitrogen) 2 %, NGF (Jena Bioscience, Australia) 10 µg/ml, Pen/Strep 100 IU/ml and Amphotericin 1 µg/ml. The culture medium

was exchanged three times a week with fresh medium and the cells were cultured 8 days under this condition for neuronal differentiation.

Identification of differentiated cells by RT-PCR and Immunohistochemistry. We selected five genes to evaluate differentiation. These genes include: Math-1, Myosin, Brn3, EPSIN and AchRa9 which are hair cells specific genes. Immunohistochemistry was used to evaluate expression of Myosin and RT-PCR for all genes. Immunohistochemistry study performed with Monoclonal anti Myosin antibody (Santa Cruz, CA, USA) and secondary anti-mouse IgG antibody conjugated with FITC.

To perform RT-PCR, RNA was extracted by chloroform (Sigma) technique and cDNA was synthetized by Reverse transcriptase enzyme (Fermentas, Canada). Sequences of forward and reverse primers of the five genes and GAPDH (housekeeping gene) were designed (Table I) and Taq DNA polymerase (Roche) was used for polymerization. PCR cycles perform by an automatic thermal cycler device (Eppendorf mastercycler gradient, Hamburg, Germany). Melting temperature and elongation temperature was 94 ºC and 72 ºC in order. Polymerization was performed for 32 PCR cycles.

Table I. Sequences of forward and reverse primers of amplified genes.

genes	Primer Sequences	bp
Brn3	F: ctctggcggcggtggata R: acggcatgcgggtgactc	325
Math-1	F: ggtaaaagagttgggggacc R: tggacagcttcttgtcgttg	545
Myosin	F: atctacacatacatacacgggttcca R: ttcttcttctcctcctcattca	527
ESPIN	F: ccctcatccccacacttgatgagc R: ctcgtcgtatcctagtgtccg	390
AchR_9	F: atcctgaagtacatgtccaggatc R: tggccttgtggtcc ttgaggcact	301
GAPDH	F: aaggtcatcccagagctgaa R: atgtagaccatgaggtccac	338

RESULTS

The cultured olfactory epithelium cells produced spheres. As described above, the isolated olfactory epithelium was digested by collagenase H then cultured in suspension condition in flask coated with poly-Hema. In first day the numerous of cells attached or dead and the other cells proliferate in culture medium. These cells were a heterogeneous population of blood cells, cartilage cells and neural stem cells (Fig 1A). Three days after primary culture some single cells begun to form small spheres and the most of adhesive cells attached to the bottom of flasks and the confluency of suspended cells decreased (Fig 1B). In day 7, some of small spheres attached to each other and formed big spheres and the confluency of single cells decreased more, which in most visual fields of microscope a combination of small and big spheres were seen (Fig 1C). In day 10, the spheres produced a noticeable cellular population by their continuous divisions and at this stage they were passaged (Fig. 1D). In next passage, the spheres formed and grew more rapid and the numerous of cells attached again. The remaining spheres produced big spheres which were bigger than previous passage. The big spheres were passaged again. In passage 2, an almost pure population of spheres produced and the spheres were bigger than the passage 1 (Fig. 1E). Spheres from this passage were selected for differentiation.

The cultured spheres expressed Nestin and Vimentin. For identification of neural stem cells, immunohistochemistry was used. Both spheres and sphere-isolated single cells from passage one and passage two expressed Nestin and Vimentin, protein markers which express in neural stem cells. (Figs. 2A and B).

The neural stem cells generated cytoplasmic projections in differentiation medium. As described before, spheres from passage two were selected for differentiation. The cells of spheres were isolated from each other by pipetting and then seeded in low density in flasks coated with Poly D-lysin (Sigma) so the cells attached to the flasks. 24 h after that, the morphology of attached cells begun to change. The most of cells became polygonal or spindle shape and some of them presented fine cytoplasmic projections, so the first degree of differentiation trigged (Fig. 3A). Differentiation procedure was observed in 10 days. In day 3, the morphologic changes were more evident. The neural projections elongated more (Fig. 3B). On day 7, neural projections interfered to each other (Fig. 3C). In second week, the cytoplasmic projections progressed more rapidly and in day 10, the final morphology of differentiated cells presented. The differentiated cells had long neural projections which interacted to each other in multi layers (Fig. 3D).

