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Hearing restoration in a deaf animal model with intravenous transplantation of mesenchymal stem cells derived from human umbilical cord blood

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ABSTRACT

Objective: This study was performed to confirm the effect of transplantation of human umbilical cord blood mesenchymal stem cells (UCB-MSCs) on hearing restoration in a sensorineural hearing loss (SNHL) animal model.

Material and methods: UCB was collected from pregnant women after obtaining consent, and mesenchymal stem cells (MSCs) were extracted. We established an SNHL model and transplanted UCB-MSCs through the brachial vein of the guinea pigs. The animals were divided into 4 groups: animals with normal hearing, animals with SNHL, animals with SNHL and injected with saline, and animals with SNHL and transplanted with UCB-MSCs. Hearing tests were conducted at 1, 3, and 5 weeks, and the results were compared by grading auditory brainstem response (ABR) recordings and distortion product otoacoustic emissions (DPOAEs) for each treatment. Lastly, cochlear pathological features were examined, and surface preparations and morphological changes in each animal model were compared using hematoxylin and eosin staining and light microscopy studies.

Results: In SNHL group, decreased DPOAEs and increased ABR threshold were noted. Furthermore, in the SNHL group, ABR hearing thresholds were unconverted and were similar to those observed in deafness. The transplanted UCB-MSC group showed a significant improvement in hearing threshold (40 dB) compared to that in all the SNHL group (80–90 dB). Examination of the SNHL animals' cochlear morphological features demonstrated a noticeable lack of spiral ganglion cells and also showed degenerated outer hair cells. However, the transplanted UCB-MSCs showed an increase in spiral ganglion and hair cells.

Conclusion: Intravenous transplantation of UCB-MSCs can enhance hearing thresholds, outer-hair cells and increase the number of spiral ganglion neurons (SGNs).

1. Introduction

Hearing loss is one of the most common diseases. The severity can range from modest difficulty with speech comprehension to profound deafness. The majority of human sensorineural hearing loss (SNHL) results from primary neuronal loss involving the degeneration of neurons in the absence of hair cell degeneration or secondary to the loss of hair cells that normally provide trophic support to spiral ganglion neurons (SGNs) [1]. There are also peerless of this sort of hearing loss, only the auditory centers of the brain are affected. Moreover, in over 80% of cases, hearing loss is related to the degeneration, death, or damage of auditory hair cells and of their associated SGNs [2]. SNHL is a type of hearing loss in which the root cause lies in the vestibulocochlear nerve or inner ear [3]. This type of hearing loss is mainly caused by damage to the cochlear hair cells, which do not regenerate and are only partially replaced. However, several recent studies have reported that adult stem cells may be connected with rare diseases. Mesenchymal stem cells (MSCs) have the capacity for self-renewal and proliferation and are multipotent; thus, they can differentiate into various specific cell types such as muscle, epithelium, and liver cells [4,5]. Recent studies have also demonstrated that neuronal cells such as Schwann cells (supporting cells) can differentiate from the endogenous stem cells in the inner ear and from MSCs [6,7]. In addition, according to recent reports, transplanted umbilical cord MSCs (UC-MSCs) accelerate recovery after CCl_4-induced liver injury [8,9] and transplanted UCMSCs reduce liver inflammation and inhibit hepatic stellate cell and myofibroblast activation. Prior to this report, our previous study had shown that human MSCs in the process of neural differentiation show MSC surface markers and are negative for the HLA marker in FACS analysis [10]. This implies that there would be no immunological problems related to the human histocompatibility complex and no graft rejection due to immune response. However, it remains uncertain whether UCB-MSCs can provide a better source for cell

