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Mimic Cochlear Implant Surgery-Induced Cochlear Infection Fails to Further Damage Auditory Pathway in Deafened Guinea Pigs

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Data Interpretation D
Manuscript Preparation E
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Background: Kanamycin and subsequent furosemide administration was applied to the healthy guinea pigs to induce deafness.

Material/Methods: Of the deafened guinea pigs, 10 were further infused with anti-infection procedures (Group B) and the other 10 animals did not undergo anti-infection procedures (Group C). In Group B, the deafened animals were able to restore cochlear and middle ear functions following the anti-infection procedure. In Group C, all animals developed cochlear and middle ear infections.

Results: Compared to the healthy guinea pigs, hair cells and spiral ganglion neurons (SGN) of deafened animals (in Group B and Group C) were severely damaged. SGN density of deafened animals was significantly lower than that of healthy control animals in all ear turns except the basal turn. There was no significant difference between Group B and Group C in SGN density. The average optical density value of neurofilaments of deafened animals was also significantly decreased after the ototoxic drug administration. Notably, the density of the neurons in the cochlear nucleus region (CNR) of the brainstem were not significantly different between the healthy control guinea pigs and deafened animals.

Conclusions: Mimic cochlear implant surgery-induced cochlear infection caused no significant damage to the auditory pathway in ototoxic drug-induced deafened guinea pigs.

MeSH Keywords: Auditory Pathways • Cochlear Implants • Deafness • Guinea Pigs

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Background

In the mammalian cochlea, hair cell loss can be due to multiple factors, including aging, loud noises, or ototoxic drugs [1–3]. Hair cell loss can further lead to spiral ganglion neuron (SGN) damages [1–3].

Cochlear implant is still the main procedure to complement cochlear function [4–6]. However, this procedure requires a sufficient number of SGNs to recover the hearing [7,8]. Certain residual contra-indications apply for the implant operation. The middle ear should be free from infection prior to the implant and infections in the cochlea and brain should also be avoided [9,10]. Therefore, a cochlear implant is not suitable for hearing loss patients with acute or chronic otitis media with otorrhea [9,10].

There is scant evidence suggesting that middle ear and/or inner ear infections can damage the auditory pathway or functions of cochlear implants [11]. Stathopoulos et al. [11] reported the potential risk of pneumococcal meningitis after use of a dexamethasone-eluting intra-cochlear electrode array.

In the present study, we explored whether the mimic cochlear implant procedure could change the auditory pathway of ototoxic drug-induced deafened animals. In line with previous findings [12–14], we showed that combined administration of kanamycin and furosemide caused permanent hearing loss in guinea pigs, which was associated with damages in the SGN and neurofilaments of habenular holes. Notably, long-term observation of these animals was not performed in the early studies [12–14]. Changes in neurofilament quantification, proximal and distal SGN filaments, and auditory neurons in the brain stem have also not been studied to date.

Intriguingly, published studies have presented inconsistent results in damage sites and severity with the various aminoglycoside drugs [15]. Certain aminoglycoside antibiotics (e.g., gentamicin) have more vestibule-toxic profiles. Others (e.g., kanamycin), on the other hand, have more cochlea-toxic profiles [16]. Here, we generated the deaf model animals by combined administration of kanamycin and furosemide. This animal model was applied to explore if the infection by mimic cochlear implant procedure could induce more damages to auditory pathways. The hair cells, SGNs, and the cochlear nuclei in the brain stem, as well as the neurofilaments connections between them, were observed. Possible brain infections were also assessed.

Material and Methods

Experimental animals

Thirty pigmented guinea pigs with body weight 250–350 g and normal prey reflex were included. Animals were purchased from the Fudan University Medical School Experimental Animal Science Department (Shanghai, China). Ten animals were assigned to the Control Group and the other 20 were assigned to the deafness groups (Group B and Group C, 10 each). The care and use of the animals and all experimental procedures were approved by the Ethics Review Committee (ERC) of Fudan University (Shanghai, China), and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23). The animals had thresholds ranging from 45 to 55 dB SPL prior to the experiment. Three days after drug administration, animals with thresholds higher than 95 dB SPL were included in the deafness groups, which were further divided into 2 groups: Group B animals underwent cochleostomy with anti-infection procedure (N=10) and Group C animals underwent cochleostomy without anti-infection procedure (N=10).