RT-PCR revealed that differentiated cells expressed hair cells specific genes. For molecular definition of differentiated cells, total RNAs of them was extracted and cDNAs was constructed and expression of five hair cells specific genes were evaluated. These genes included: Math-1, Myosin, Brn3, EPSIN, and AchRa9. RT-PCR of these genes revealed that differentiated cells expressed all of them (Fig. 4).

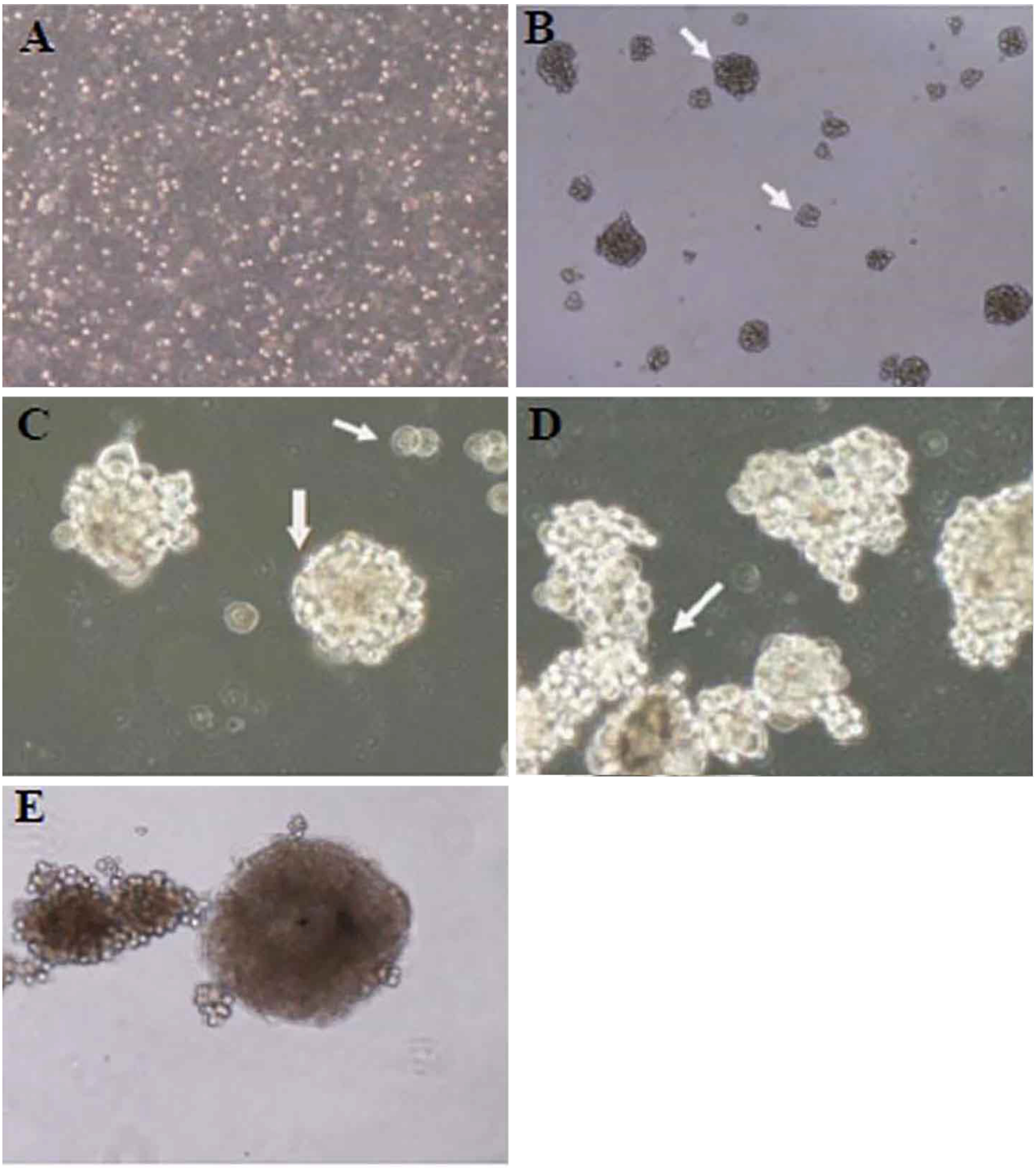

Fig. 1. Microscopic view of primary culture of rat olfactory epithelium. 4 h after culture (A), 3 days after culture and formation of spheres (B), 7 days after culture and enlargement of spheres (C), and 10 days after culture and attachment of spheres to each other (D). Spheres in passage two was bigger and tended to attach to flask bottom (E). Arrows show spheres.