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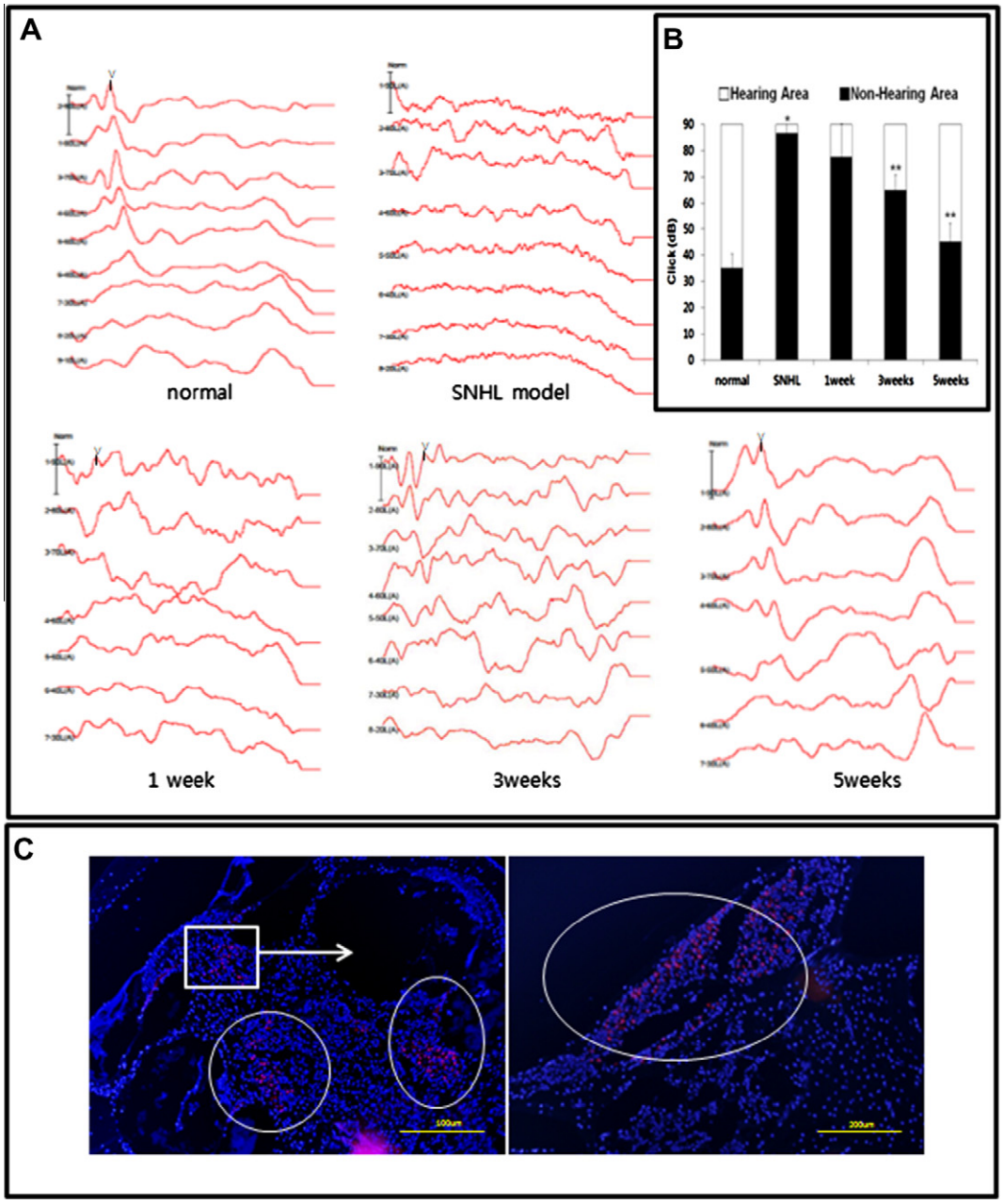

Fig. 1. (A) Auditory brain response (ABR) results compared between normal hearing, SNHL, and UCB-MSC transplantation groups. Click-evoked ABR waves were recorded up to 10 dB in guinea pigs with normal hearing. Three days after application of ouabain and neomycin to the middle ear, an increase was noted in the ABR threshold. After intravenous injection, the UCB-MSC group showed a significant improvement in hearing threshold compared to that for the SNHL group. (B) Representative graph showing the hearing and non-hearing area of each group. (C) Histological findings in cochleae after stem cell transplantation. Numerous PKH26-tagged UCB-MSCs were observed in all the turns of the cochleae from the first to the fifth week after stem cell grafting. Staining with only DAPI (Left: 200×; $n = 5$; bars, 100 μm. Right: 400×; $n = 5$; bars, 200 μm) ($^{*}p < 0.01$, normal model vs. SNHL model, $^{**}p < 0.01$, SNHL vs. after 1, 3, and 5 weeks).

replacement, trophic support, or cell-to-cell-contact therapy in cochlear injuries such as SNHL. The results of this study indicate that the use of human UCB-MSCs may aid in replacing damaged hair cells and SGNs and that intravenous transplantation of UCB-MSCs may also promote hearing restoration in SNHL animals.

2. Material and methods

2.1. Isolation of MSCs from human UCB

UCB was collected from pregnant women after obtaining consent and was placed in a heparin-treated vessel for storage. MSCs were isolated from a total of 10 samples of human UCB by using a centrifuge gradient method with Histopaque (Sigma–Aldrich, St. Louis, MO) [11]. All the MSCs were isolated from UCB using our previous study according to the isolation protocol [10].

