Original Article

A study of gentamicin injury mechanisms using cultured mouse cochlear spiral ganglion cells

GU Xi, LIN Chang, ZHANG Rong

Department of Otolaryngology-Head and Neck Surgery, First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian 350005, China

Abstract Objective To study gentamicin injury mechanisms using postnatal mouse cochlear spiral gangcells (SGC). **Methods** SGCs were isolated using a combinatorial approach of enzymatic digestion and mechanical separation from P2 ~ 6 Kunming mouse cochleae. After 4 days, cultured SGCs were fixed with 4% paraformaldehyde at room temperature for immunocytochemical examination using the methods of S-P and the monoclonal antibody against mouse neurofilament protein (Neurofilament-68/200Kda, NF-L+H). SGCs were randomly divided into a blank control group and three gentamicin treatment groups (medium gentamicin concentration at 50 mg/L, 100 mg/L and 150 mg/L respectively), SGCs were collected and examined under a transmission electron microscope after being cultured for 48 h. **Results** SGC primary culture was successful. SGC cytoplasm and neurites were dyed brownish yellow by the monoclonal mouse neurofilament protein antibody. SGCs showed classical bipolar neuron appearance. Under the transmission electron microscope,.gentamicin treated SGCs showed morphological features different compared to those in the blank control group, which might indicate apoptosis. **Conclusion** Our results indicate that gentamicin has direct toxic effects on cochlear SGCs in mice and the injury mechanism is closely related with apoptosis. Damage to mitochondria may play an important role in the process.

Key words gentamicins; spiral ganglion; cells, cultured; microscopy; electron; transmission; apoptosis

Aminoglycoside antibiotics has been used clinically for more than half a century. Gentamicin is one of the aminoglycoside antibiotics that is more often used than others and its ototoxicity has been generally acknowledged. The mechanisms by which gentamicin induces inner ear injury are not completely understood. SGCs from the cochlea are the primary neurons of the auditory system, which are well differentiated and show little repair after injury. Recently, researchers have started to focus on the mechanisms of SGC injury in ototoxic deafness caused by aminoglycoside antibiotics, although the extent of studies is limited.

In this study on gentamicin ototoxicity, we used cultured SGCs to eliminate the influence from various internal environment factors, and used transmission electron microscopy to study SGC changes at the ultrastructural level. Our study aims at determining whether gentamicin has direct toxic effects on cochlear SGCs and whether the injury mechanism concerns apoptosis.

Correspondence author: LIN Chang, M.D., Ph.D., Professor, Department of Otolaryngology-Head and Neck Surgery, First Affiliated Hospital of Fujian Medical University. 20 Chazhong Road, Fuzhou, Fujian 350005, China. Email: linc301@yahoo.com

Materials and Methods

Animals

Kunming normotrophic suckling mice of 2 to 6 days of age (both genders) were used.

Drugs and Reagents

Reagents included poly-L-lysine (Sigma, USA); Penicillin G potassium salt, and Gentamicin solution (Sigma, USA), Trypsin and DMEM/F12(1∶1) culture medium (Gibco, USA), Fetal Bovine Serum(FBS), (Hyclone USA), Ara-C (Fluka, USA), Neurofilament-68/200KDa (NF-L+H) monoclonal antibody (Lab Vision-Neomarkers, USA), and SP immunohistochemical kit (Zymed, USA).

Culture medium preparation: We chose Dulbecco's modified Eagle's medium (DMEM)/F12 synthetic medi-

um which is fit for nervous tissue culture and supplemented it with 10% fetal bovine serum, 100 U/ml penicillin and 4 mmol/L glutamine. The pH value was adjusted to between 7.2 and 7.4 with NaHCO3. Finally, the culture medium was filter sterilized with a 0.22 μm microporous filter membrane.

Experimental methods

SGC primary culture　Neonatal Kunming mice (postnatal day 2 to 6) were executed by dislocation of cervical vertebra and were decapitated after being soaked in 75% alcohol. The cerebrum and cerebellum were removed and middle and posterior cranial fossa exposed. Both cochleae were removed from the cranium directly over the foramen magnum and soaked in the 4℃ pH7.2 D-Hanks solution. Under a stereomicroscope, the de-ossified cochlea was opened and the modiolus containing spiral ganglion was isolated by carefully removing the lateral vascular stria, spiral ligament and basilar membrane with microforceps. With this method, abundant modiolus tissue was acquired. And the whole process was under axenic conditions. The isolated modiolus was cut into about $1mm^3$ specimens, digested with 0.25% trypsin, in a 37℃ water bath for 30 minutes, and then dispersed by blowing the solution. The procedure of digestion was terminated by FBS. After centrifugation of the solution at 1000 rpm for 10 minutes, supernatant was discarded and the precipitate was transferred into the serum-containing medium. The medium was gently blown with a lambda pipet to disperse the cells evenly. Medium cell density was adjusted to 1×10^5/ml and the cells were moved into 6-pore culture plates coated with poly-L-lysine and containing 2 ml medium in every pore. Neurons were maintained in the culture at 37℃ in a humidified incubator with 5% CO^2. After 24 hours, the medium in the pore was replaced with fresh serum-free medium and 5 μmol/L Ara-C was added in the medium for 48 h to inhibit over-proliferation of non-neurons.