Differentiated cells were Myosin positive in immunohistochemistry. Expression of Myosin was also evaluated by immunohistochemistry. The differentiated cells were positive for Myosin marker (Fig. 5).

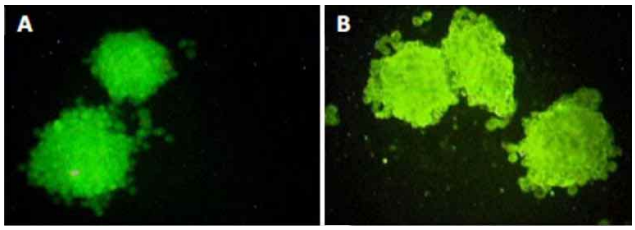

Fig. 2. Immunohistochemistry evaluation of spheres cultured from rat olfactory epithelium for neural stem cells markers Nestin (A) and Vimentin (B).

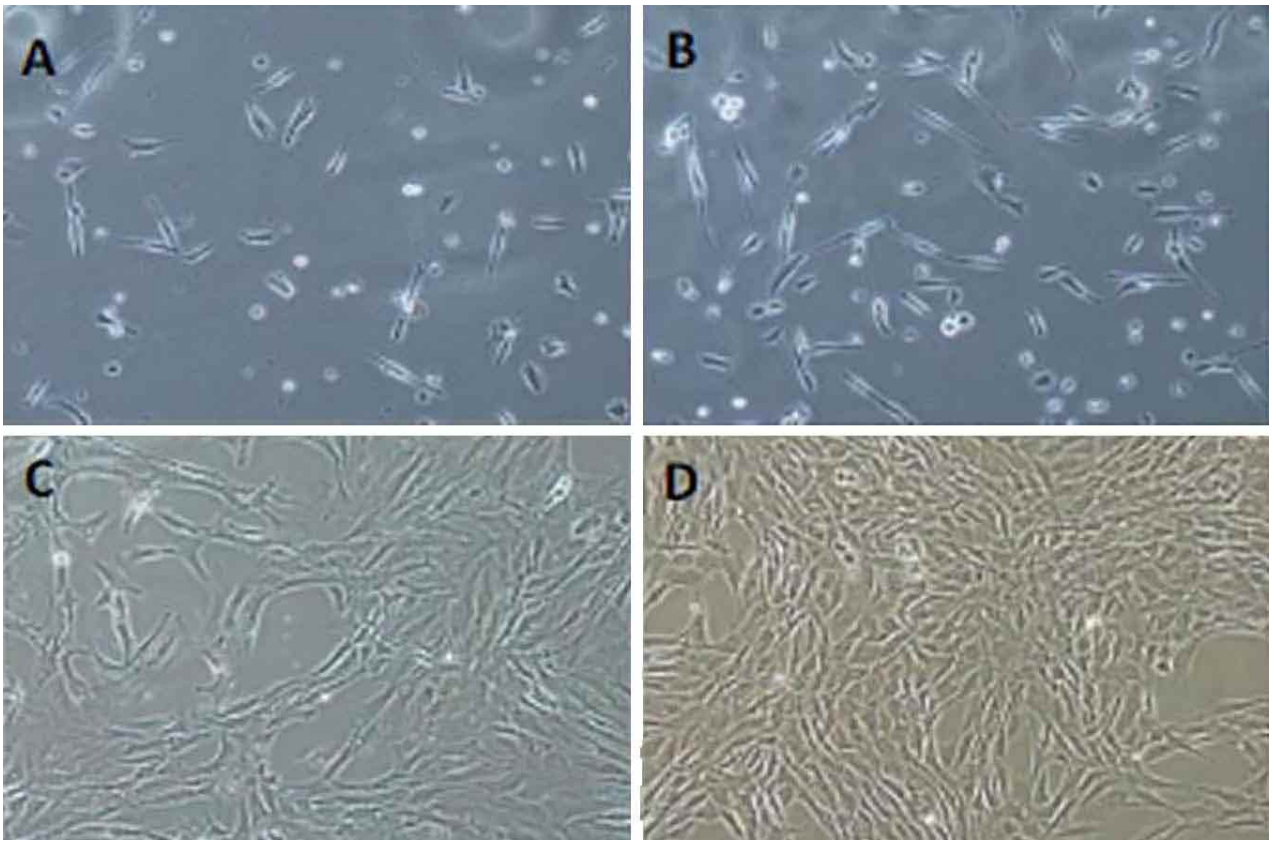

Fig. 3. Morphology of differentiated neural stem cells in differentiation process. 24 h after culturing cells in differentiation medium morphology of cells begun to change (A). In day 3 neuronal projections were more sensible (B). In day 7 neural projections elongated more and interfered to each other (C). In day 10 projection interfered to each other in multi layers (D).

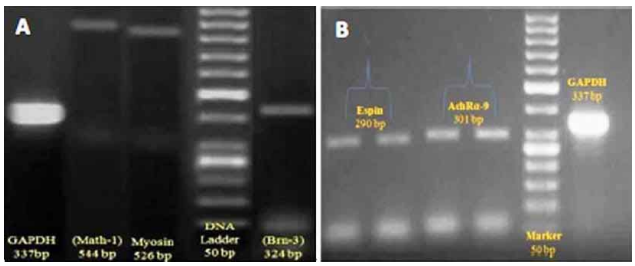

Fig. 4. RT-PCR revealed that differentiated cells expressed hair cells specific genes: Math-1, Myosin, Brn-3 (A), EPSIN, and AchRa9 (B). GAPDH is a house keeping gene.

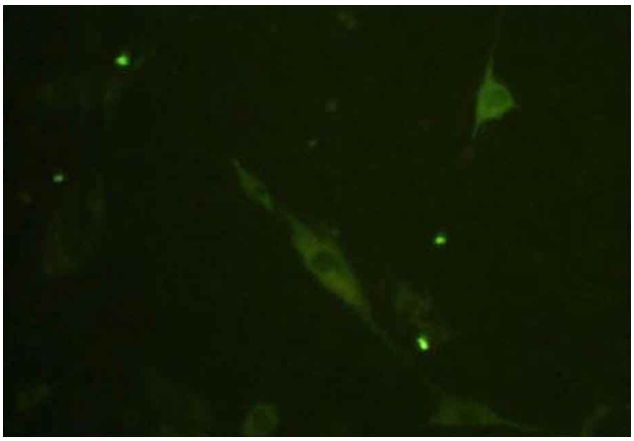

Fig. 5. Immunohistochemistry of differentiated cells for Myosin marker.

DISCUSSION

In this study, we successfully isolated and cultured neural stem cells from the olfactory epithelium of rat and then differentiated to hair cells of inner ear. Indeed, it is presented that olfactory epithelium, as an easy accessible and infinitive source of neural stem cells, can be a proper potential for cell therapy of sensory-neural hearing loss.