2.2. Tagging PHK26 on UCB-MSCs

For tracking of injected UCB-MSCs, we tagged the cells *in vivo* with the commercially available PKH26 stain. To prepare the UCB-MSCs for intravenous transplantation, they were detached from the plates by using trypsin EDTA (Gibco). Approximately 1×10^7 single cells were placed in a conical bottom polypropylene tube and washed once with serum-free medium. UCB-MSCs

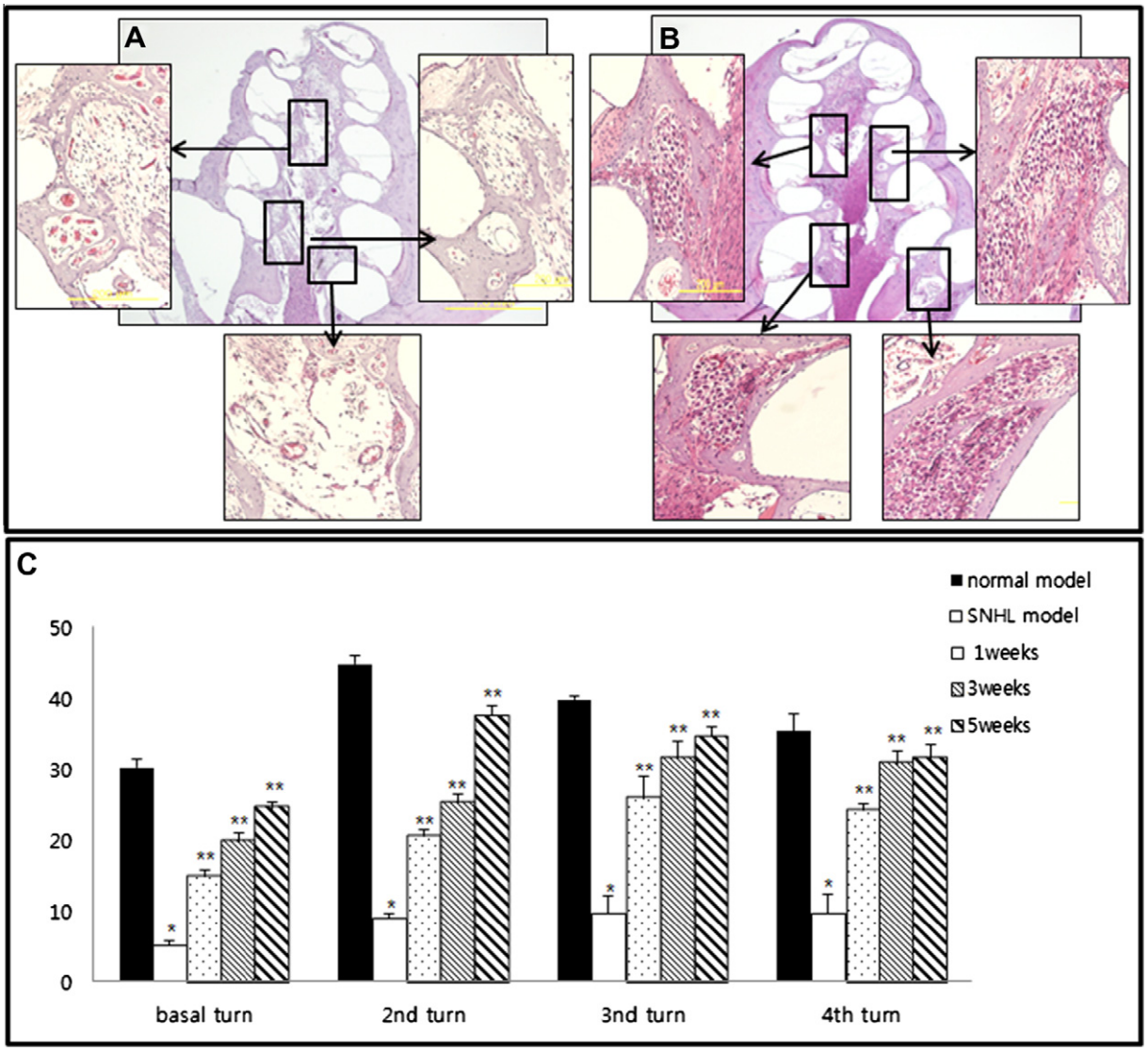

Fig. 2. Regeneration of SGNs after UCB-MSCs transplantation. (A) Severe loss of SGNs from the basal to the apical turn of the cochlea was observed in the SNHL group. (B) Five weeks after transplantation of UCB-MSCs, SGNs were regenerated in all the turns of the cochleae (H&E staining [inside: 40×; $n = 5$; bars, 1.0 mm. outside: 200×; $n = 5$; bars, 100 μm]). (C) Average neuron counts in each turn of the spiral ganglion for each treatment after transplantation. The average numbers of SGNs were significantly greater in the stem cell transplantation group than in the SNHL group (*$p < 0.01$, normal model vs. SNHL model; **$p < 0.01$, SNHL vs. after 1, 3, and 5 weeks).