Drug administration

Kanamycin (500 mg/kg, Shanghai Asia Pioneer Pharmaceutical Co., Shanghai, China) was injected to the leg muscle using a hypodermic needle. After 2 h, intravenous injection of furosemide (100 mg/kg, Nantong Jinhua Pharmaceutical Co, Nantong, China) was performed [17]. The animals were anesthetized with xylazine (10 mg/kg, *i.m.*, Nanjing Pharma Chemical Plant, Nanjing, China) and Ketamine (40 mg/kg, *i.m.*, Jiangsu HengRui Medicine Co, Lianyungang, China) [18].

Auditory brainstem response (ABR)

ABR was tested prior to drug administration, as well as 3 days and 8 weeks after drug administration. Biologic auditory brainstem response potential AEP Windows V1.3.0 was utilized. Animals were anesthetized with the method detailed above. ABR was evoked through tone burst and recorded via subcutaneous electrodes placed in the ipsilateral pinna and vertex, with the ground electrode was placed by the contralateral pinna. The stimuli were tone bursts. The response was amplified (100 000×) and filtered (100 Hz–1.5 kHz). The sound level was raised from 15 dB SPL to 95 dB SPL in 5-dB steps. At each sound level, 1000 responses were averaged. Response waveforms were rejected if the peak-to-peak voltage exceeded 23.5 mV. We tested responses at 2, 4, 6, and 8 kHz for ABR threshold. Thresholds were determined by a single observer, who recorded the lowest sound level at which a recognizable waveform was observed on a screen of tracings stacked from lowest to highest sound level.

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Mimic cochlear implant operation with/without anti-infection procedures

The mimic cochlear implant procedure was described in previous studies [19,20] and were performed on the fifth day after drug administration. After anesthesia, the guinea pigs were kept in supine to expose the ventral skin of the neck. The tympanic bulla was exposed, and a 1.5-mm-diameter hole was drilled in the bony bulla. A small hole was made on the bone wall in the pigmented area of the striavascularis of the second turn of the left cochlea, and polyimide tubing (code039-I, Mmicrolumen company, Tampa, FL, USA) was inserted into the scala media through the perforation. Over a period of 5 min, 5 μL of saline was injected slowly. After inoculation, the anti-infection procedure in Group B was performed by sealing the opening on the bony bulla with dental zinc phosphate cement, and by covering the cochleostomy hole on the lateral wall of the cochlear with tissue mass. In Group C, the holes on the bony bulla and the cochlear lateral wall were left open, which allows the tissue fluid to filtrate into the bulla. This then induced infections in middle and inner ears.

Histological preparation

At the end of the cochleostomy, animals were injected with anesthetic agents and perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) via transcardiac puncture. Upon cervical dislocation and decapitation, the temporal bones with the inner ear and the brainstem were separated. Both cochleae were further micro-dissected from the temporal bone, which were opened at the apical tip as well as the round and oval windows. The dissected tissues were then fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C overnight. Afterwards, the bony wall of 1 cochlear was removed to reveal the surface of the sensory epithelium, and the whole mount of the basilar membrane was performed for immunohistological staining. The brainstem sections were fixed in 4% paraformaldehyde. Another cochlea was decalcified by immersion in a 10% ethylene diamine tetra-acetic acid (EDTA)-buffered solution for 10 days at 4°C. Afterwards, the specimens (including the brainstem) were incubated in 15% sucrose for 2 h, then 30% sucrose overnight at 4°C. The cochleae and brainstems were positioned on the cryostat plate at 20°C after incubation in optimum cutting temperature compound for 2 h at 4°C. The cochleae blocks were sectioned at a thickness of 8 μm parallel to the modiolus, and the brainstem was serially sectioned at a thickness of 50 μm with a Leica cryostat (Leica CM3050S, Leica Microsystems, Wetzlar, Germany). All of the sections were mounted on glass slides coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) and stored at 80°C until further use.