Due to our literature review, there is just one similar study to this study reported by Doyle *et al*. (2007). They isolated and cultured stem cells from an olfactory epithelium of inbreed mice and differentiated these cells into hair cells of inner ear. They cultured the neural stem cells in suspension method and produced spheres like our study, using a different method for differentiation. They cultured cochlear cells from inner ear of mice separately and then used two techniques for differentiation. One co-culturing of neural stem cells spheres with primary cultures of cochlear cells and the other one exposure of neural stem cells spheres with cochlear cells supernatant. Both techniques led to successful differentiation of hair cells. In contrast, we used a defined cultured medium for differentiation addressed in materials and methods.

Generation of hair cells was investigated during recent years in some study with other cell sources. Koehler *et al*. and Ouji *et al*. (2012) worked on differentiation of mouse embryonic stem cells into hair cells of inner ear *in vitro*. The first group done this work in 3D culture. Durán Alonso *et al*. (2012) and Qin *et al*. (2011) investigated mesenchymal stem cells (MSCs) potential to differentiate into hair cells. First study was on human and the second one was on rat. In both studies, first MSCs differentiated into neural precursor cells and then differentiated into hair cells by defined medium. Wang & Dong (2011) isolated neural stem cells from newborn guinea- pig hippocampus and differentiated them into hair cells both with defined medium and artificial perilymph techniques.