were centrifuged at 1000 rpm for 5 min to form a loose pellet. After centrifugation, we carefully aspirated the supernatant and then added 1 ml of Diluent-C solution after suspending the pellet with gentle pipetting to insure complete dispersion. Immediately before staining, 2 μl of PKH26 dye was prepared in polypropylene tubes by using 1 ml of Diluent-C. The cells were then mixed and incubated at 25 °C for 5 min. The staining reaction was stopped by adding an equal volume of culture medium. The cells were centrifuged at 1000 rpm for 10 min at 25 °C to remove the supernatant; subsequently, the pellet was resuspended in 1 ml of culture medium, transferred to a fresh polypropylene tube, and centrifuged at 1000 rpm for 5 min at 25 °C. The cell pellet was washed 2 more times with 200 μl of physiological saline and transferred to a Hamilton syringe (Hamilton Company, Nevada, USA).

2.3. Establishment of SNHL and transplantation

Guinea pigs were purchased from the Central Lab Animal Incorporation (Central Lab. Animal Inc. Seocho-gu, Korea). On an average, the guinea pigs used in this study were 3 weeks old and weighed 250–300 g. The animals were divided into 4 groups ($n = 5$ per group), as follows: animal with normal hearing, animal with SNHL and injected with saline, and animal with SNHL and transplanted with UCB-MSCs. All the surgical procedures were performed under sterile conditions. The animals were anesthetized with a mixture of xylazine (40 mg/ml; Bayer, Dongjak-gu, Korea) and zoletil (60 mg/ml; Virbac, Songpa-gu, Korea) by intramuscular injection. The microsurgical techniques were performed under a Zeiss Universal S2 surgical microscope (Carl Zeiss, Goettingen, Germany). To produce the SNHL models, a mixture of 10% neomycin and 5 μM ouabain octahydrate (both from Sigma–Aldrich) was injected into the middle ear in gel form. One SNHL group was injected ouabain and neomycin mixture, to keep long-term counterpoise. Another SNHL group was injected with saline at 3 d after being injected with the neomycin and ouabain mixture, to establish long-term counterpoise. The other experimental group was injected via the brachial vein with PKH26-tagged UCB-MSCs (1×10^7 cells in 100 μl) by using a Hamilton syringe and a microinfusion pump at a speed of 20 μl/min. The hearing threshold for the SNHL