Immunohistochemistry and scanning electron microscopy

Following pre-incubation in 10% goat serum (BOSTER Biological Technology, Ltd., Wuhan, China), tissue sections were stained with the primary antibody, anti-Tuj1 (1: 1000, Abcam Inc., Cambridge, MA, USA), overnight at 4°C. This was followed by incubation with the secondary Alexa fluor-555 goat anti-rabbit IgG (H+L) antibody (Molecular Probes, Eugene, Oregon; 1: 400) at 37°C for 1 h. Counterstaining with 4, 6-diamino- 2-phenylindole (DAPI, Molecular Probes, 1: 600) was also conducted. The cochlea and brainstem sections were then air-dried and stained with toluidine blue. Sections were incubated in 1.3% toluidine blue solution for 5 min at 55°C, rinsed in distilled water for another 20–30 min, rinsed in 70% ethanol for 10 min, and then mounted with 10% glycerol and sealed by nail polish. The brainstem sections were selected according to a brain atlas. The whole mount membrane was incubated in 0.3% Triton-100 for 40 min, followed by Phalloidin-TRITC (1: 600, Sigma, St. Louis, MO, USA) for 40 min at 37°C. Next, the specimens were mounted by 10% glycerol and sealed with nail polish. Sections stained without primary antibody application were utilized as the negative control. Specimens were observed with a Leica DMR fluorescence microscope and Leica DFC 300 FX image processing system, or a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems, Inc., Shanghai, China). Cochlea (2 cochleae of control animals and 4 cochleae of deafness animals) for scanning electron microscopy were first trimmed to expose the organ of Corti, and then fixed in a mixture of 4% paraformaldehyde and 2.5% glutaraldehyde at pH 7.3, and post-fixed in 1% osmium tetroxide in cacodylate buffer. Dehydrated cochleae were sputter-coated with palladium gold and observed with a HITACHI S-520 (Hitachi, Tokyo, Japan) scanning electron microscope.

Determining density of the SGN and brainstem cochlear nucleus neurons, as well as the average optical density (OD) value of neurofilaments in Rosenthal's canal and modiolus

All counts of cells in sections of brainstem were based on the average of total SGN in 7 canals from 4 serial sections taken at 1-slide interval. The density (number of cells 10 000 μm^2) of the cells in the area of cochlea nuclear and Rosenthal's canal were determined using Image Pro Plus software and ImageJ image processing software (NIH). The average OD value of neurofilaments in each cochlea habenular hole and modiolus region was determined using ImageJ Pro Plus image analysis software. All of the process was performed by the same researcher who was blinded to the sample groups [21].

Statistical analysis

To quantify SGN and neurons in the brainstem cochlear nucleus, the average density for all 4 sections was analyzed and compared between deafness groups and the Control Group using the SAS8.2 Wilcoxon rank sum test. Differences between average optical density of neurofilaments were compared using SAS 9.13 ANOVA (mean ±SD).

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Results

Combined administration of kanamycin and furosemide causes deafness in guinea pigs

Auditory brainstem response (ABR) test results showed that co-administration of kanamycin and furosemide efficiently induced deafness in guinea pigs at an efficiency rate of 80% (16/20). The whole-mount staining of basal membrane by phalloidin showed severe damage to inner and outer hair cells. Scanning electron microscope (SEM) image results showed the villi of the outer hair cells were lying flat after 3 days of deafness induction. Meanwhile, the first row of hair cells was severely damaged and there were few villi left after 8 weeks of deafness. No animals recovered from deafness, which was confirmed by ABR 8 weeks after drug administration (Figure 1).

The density of SGN and brainstem cochlear nucleus neurons and the average optical density value of neurofilaments in habenular holes and modiolus were reduced by kanamycin and furosemide co-administration

The average optical density (OD) value of neurofilaments in the cochlear modiolus and habenular hole are shown in Figure 2, Table 1, and Figure 3. There were significant differences in neurofilament density between Group B and the Control Group in the cochlear modiolus (Figure 1), basal turn (Figure 2A, 2B), second turn (Figure 2C, 2D) and third turn (Figure 2E, 2F), but not in the apex turn (Figure 3). There were no significant differences between Groups B and C (basal turn 1467.4±465.5, 1497±273.1, P=0.856; second turn 1164.5±574.6, 1353.1±238.8, P=0.314; third turn 1025.7±477.4, 1184.6±263.9, P=0.331; apex turn 1081.9±606.7, 1065.6±370.0, P=0.945) (Table 1). The SGN density in the basal turn in the Control Group and Group B were 1682.2±260.15 and 1644.8±377.48, respectively (Table 1). No statistically significant difference was observed (P=0.1553).

The intensity of SGN was significantly reduced at all the turns in Group B (Figure 1, Figure 4), as compared with the control animals: the second turn was 1761±266.14 *vs.* 1254.7±447.54 (P=0.0036), the third turn was 1625.0±299.34 *vs.* 1101.7±390.12 (P=0.0011), and the apical turn was 1584.9±452.77 and 1074.2±494.93 (P=0.0146) (Figure 4). There was no significant difference between Group B and Group C in any turns (P>0.05) (Table 1). There were no significant differences in the quantity of brainstem cochlear nuclear neurons in any groups (Group B 758.15±176.07, Group C 782.7±90.4 and Control Group 682.7±111.3, P=0.2134) (Figure 1).