Immunocytochemistry identification of SGC　After 4 days of culture, the medium was removed, the plate rinsed with PBS, and the cells fixed for 30 minutes with 4% paraformaldehyde at room temperature. Mice Neurofilament-68/200KDa (NF-L + H) monoclonal antibody served as the first antibody and SGCs were identified with SP staining. After sufficient development of DAB and mild re-dyeing with haematoxylin, the cells were examined under a light microscope. PBS was used instead of the first antibody in blank control group and the rest of the steps were the same.

Experimental groups　On the fourth day of culturing, the cells in the plates were divided into 4 groups in accordance with the principles of randomization. One group was regarded as blank control whose medium was changed to DMEM/F12, while the other three groups served as experimental ones whose medium contained gentamicin at 50 mg/L, 100 mg/L and 150 mg/L respectively. Each group had five pores. After 48 h, the cultured cells of each group were collected and prepared for electron microscopic studies.

Morphological changes of SGC under the TEM (transmission electron microscope)　On the sixth day of culturing, the medium was discarded and SGCs were digested with 0.25% trypsin. The digestion time was controlled by monitoring morphological change of the cells under an inverted phase contrast microscope to avoid over-digestion. Fetal bovine serum was added to terminate digestion when most of the cells had turned spherical and floated upon gently shaking of the fluid. The cells of each group were transferred to corresponding centrifuge tubes. After centrifugation, the supernatant fluid was discarded and cells were prefixed with 3% glutaraldehyde-1.5% paraformaldehyde followed by 1% osmium tetroxide-1.5% potassium ferrocyanide. The cells were dehydrated using alcohol-acetone, embedded in epoxy resin 618, sliced into ultrathin sections and stained with uranyl acetate and lead citrate. The specimens were then studied and photographed under a HITACHI Hu-12A TEM.

Results

SGC growth

Primary culture of mouse cochlear SGCs was successful. At the beginning of inoculation, the cells were spherical with few distinguishable morphological characteristics. After 24 h, all cells were adherent. SGCs were elliptic with, smooth-edge and strongly refractive. The edge of the cell showed obvious halation with fibrous processes extending from the body. Most SGCs were typical bipolar neurons with two processes growing in opposite directions while a few were unipolar or multipolar neurons. Besides SGCs, there were also astroids or polygo-

nal fibroblasts with wide cytoplasm and irregular cell body. There were also spindle shaped Schwann cells with shorter process time increased, while the cyton showed little change, the process kept growing and gradually formed a nerve fiber network. After eight days of culturing, the cells gradually degenerated.

Immunocytochemical identification of SGCs

The cultured SGC was stained with NF-L+H antibody (Figure 1a). The NF-positive cells had a round or oval body with brown cytoplasm and processes growing in opposite directions, and large and round nucleus dyed purple by hematoxylin, showing typical bipolar neuron morphology. A few fibroblasts were visible, which were large in volume, irregular in shape, hardly refractive under the stereo microscope and NF-negative in cytoplasm staining, with flat or short processeses. A few Schwann cells were also present with typical spindle shaped cell body, wide initial segment of process and NF-negative cytoplasm. No cells in the negative control group were stained (Figure 1b).

SGCs under TEM

Under the TEM, compared to the blank control group, the SGCs of experimental groups were smaller in size with cell shrinkage. The cytoplasm was dense with nuclear chromatin pyknosis that aggregated peripherally under the nuclear membrane and condensed to mass. The shape of nucleus was irregular, although the organelles were well-preserved. There were also mitochondria swelling and decreased mitochondrial cristae. Cytoplasm vacuoles increased along with increasing medium gentamicin concentration. Extensive plasma membrane blebbing during a process called "budding" and apoptotic bodies consisting of cytoplasm with tightly packed organelles with or without a nuclear fragment were visible. Organelle integrity was however maintained and all of this was enclosed within an intact plasma membrane. Only a few cells in the blank control group displayed slight peripheral nuclear chromatin aggregation under the nuclear membrane. Compared to the blank control group, morphological changes in SGCs from the experimental groups were more profound and might suggest apoptosis (Figure 2).