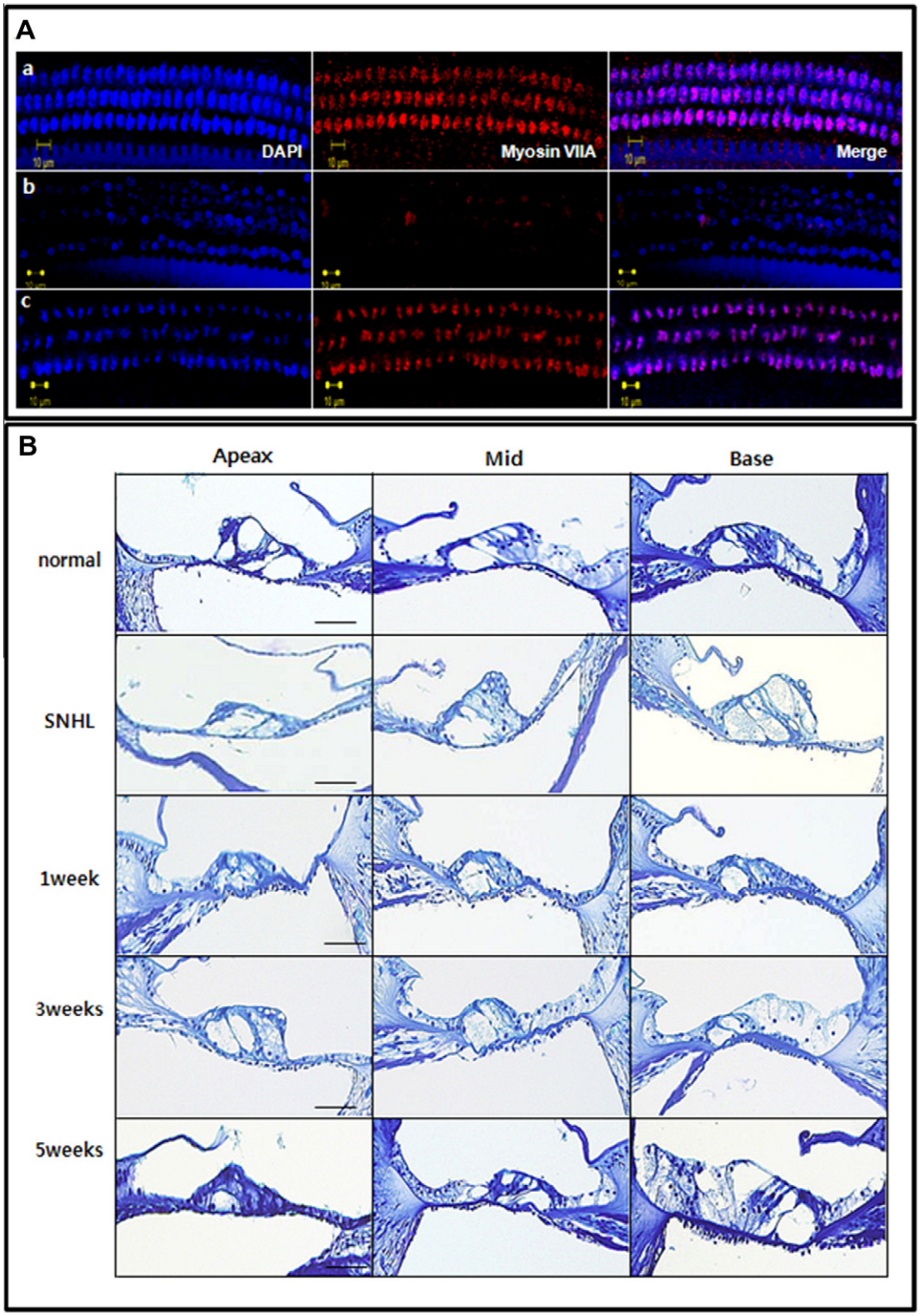

Fig. 3. (A) The hair cells of normal hearing guinea pig cochleae were intact from the base to apex when stained with the hair cell marker myosin VIIA (a). In the SNHL group, multiple hair cells were lost from the basal to the apical turn of the cochleae (b). Five weeks after transplantation of UCB-MSCs, hair cells were visible (c). (×400, $n = 3$, bars, 100 μm). (B) Serial microscopy sections of the organ of Corti (OC) from normal hearing versus SNHL and transplanted UCB-MSCs groups from the basal, middle, and apical regions of the cochleae. Normal state (A): the morphological features of OC did not indicate damage. However, the SNHL group showed damage from ouabain and neomycin application and degeneration of outer hair cells at all turns of the cochlea (B). However, after transplantation of UCB-MSCs, the OC was regenerated in comparison with the SNHL state (400×; $n = 5$; bars, 100 μm).

model and the experimental group were compared and graded using auditory brainstem response (ABR) recordings and the distortion product otoacoustic emission (DPOAE) test for outer hair cell functions at 1, 3, and 5 weeks.

2.4. ABR recordings and the DPOAE test

Hearing thresholds were evaluated by ABR recordings, and outer hair cell functions were estimated by the DPOAE test for each