The most important advantage of this study is establishing required *in vitro* evidence for animal model study and then clinical study for treatment of sensory-neural hearing loss by cell therapy with neural stem cells derived from the olfactory epithelium. Another important advantage of this study compared to previous similar study (Doyle *et al*.) is differentiation of neural stem cells into hair cells by defined culture medium instead of co-culturing with cochlear cells or supernatant of cultured cochlear cells facilitated differentiation process.

We just evaluated morphologic and genetic differentiation of olfactory epithelium neural stem cells into hair cells and did not evaluate physiological differentiation of hair cells, which is the most important limitation of our study. Doyle *et al*. also did not evaluate the physiological differentiation of hair cells. So further studies are needed to assess whether the differentiated hair cells are functional or not.

In conclusion, our study provides another *in vitro* evidence of capability of usage of olfactory epithelium as an easily accessible and high regenerative source of neural stem cells for treatment of sensory neural hearing loss. But further *in vitro* and animal model studies are needed for designing clinical trials. Finally, with performing high quality clinical trials in future, it is anticipated that by the usage of a piece of the olfactory mucosa of a person his/her hearing loss can be treated.

ACKNOWLEDGMENTS

We appreciate Dr. KirollahYari, Dr Maryam Mehrabi and Mr Kaikawoos Gholami (Medical Biology Research Center, Kermanshah University of Medical Sciences) for their technical supports. We also thank research Deputy of Kermanshah Medical University for financial support.

BAHMANI, T. ; GHOLAMI, S.; MOSTAFAIE, A.; KARIMI, E.; HOSEINKHANI, Z.; AZAD, T.; MAHDIZADEH, H. & MANSOURI, K. El epitelio olfatorio como fuente infinita de células madre neurales para la derivación de los vestibulocitos del oído interno. *Int. J. Morphol., 35(2)*: 413-419, 2017.

RESUMEN: Las técnicas quirúrgicas para el tratamiento de la pérdida auditiva neural sensorial (PANS) tienen resultados impredecibles y en los últimos años la terapia celular ha sido investigada para su tratamiento. El epitelio olfatorio se forma durante la neurogénesis y proporciona una fuente fácilmente accesible de células madre neurales. El objetivo de este estudio fue aislar las células madre neurales del epitelio olfativo de la rata y la diferenciación de estas células en vestibulocitos del oído interno *in vitro*. Se retiró el tejido del epitelio de la mucosa olfatoria de ratas y fue digerido con colagenasa H. El tejido se cultivó en forma de suspensión para crear esferas. Se seleccionaron dos esferas para la diferenciación. En esta fase, las células de esferas fueron aisladas unas de otras y colocadas en un medio de diferenciación definido. Células en esta etapa fueron cultivadas en forma adhesiva. Inmunohistoquímica y RT-PCR se utilizó para las células madre neurales y la identificación de células ciliadas. Las esferas formadas a partir del cultivo del epitelio olfatorio y la inmunohistoquímica revelaron que las células de esferas en etapas uno y dos expresaban los marcadores de células madre neurales. Se observaron cambios en la morfología de las células después del cultivo de células aisladas. RT-PCR e inmunohistoquímica revelaron que las células diferenciadas expresaron células específicas de gen de vestibulocitos. Se observó que las células madre neuronales de

epitelio olfatorio puede diferenciarse en células en forma de cabello del oído interno y por lo tanto puede ser utilizado para el tratamiento de PANS.

PALABRAS CLAVE: Mucosa olfatoria; Células madre; vestibulocitos; Diferenciacion; Rata.

REFERENCES

Beites, C. L.; Kawauchi, S.; Crocker, C. E. & Calof, A. L. Identification and molecular regulation of neural stem cells in the olfactory epithelium. *Exp. Cell Res., 306(2)*:309-16, 2005.

Calof, A. L.; Bonnin, A.; Crocker, C.; Kawauchi, S.; Murray, R. C.; Shou, J. & Wu, H. H. Progenitor cells of the olfactory receptor neuron lineage. *Microsc. Res. Tech., 58(3)*:176-88, 2002.

Doyle, K. L.; Kazda, A.; Hort, Y.; McKay, S. M. & Oleskevich, S. Differentiation of adult mouse olfactory precursor cells into hair cells *in vitro*. *Stem Cells, 25(3)*:621-7, 2007.