Manifestations of deafened guinea pigs with middle ear and cochlea infection

All the animals in Group C had infections in the middle and inner ear but Group B animals showed no infections at all. The space of the acoustic bulla and the cochlea were infused with pus in Group C animals. The bones of the bulla and cochlea became thicker. The Group C animals with infection showed tilt head only without sag, hair-slip, inappetence, or weight loss. There was no significant difference between Group B and Group C in SGN density. Further, there was no significant difference in filament density between Group B and C (Table 1). No brain infection was observed in any of the animals from Group C (data not shown).

Discussion

Hypodermic injection of kanamycin plus subsequent intravenous injection of furosemide effectively induces deafness, and the peak administration time of kanamycin is 1–2 h after injection [13]. Following administration of furosemide, kanamycin enters cochlear endolymph, and kanamycin will reach the highest concentration in the striavascularis [13]. This will eventually lead to deafness in the most effective manner [13]. Results of the present study demonstrated that kanamycin with subsequent furosemide administration induced profound deafness in 80% of tested guinea pigs. The auditory thresholds of these guinea pigs were up to 95 dB SPL. In comparison of our results with previous findings, we showed that the time interval between kanamycin and furosemide administration could be the key factor determining the severity of cochlear damage [22].

In the present study, administration of kanamycin and furosemide led to severe hair cell damages, which was most dramatic in the outer hair cells. Notably, the damage severity of hair cells and the support cells increased from the inner row to outer row. Remarkably, the loss of hair cells and support cells also induced collapse of the organ of Corti. The co-administration method caused more profound damage in the cochlear than reported in a previous study [23], possibly because guinea pigs in our study were more sensitive to ototoxic drugs.

Besides damage to the hair cells and support cells, direct or indirect toxicities to the SGN and their connected neurofilaments were also observed the drug-treated guinea pigs. The loss of SGN in deafened animals was more severe than in control animals in almost all turns except at the basal turn. It was, however, reported that gentamycin and furosemide co-administration in rats can lead to more severe damage of SGN in basal turns than in other turns [24].

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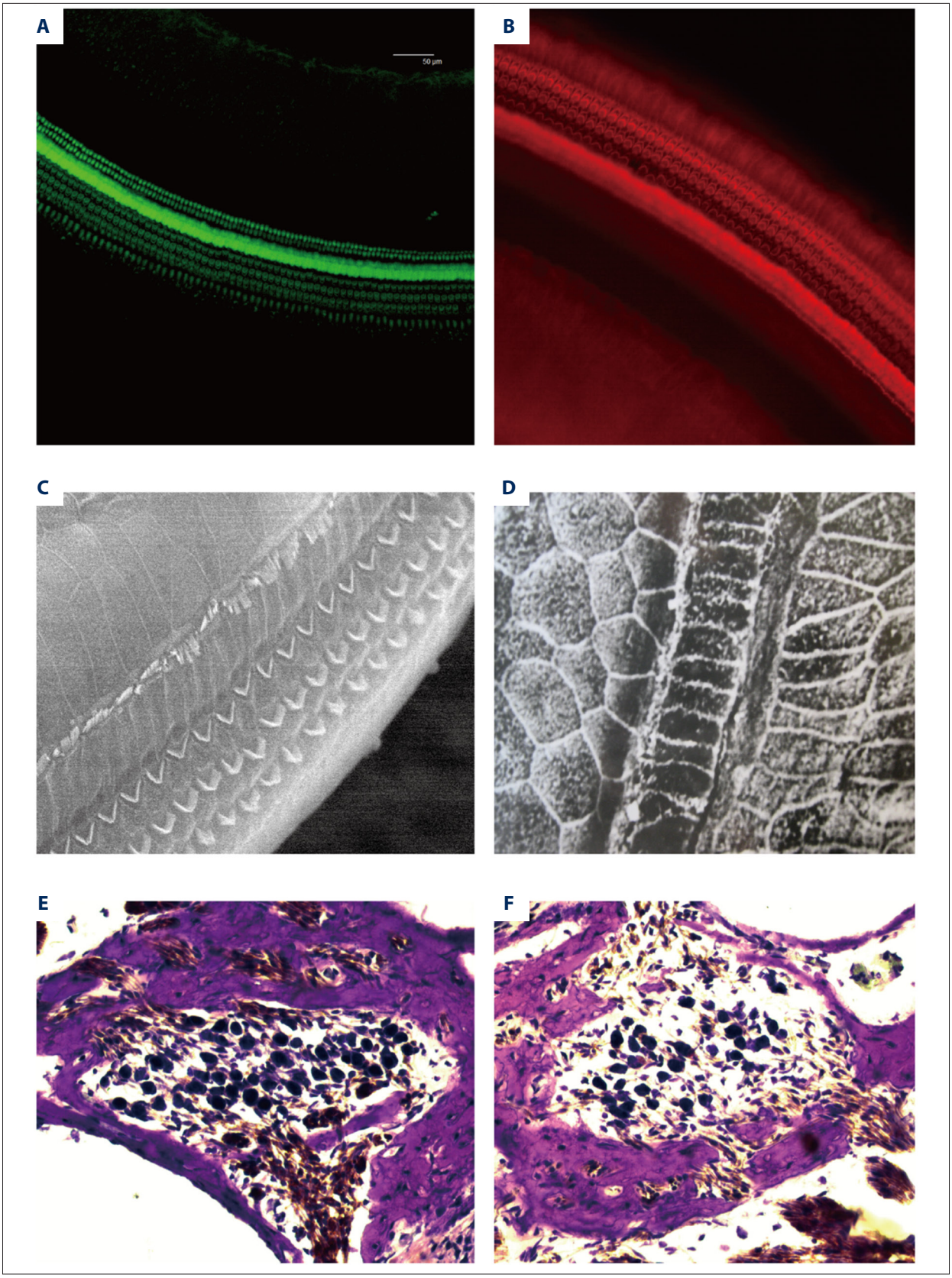