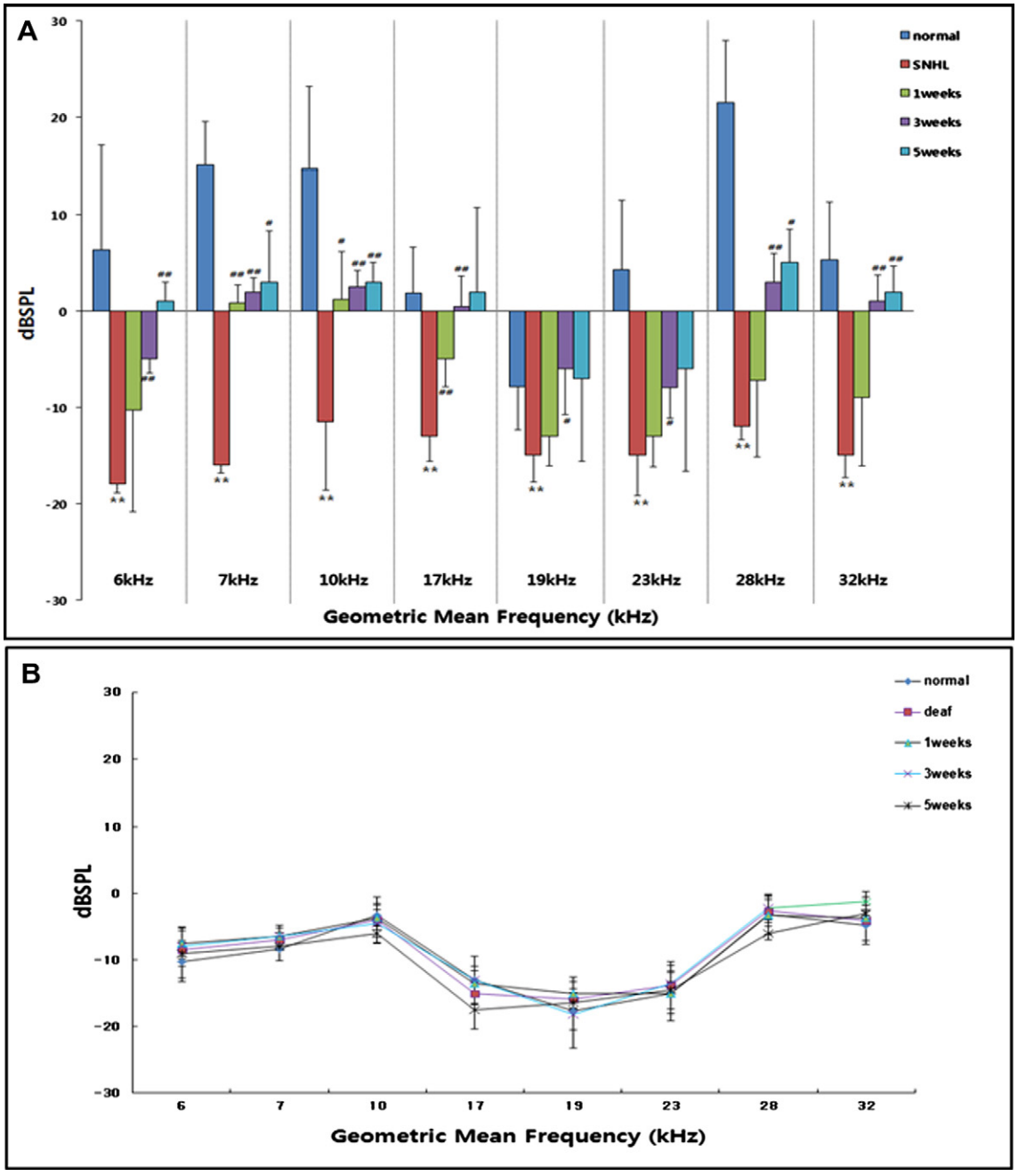

Fig. 4. (A) The SNHL group showed clear defects in outer hair cell function from the mid-to-high frequency region, which has been observed as a low-distortion product of the DPOAE level compared with the normal hearing and UCB-MSC transplanted groups. After UCB-MSC transplantation, the DPOAE values approached those of the normal state. (B) Noise floor ($^{**}p < 0.01$ vs. normal; $^{\#}p < 0.05$ vs. SNHL, $^{\#\#}p < 0.01$ vs. SNHL).

group. The ABRs evoked were recorded from the scalp of the guinea pigs. The responses were recorded using sub-dermal needle electrodes at the vertex, below the pinna of the left ear (reference) and below the contralateral ear (ground). The sound stimuli included clicks (100-ms duration; 8 Hz), and ABR measurements were obtained with an Intelligent Hearing System (IHS), that is, the Smart EP System, running the IHS high-frequency software (ver. 2.33) and using IHS high-frequency transducers. Acoustic stimuli were presented directly to the ear canal. Stainless steel needle electrodes were placed subcutaneously at the vertex and over the bullae, with a reference electrode at the occiput. The responses were digitally band-pass filtered (100–3000 Hz), amplified, and time averaged (sweep = 512–1024) for a post-stimulus time window of 50 ms. A stimulus intensity ranging from 90 decibels (dB) sound pressure level (SPL) down to a threshold of 10 dB was used, with 10-dB intervals near the threshold level. The ABR thresholds were measured by detecting the presence of wave V. At the threshold intensity, at least 2 sequences of recordings were made to confirm response reproducibility.

DPOAEs were recorded using the HIS of smart OAE 4.26(HIS). DPOAE measurements were conducted for pure tones ranging from 6 to 32 kHz. An Etymotic10B+ probe was inserted into the external ear canal and used in conjunction with 2 different types of transducers, depending on the range of the stimulation frequency. An EtymotionER2 stimulator was used for frequencies ranging from 6 to 16 kHz. For frequencies ranging from 16 to 32 kHz, an HIS high-frequency transducer was used. Stimulus response signals were sampled at a rate of 128 kHz by using a 16-bit D/A converter.

The L1 amplitude was set to 65 dB SPL, and the L2 amplitude was set to 55 dB SPL. Frequencies were acquired with an F2:F1 ratio of 1.22. Five stimulation levels, ranging from 65 to 25 dB SPL in 10-dB steps, were used. In total, 4 blocks were acquired, with each block consisting of 32 sweeps. The mean ABR threshold and DPOAE values for the SNHL and intravenous transplanted UCB-MSC groups were compared using the Student's *t*-test. A *p*-value of <0.01 was deemed to indicate statistical significance.