Durán Alonso, M. B.; Feijoo-Redondo, A.; Conde de Felipe, M.; Carnicero, E.; García, A. S.; García-Sancho, J.; Rivolta, M. N.; Giráldez, F. & Schimmang, T. Generation of inner ear sensory cells from bone marrow-derived human mesenchymal stem cells. *Regen. Med., 7(6)*:769-83, 2012.

Evgrafov, O. V.; Wrobel, B. B.; Kang, X.; Simpson, G.; Malaspina, D. & Knowles, J. A. Olfactory neuroepithelium-derived neural progenitor cells as a model system for investigating the molecular mechanisms of neuropsychiatric disorders. *Psychiatr. Genet., 21(5)*:217-28, 2011.

Hahn, C. G.; Han, L. Y.; Rawson, N. E.; Mirza, N.; Borgmann-Winter, K.; Lenox, R. H. & Arnold, S. E. In vivo and *in vitro* neurogenesis in human olfactory epithelium. *J. Comp. Neurol., 483(2)*:154-63, 2005.

Koehler, K. R.; Mikosz, A. M.; Molosh, A. I.; Patel, D. & Hashino, E. Generation of inner ear sensory epithelia from pluripotent stem cells in 3D culture. *Nature, 500(7461)*:217-21, 2013.

Limb, C. J. When inner ear stem cell therapy becomes a reality. *Trends Amplif., 16(1)*:3, 2012.

Lopez, I. A.; Zhao, P. M.; Yamaguchi, M.; de Vellis, J. & Espinosa-Jeffrey, A. Stem/progenitor cells in the postnatal inner ear of the GFP-nestin transgenic mouse. *Int. J. Dev. Neurosci., 22(4)*:205-13, 2004.

Mackay-Sim, A. Concise review: Patient-derived olfactory stem cells: New models for brain diseases. *Stem Cells, 30(11)*:2361-5, 2012.

Okano, T. & Kelley, M. W. Stem cell therapy for the inner ear: recent advances and future directions. *Trends Amplif., 16(1)*:4-18, 2012.

Oshima, K.; Grimm, C. M.; Corrales, C. E.; Senn, P.; Martinez Monedero, R.; Géléoc, G. S.; Edge, A.; Holt, J. R. & Heller, S. Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. *J. Assoc. Res. Otolaryngol., 8(1)*:18-31, 2007.

Ouji, Y.; Ishizaka, S.; Nakamura-Uchiyama, F. & Yoshikawa, M. *In vitro* differentiation of mouse embryonic stem cells into inner ear hair cell-like cells using stromal cell conditioned medium. *Cell Death Dis., 3*:e314, 2012.

Patel, N. P.; Mhatre, A. N. & Lalwani, A. K. Biological therapy for the inner ear. *Expert Opin. Biol. Ther., 4(11)*:1811-9, 2004.

Qin, H.; Zhao, L. D.; Sun, J. H.; Ren, L. L.; Guo, W. W.; Liu, H. Z.; Zhai, S. Q. & Yang, S. M. The differentiation of mesenchymal stem cells into inner ear hair cell-like cells *in vitro*. *Acta Otolaryngol., 131(11)*:1136-41, 2011.

Ryals, B. M. & Rubel, E. W. Hair cell regeneration after acoustic trauma in adult Coturnix quail. *Science, 240(4860)*:1774-6, 1988.

Suzuki, J.; Yoshizaki, K.; Kobayashi, T. & Osumi, N. Neural crest-derived horizontal basal cells as tissue stem cells in the adult olfactory epithelium. *Neurosci. Res., 75(2)*:112-20, 2013.

Wang, Y. & Dong, M. M. *In vitro* induction and differentiation of newborn guinea pig hippocampus neural stem cells into cells resembling inner hair cells, using artificial perilymph. *J. Laryngol. Otol., 125(8)*:771-5, 2011.

White, P. M.; Doetzlhofer, A.; Lee, Y. S.; Groves, A. K. & Segil, N. Mammalian cochlear supporting cells can divide and trans-differentiate into hair cells. *Nature, 441(7096)*:984-7, 2006.

Corresponding author:
Kamran Mansouri
Medical Biology Research Center
Kermanshah University of Medical Sciences
Sorkheh Lizheh
P.O. Box 6714869914
Kermanshah
IRAN

Telephone: +98 831 4276473

E-mail: kmansouri@kums.ac.ir
amostafaie@kums.ac.ir

Received: 08-08-2016
Accepted: 06-01-2017