Discussion

SGCs are well differentiated cells which are well developed in mammals at birth and show little repair after injury. Damage to these cells caused by noise, ototoxic drugs, aging and other factors is unrecoverable and accumulates over time. It has been reported in the literature [1] that damage to hair cells by aminoglycoside antibiotics happen before the decrease of SGCs. Some view the decrease of SGCs as a result of degeneration, while others think [2] that SGCs are damaged first before hair cells and the damage to SGCs may be caused by drugs directly. Roehm et al [3] discovered using immunohistochemistry and radioautography that high-level gentamicin congregated in SGC after intratympanic injection and concluded that aminoglycoside antibiotics had direct effects on SGCs. It is disputed as to whether gentamicin has direct toxic effects on cochlear SGCs and whether the damage to SGCs by aminoglycoside antibiotics is a result of degeneration secondary to hair cell injury. There lacks direct proof on this. The primary culture of SGCs in our study eliminates the influence from various internal environment factors and other cochlear tissues, allowing direct study on how gentamicin impairs SGCs. The morphological changes of SGCs as revealed by TEM indicate that gentamicin can induce apoptosis in SGCs, as represented by swelling of mitochondria and decrease or disappearance of mitochondrial cristae. These findings imply direct toxic effects on SGCs by gentamicin.

Generally, apoptosis is the reaction of cells to toxic stimulation which doesn't induce death of cells immediately. The occurrence of apoptosis is triggered by a series of biochemical processes that include activation of numerous enzymes such as endonuclease and protease, DNA cracking caused by activating enzymes, chromatic aggregation and reorganization and bubbling of cytoskeleton. Mitochondria is the main ATP-producing center in the cell. Most of oxygen serves as terminal electron acceptor in respiratory chain, participates in ATP-producing reaction of oxidative phosphorylation and maintains the process of energy metabolism. However, a little oxygen takes part in the production of superoxide anion through the pathway of electron leak in respiratory chain which is the main pathway of producing intracellular reactive oxygen species (ROS). A certain amount of ROS can be removed by the antioxidative defense mechanism such as superoxide dismutase. But when ROS are generated excessively and oxidative stress goes to certain ex-