2.5. Light microscopy and SGN cell counts

Guinea pigs were anesthetized, and their cochleae were isolated and dissected. The cochleae were perfused through the round and oval windows with both 2% paraformaldehyde (PFA) and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and were incubated in the same fixative overnight at 4 °C. The cochleae were rinsed with 0.1 M PBS and incubated in 1% osmium tetroxide overnight, followed by immersion in Calci-Clear (National Diagnostics Inc, Atlanta, USA) for 3 d. The calcified cochleae were then dehydrated in ethanol and propylene oxide and embedded in Araldite 502 Resin (Electron Microscopy Sciences, Fort Washington, USA). The cochleae were sectioned at 5 μm, stained with toluidine blue, and mounted in permount on microscope slides. The image processing and analysis capabilities of Java (ImageJ; National Institute of Health, Bethesda, MD, USA) were used for morphological observations. The SGNs were counted from the same section used for the morphometric study of the organ of Corti (OC). Rosenthal's canals from the basal, mid, and apical turns were circled to determine the total area, and all the spiral ganglion cells were counted. The number of SGNs was divided by the area measured to obtain SGN density. Comparison of mean SGNs counts was performed using the Student's *t*-test. A *p*-value of <0.01 was deemed to indicate statistical significance.

2.6. Surface preparation

The cochleae of guinea pigs were perfused with 4% PFA and retained in fixative overnight at 4 °C. After rinsing with PBS, the cochleae were decalcified for 3 d. The otic capsule was then removed, followed by the removal of the lateral wall, Reissner's membrane, and tectorial membrane. The remaining portion of the OC was stained with the hair cell marker myosin VIIA (1:200) overnight. After rinsing with PBS, each turn of the cochlea was whole-mounted. Hair cells in the OC were visualized under a microscope equipped with epifluorescence. Fluorescent images were obtained using a Zeiss LSM510 META confocal microscope (Carl Zeiss). The captured images were processed using Zen 2009 Light Edition (Carl Zeiss).

2.7. Statistical analysis

All results have been reported in terms of mean ± standard error of the mean values. Statistical analysis was performed with an independent sample *t*-test comparison of the 2 experimental groups by using SPSS (version.15.0, SPSS Inc., Chicago, IL, USA). A *p*-value of <0.01 was deemed to indicate statistical significance.

2.8. Ethics statement

This study protocol and procedures were reviewed and approved by the institutional review board (2011-0137-02) and the Institutional Animal Care and Use Committee (IACUC) of the Department of Laboratory Animals at the School of Medicine, Catholic University of Korea.

3. Results

3.1. Isolation of UCB-MSCs

We isolated UCB-MSCs from post-parturition placenta. After culture for 5 d, the culture contained mononuclear cells that were attached to the dish surface and suspended. As the cell number in the subcultures increased, the cells became elongated and resembled fibroblasts (data not shown).

3.2. Hearing restoration with transplanted UCB-MSCs

To evaluate the effects of the UCB-MSC transplantation on the restoration of hearing in deaf animals, we performed the ABR test and compared the hearing capacity among the normal, SNHL, SNHL-saline, and UCB-MSC transplantation groups at 1, 3, and 5 weeks. Stimulus intensity ranging from 90 dB SPL to a threshold of 10 dB was used Fig. 1(A and B).ABR and OAE measurements showed a significant difference in hearing in the SNHL group in comparison with normal hearing Figs. 1(A) and 4(A). In addition, SNHL and SNHL injected with saline were identified the ABR threshold testing trough at 1, 3 and 5 weeks, indicating that the hearing level was preserved in the deaf state. The DPOAE levels and the recorded thresholds were similar between the animals with SNHL and the animals with SNHL injected with saline. Therefore, we combined the data for these 2 groups and presented a single result. The DPOAE measurements demonstrated a defect in the function of outer hair cells, indicated by the fact that this group had lower DPOAE levels than the transplanted UCB-MSC group Fig. 4(A and B).However, in the transplanted UCB-MSC, the ABR threshold testing trough at 1, 3, and 5 weeks showed improved hearing as time passed. In particular, at 5 weeks after transplantation, ABR threshold data showed a hearing improvement of up to 40 dB compared to the level observed for the SNHL (80–90 dB) Fig. 1(A). We also examined the localization of human UCB-MSCs after transplantation into the brachial vein. Transplanted UCB-MCSs tagged with PKH26 fluorescent cells were observed to be located near the spiral ganglion, as well as near sensory hair cells from the basal to the apical turn of the cochleae Fig. 1(C). There was no evidence of acute rejection, and no noteworthy inflammation response was observed in the stem cell transplantation group (i.e., sudden deafness, lack of appetite, seizure, convulsions, and weight loss).