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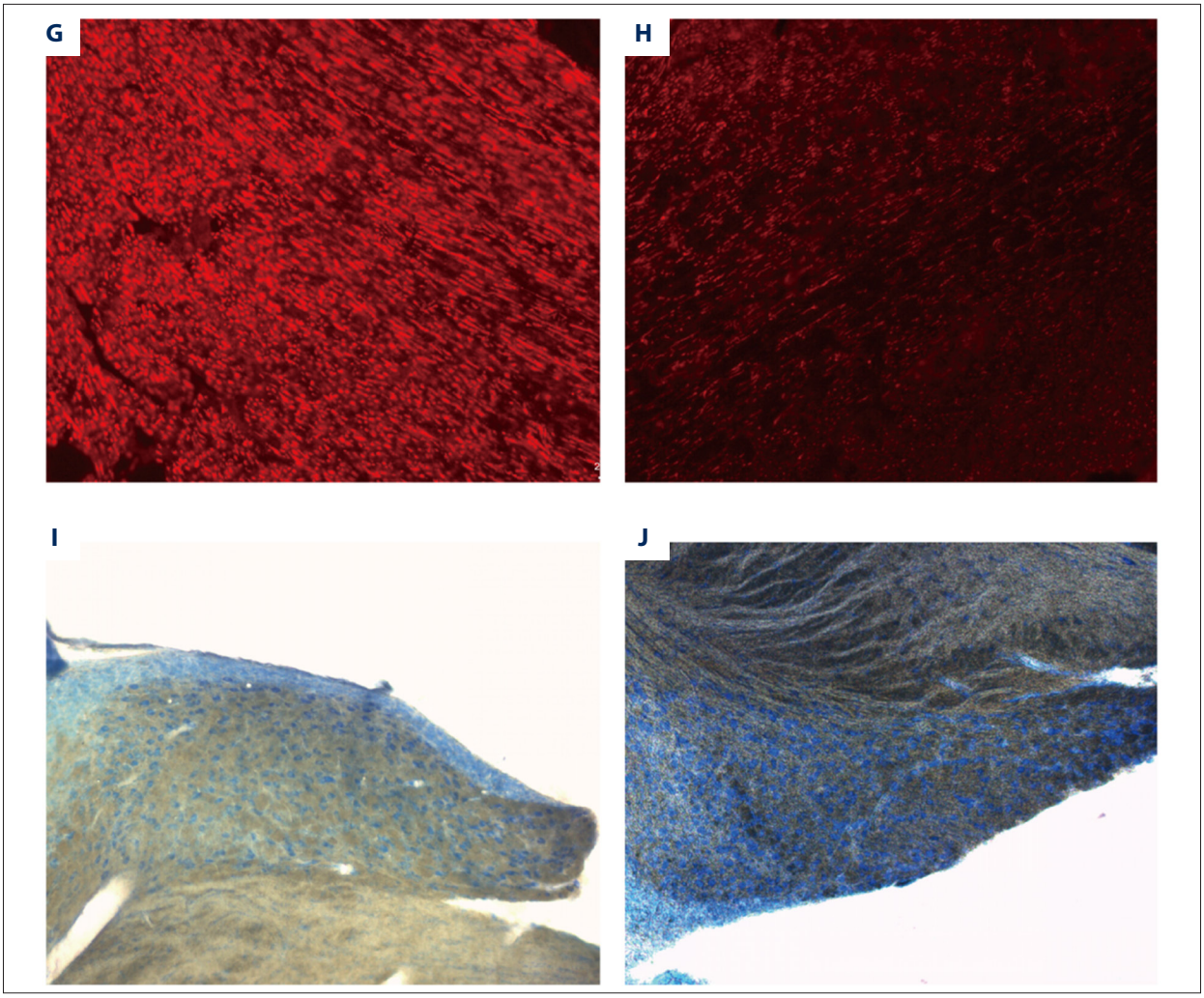