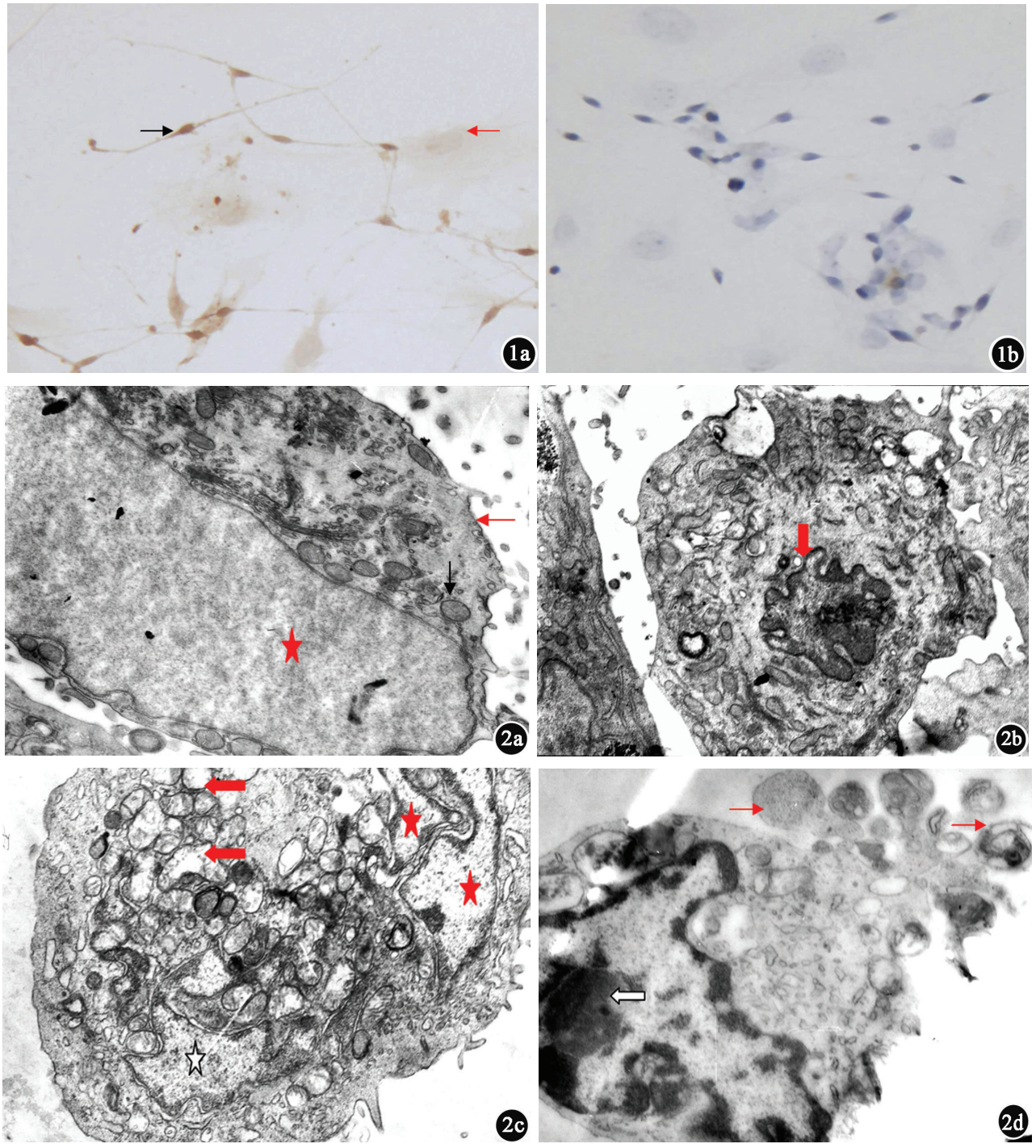

Figure 1a Immunocytochemical identification of SGC ×400 SGC(→); fibroblast (←); **Figure 1b** Micrograph from blank control group ×400 No cell is dyed brown and the nucleus is dyed amethyst by hematoxylin. **Figure 2a** TEM graph of a cell in the blank control group (×8000): The membrane is intact,with profuse microvilli (←). Organelles are well-preserved and mitochondrial cristae are clear (↓), Nuclear chromatin is well-distributed and nucleolus is obvious (★); **Figure 2b** A cell treated with 50mg/L gentamicin (×13000): The cell is small in size indicating cell shrinkage. Cytoplasm and nuclear chromatin are dense and the nucleus is small and deformed (↓) with intact membrane. **Figure 2c** TEM image of a cell treated with 100 mg/L gentamicin (×17000): There are increased vacuoles in cytoplasm (⇦), There is some mitochondria swelling (★), with decreased or diminished mitochondrial cristae. **Figure 2d** TEM image of a cell treated with 150mg/L gentamicin (×11000): There is plasma membrane budding (→) and the nuclear chromatin pyknosis which aggregates peripherally under the nuclear membrane and condenses to mass (⇦).

tents, ROS can induce mitochondria swelling, changes in mitochondria membrane permeability and release of cytochrome c and other apoptosis-related factors [4]. The mitochondria is the place of cell energy metabolism that can induce apoptosis, as well as the executor of apoptosis. Dai De[5] discovered that gentamicin significantly reduced the activity of total superoxide dismutase and glutathione peroxidase in the cochlear SGC and raised the level of malonaldehyde. Malonaldehyde is the product of lipid peroxidation which can cause cross linking of amino compounds such as in proteins, nucleic acid and more. Besides breaking the normal trans-membrane ion gradient by injuring cellular membrane, MDA also obstructs oxidative phosphorylation by injuring mitochondria membrane. The MDA level reflects how severely the cell is attacked by ROS. Some studies [6] have suggested that lipid peroxidation may induce apoptosis. Based upon the results of our study, we propose that through some undefined mechanisms gentamicin may induce excessive production or compromised removal of ROS which can cause lipid peroxidation. This can lead to damage to SGC mitochondria manifested as swelling of mitochondria, change of permeability of mitochondria membrane, release of cytochrome c and other apoptosis-related factors and initiation of series of apoptosis-related biochemical processes.

It remains unclear as how aminoglycoside antibiotics induce apoptosis in cochlear SGCs. It may come from coaction of multiple factors. It is necessary to determine whether aminoglycoside antibiotics cause damage to mitochondria and trigger apoptosis cascade through breaking the balance between oxidation and antioxidant activities in SGCs and how. TEM examination is the most reliable method in determining apoptosis. However, because limited number of cells can be examined using TEM, this method is used as qualitative investigation rather than quantitative study. To conclude that gentamicin causes SGC apoptosis and to decide whether the process is concentration dependent, future studies need to demonstrate apoptosis-related indicators with quantitative analysis.

The transmission of human auditory information and the ability of speech recognition is closely related with the amount of living SGCs in the cochlea.[7] Studying the mechanism of SGC damage will help provide new targets for drug treatment and strategies of gene therapy to prevent and treat ototoxic deafness.

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