3.3. Surface preparation and morphological changes in the organ of Corti

To produce the SNHL models, we used ouabain and neomycin application. After treatment with this mixture, severe damage to the SGNs and outer hair cells from the basal to the apical turn of the cochleae was observed in all SNHL Fig. 3(B). At this time, we confirmed the loss of SGNs and abnormal sensory hair cells from the basal to the apical turns of the cochleae Figs. 2(A and B) and 3 (A–b). In the SNHL and SNHL-saline, all turns were observed to suffer a substantial loss of afferent innervation and showed damaged hair cells Fig. 3 (A–b and B). However, in the transplanted UCB-MSC group, the morphological features of SGNs improved, and the SGNs were formed normally at all turns of the cochleae at 1, 3, and 5 weeks as time passed after cell grafting Figs. 2(B) and 3(B). The transplanted UCB-MSC group showed several cases of regeneration of sensory hair cells at the middle and base of the cochleae Fig. 3(B). We also reconfirmed the SGN cell counts, which were averaged to obtain a value representative of all the turns Fig. 2(C).

4. Discussion

Many advances have been achieved in the field of MSCs; recently, many stem cell researchers have demonstrated that various types of stem cells can differentiate into different kinds of cells such as cartilage cells, insulin-processing cells, and muscle cells [7,12,13]. In addition, bone marrow MSCs have been reported to differentiate into myocytes, cardiocytes, and neuron-like cells [4,6,13]. Thus, MSCs have attracted much attention as candidates for replacement or regenerative therapy of disease [2,5]. However, lately, MSCs have been isolated from alternative sources, including adipose tissue, epithelial cells, and umbilical cells [9,14,15]. Also, we confirmed that a subset of the UCB-MSCs could differentiate into a neuronal cell and alternative supporting cells phenotype [10]. As shown above, it has been documented that multipotent MSCs synthesize a wide variety of growth factors and cytokines that can have a paracrine effect on local cellular vitals [16]. Such trophic effects may be observed irrespective of the direct differentiation of transplanted cells into lineages of the respective tissue, as demonstrated earlier in an ischemic acute renal failure model [16,17].

The application of neomycin to inner ear hair cells is a well-known method that induces the death of hair cells. Also, neomycin treatment is generally known to induce the decrease or death of most SGNs [18]. In our study, the final goal of the stem cell therapy in SNHL patients is hearing normalization, that is, not only restoration of missing SGNs but also an increase in other hearing functions. So, we produced the SNHL model by application of a mixture of ouabain and neomycin. And we expected to find that MSCs have some practicability when compared with other stem cells. First, a significant change in threshold was observed when comparing the results related to SNHL and UCB-MSC transplantation. The SNHL model showed a defect in the function of outer hair cells, indicated by a decrease in DPAOE levels compared with those of the normal hearing and UCB-MSC transplantation groups. However, in the UCB-MSC transplantation group, we observed that UCB-MSCs had integrated into the inner ear, and the ABR threshold and DPOAE test trough data indicated an improvement in hearing levels. Second, the number of SGNs increased at all turns of the cochleae after UCB-MSC transplantation, and the PKH26-tagged UCB-MSCs were located near the basal to apical turn of the OC. This suggests that the UCB-MSCs enhance the lost or damaged neural cells in the spiral ganglion and implies that the migration and differentiation of grafted stem cells are influenced by signals in the microenvironment of damaged tissue. Some *in vivo* experiments have conclusively shown that UCB-MSCs can induce total liver recovery by acting as an adjuvant [9]. Buraa et al. have also demonstrated that stem cells act through a dual mechanism: cell-to-cell contact and modulation mediated by soluble factors produced by the cells themselves. It is possible that the cells can also act while they are in the bloodstream and complete their action upon reaching the neuronal support cells [9].

In recent years, research on stem cells has suggested the practicability of the clinical use of stem cell transplantation for intractable diseases and neurodegenerative disorders [19]. It has been suggested that the migration and improvement of grafted stem cells are influenced by the cell conditions of damaged tissue. Restoration or replacement of SGNs is one of the best targets for stem cell therapy in the treatment of hearing loss [20]. Besides, the implications of the interplay of stem cells would enable the control of therapeutic cells and effective regeneration (growth factors, cell-to-cell contact, and cell matrix interaction) of functional tissue [21]. Thus, these data suggest that UCB-MSCs could cause trophic effects through cell-to-cell contact and modulation mediated by soluble factors produced by multipotent cells. However, whether human UCB-MSCs or any other stem cells can provide the best source for cell-replacement therapy remains unclear.

The present study provides the scientific basis for the requirements for UCB-MSCs in order to ensure better treatment of therapeutic agent sources. This result indicates that there are several possibilities for treating intractable SNHL with MSCs by regenerating the hair cells and spiral ganglion of the inner ear. Furthermore, the use of multipotent and self-renewable MSCs for inner ear development will help in determining whether MSCs could give rise to therapeutic resources. We believe that these findings reinforce the conviction that UCB-MSCs have strong potential for differentiating into neurological lineage cells *in vitro*.

Lastly,Based on these results, UCB-MSCs could be used to treat intractable inner ear disease, especially in SNHL, at the cellular level.

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