Figure 1. Guinea pigs were treated with vehicle control or kanamycin plus furosemide co-administration, whole amount and scanning electron microscopy (SEM) showing hair cells on the basal members, SGN in the Rosenthal canal, neurofilaments in the cochlear modiolus, and neurons in the cochlea nuclear region. (**A**) Phalloidin staining normal outer and inner hair cells in the second basal member by phalloidin staining; (**B**) Loss of cilia of 3 rows of outer hair cells as well as inner hair cell loss in the second turn basal member 8 weeks after deafness; (**C**) Scanning electron microscope (SEM) image (1500×) showing normal hair cells at second turn; (**D**) SEM image (1500×) showing complete outer hair cells loss and inner hair cells with cilia lying down 8 weeks after deafness induction; (**E**) Toluidine blue staining showing SGN in Rosenthal's canal of the second turn; (**F**) SGN intensity 8 weeks after deafness induction; (**G**) Tuj1 staining (red color) showing the neurofilaments in the normal cochlear modiolus; (**H**) Neurofilaments in the normal cochlear modiolus were significantly reduced 8 weeks after deafness induction; (**I**) Toluidine blue staining of the normal neurons in the cochlea nuclear region; (**J**) Toluidine blue staining of neurons in the cochlea nuclear region 8 weeks after deafness induction.

Presence of spiral vessels in guinea pigs (but not in adult rats [25]) may protect SGN in the basal turn, possibly because spiral vessels maintain better blood flow in guinea pigs than in rats in these particular regions. In addition, our results showing SGN damage were slightly different from those of a previous study [26]. It had been demonstrated that noise-induced SGN loss was most significant in the second turn in the first month [27]. However, in the present study, significant SGN loss was observed in second, third, and apex turns of co-administration-induced guinea pigs. These results suggest that noise and the ototoxic drug have distinct effects on the site of damage and SGN injuries. Notably, infection in the middle ear and cochlea had no further impact on the SGN in any turns.

In other studies, furosemide was applied 15–60 min after kanamycin administration, and the most severe loss of SGN and hair cells was observed in basal turns [22]. The only difference between those results and those of the present study was the time period of infusion and the dose of furosemide [22]. Thus, the time interval between administration of the 2 drugs

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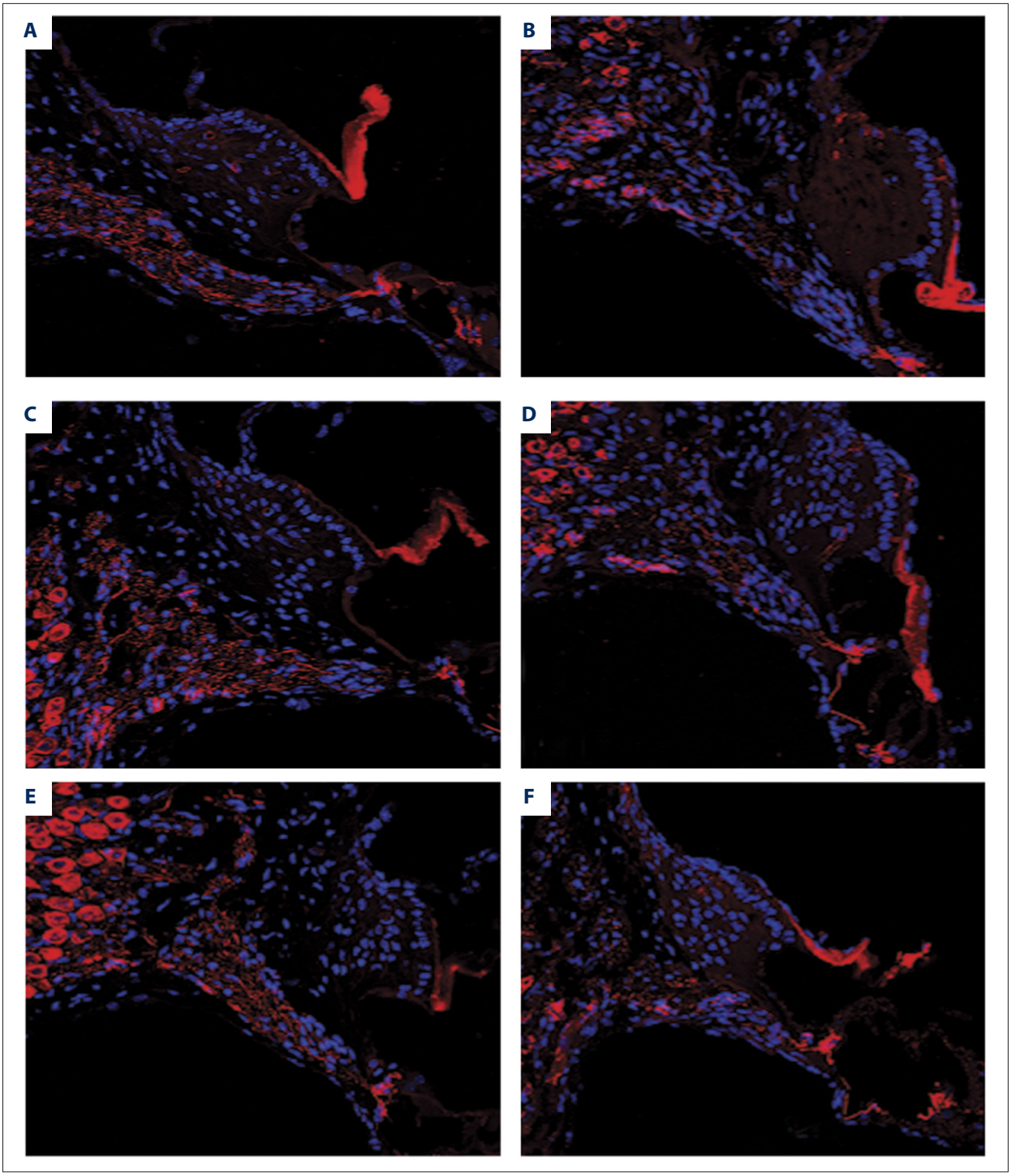

Figure 2. Comparison of the neurofilaments staining in the habenular holes in the Control Group (**A, C, E**) and Group B (**B, D, F**). (**A**, **B**) show the basal turn; (**C, D**) show the second turn; and (**E, F**) show the third turn. The red color is Tuj1 staining and the blue color is DAPI nuclear staining. Tuj1 staining results demonstrated a significant decrease in the number of neurofilaments in Rosenthal's canal. Bar=50 μm.

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Table 1. Comparison of the average optical density value of the neural filaments in habenular hole and modiolus between normal and deafness groups (surgery sides and nonsurgery sides). (X (—)$\bar{X}$±SD).

Group	N	Basal burn	2nd turn	3rd turn	Apex	Modiolus
1 Normal	7	0.481±0.075	0.471±0.095	0.441±0.098	0.414±0.125	0.288±0.089
2 Deafness	7	0.845±0.253	0.720±0.182	0.681±0.229	0.541±0.097	0.605±0.145
3 Infection	7	0.864±0.044	0.656±0.082	0.576±0.163	0.446±0.008	0.507±0.071
1, 2 P value 2, 3 P value	-	0.003* P>0.05	0.008* P>0.05	0.025* P>0.05	0.068 P>0.05	<0.001* P>0.05

* *P*<0.05.

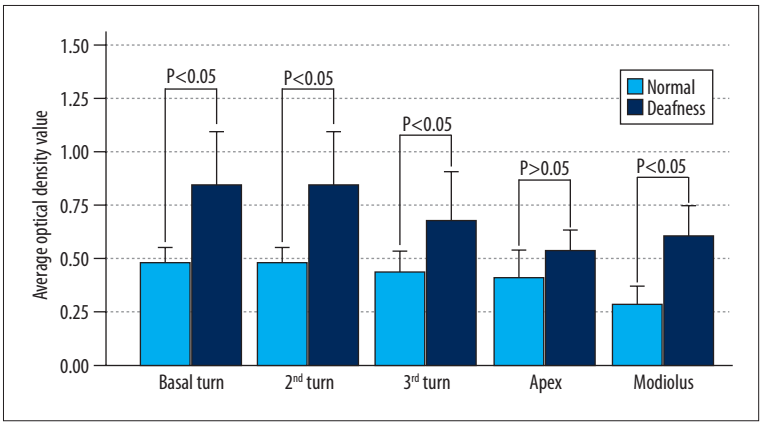

Figure 3. Average optical density of the neurofilaments in the habenular holes and modiolus in Group B (8 weeks after deafness) and Control Group. There were statistically significant differences between Group B and the Control Group in basal turn, second turn, third turn, and cochlear modiolus (*P*<0.05) but no significant difference was observed in apex turn (*P*=0.0683). A higher value of average optical density was associated with fewer neurofilaments.

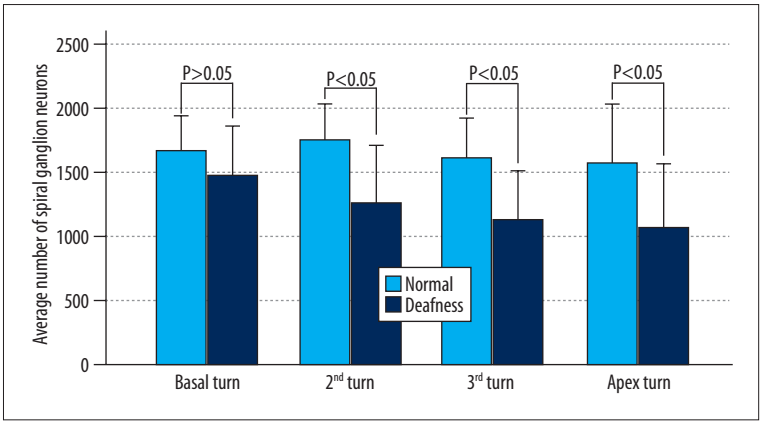

Figure 4. In comparing SGN densities between Group B (8 weeks after deafness) and the Control Group, statistically significant differences were observed in the second turn, third turn, and apex turn, but not in the basal turn.

is an important determinant of SGN damage. In our system, although inner and outer hair cells in the second turn were completely lost after furosemide plus subsequent kanamycin administration, the inner hair cells in the third turn remained.

In the present study, significant damages were observed in auditory pathways, including hair cells, SGN, and neurofilaments. Yet, there was no significant neuronal changes in the brainstem of the CNR. This could possibly explain why patients with total deafness can still partially recover by implantation of an electric brainstem. It should be noted that the loss of SGN in the basal turn was less significant than in other turns, and this could be due to the fact that the neurons in basal turns may receive nourishment from the brainstem auditory center.

The complementary changes in expression of vesicular glutamate transporter 1 (VGLUT1) and VGLUT2 correspond to the down- and up-regulation of co-labeled auditory nerve fibers (ANF) and somatosensory terminals, respectively. This pattern of redistribution persists for at least 6 weeks after cochlear ablation [28]. These results suggest that somatosensory inputs compensate for ANF deafferentation and the upregulation of excitatory neurotransmission from somatosensory projections. These findings also support our results that the ANF

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deafferentation does not inhibit the cochlear neurons. This might possibly also explain why a cochlear implant is clinically efficient, as the cochlear implant does not need the distal neurofilaments of the SGN. No significant difference was observed in the apex turn neurons, possibly due to the difficulty in obtaining adequate sections.

We did not observe any differences in auditory pathway damage between Groups B and C, except for the presence of pus in the middle and inner ears in Group C. The basal structure and function of the cochlear implant was intact, and it was not damaged by infection from the cochlea and middle ear. The structure of the deafness cochlea and middle ear prohibits the diffusion of the infection. Although the structures of the guinea pig middle ear and inner ear are different from those of humans, the results of the present study are still translatable to clinical practice since antibiotics are being administered to patients to prevent and control the infection by implantation procedures.

Conclusions

The induction of deafness by furosemide plus kanamycin administration was successful in guinea pigs, showing profound damage to hair cells, support cells, SGN, and neurofilaments. Neurons in the CNR of the brainstem were not injured. The infection induced by the mimic cochlear implant procedure did not cause further damage to the auditory pathway.

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