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EGb 761 (*Ginkgo biloba*) protects cochlear hair cells against ototoxicity induced by gentamicin via reducing reactive oxygen species and nitric oxide-related apoptosis☆

Ting Hua Yang[a,b], Yi Ho Young[b], Shing Hwa Liu[a,*]

[a]*Institute of Toxicology, College of Medicine, National Taiwan University, Taipei 10051, Taiwan*
[b]*Department of Otolaryngology, National Taiwan University Hospital, Taipei 10051, Taiwan*

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Abstract

Gentamicin is an effective and powerful antibiotic. Extended use or excessive dosages of which can result in irreversible damage to the inner ear. The development of otoprotective strategies is a primary and urgent goal in research of gentamicin ototoxicity. *Ginkgo biloba* leaves and their extracts are among the most widely used herbal products and/or dietary supplements in the world. We investigated the protection of EGb 761 (a standardized preparation of EGb) on gentamicin ototoxicity and the involvement of reactive oxygen species (ROS) and nitric oxide (NO)-related mechanisms using *in vitro* organ cultures and an *in vivo* animal model. Gentamicin induced hair cell damage in cochlear cultures that could be prevented by EGb 761. EGb 761 also significantly reduced gentamicin-induced ROS and NO production. Furthermore, EGb 761 inhibited cellular apoptosis in cultured cochleae treated with gentamicin. In guinea pigs with gentamicin application onto the round window membrane, the mean auditory brain stem response threshold, ratio of cochlear hair cell damage and apoptosis were significantly elevated compared with those in the control group, and this could be prevented by oral administration of EGb 761. Individual EGb 761 components quercetin, bilobalide, ginkgolide A and ginkgolide B, but not kaempferol, significantly prevented gentamicin-induced hair cell damage. These results indicate that EGb 761 has a protective effect against gentamicin ototoxicity through a reduction in the formation of ROS and NO and subsequent inhibition of hair cell apoptosis in the cochlea.

Keywords: Cell death; Cochlear culture; EGb 761; Hearing; Ototoxicity

1. Introduction

Gentamicin, an aminoglycoside antibiotic, is widely used to treat infection caused by Gram-negative bacteria. However, the negative aspect of gentamicin therapy after long-term use refers to its adverse effects, which are mostly nephrotoxicity and ototoxicity. The manifestations of gentamicin-induced ototoxicity consist of hearing loss, tinnitus and vertigo. Since gentamicin is effective, of low cost and broadly used worldwide, gentamicin-induced ototoxicity is widespread. Therefore, the development of otoprotective strategies is a primary and urgent goal in research on gentamicin-induced ototoxicity. Many research studies have suggested that gentamicin-induced ototoxicity is commonly regarded to be mediated by reactive oxygen species (ROS) and reactive nitrogen species [1–6]. Overproduction of ROS triggers signaling pathways of cellular apoptosis, resulting in inner ear damage. Additionally, gentamicin also enhances the formation of nitric oxide (NO) in the inner ear through regulating the inducible NO synthase (iNOS) expression and later forms the destructive peroxynitrite with ROS [7]. Several agents that scavenge ROS or block their formation [8–12] or reduce apoptosis via inhibiting various stages of the cell death pathway have been proposed to protect the inner ear [13–17]. Nevertheless, most agents have not yet been used in clinical practice, and the effectiveness of these agents should be weighed against potential hazards before clinical usage.

Ginkgo biloba leaves and their extracts are among the most widely used herbal products and/or dietary supplements in the world. The extract of *G. biloba* (EGb) has long and safely been used to treat patients with neurodegenerative, vascular and audiovestibular disorders [18–21]. Pharmacologically, EGb is able to modulate the hemodynamics in the cerebrum under normal and ischemic conditions [22,23]. In aging rats, EGb treatment has been found to lower circulating free cholesterol and inhibit the production of brain β-amyloid precursor protein and amyloid β-peptide [24]. Moreover, EGb has been shown to possess NO-scavenging ability [25,26], which inhibits the expression of iNOS in several tissues [27–29]. A recent study has also shown that EGb prevents the formation of experimental myringosclerosis by scavenging free oxygen radicals [30]. EGb 761 is a standardized formula of EGb that contains terpenoids (6%, including ginkgolides and bilobalides) and flavonoid glycosides (24%, such as kaempferol and quercetin) [31]. These active ingredients in EGb 761 help prevent platelet aggregation and act as potent antioxidants [32–34]. They reacted with several free radicals,

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* Corresponding author. Fax: +886 2 23410217.
E-mail address: shinghwaliu@ntu.edu.tw (S.H. Liu).

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including superoxide, hydroxyl and peroxyl radicals, to protect tissues from oxidative stress [35,36]. Hence, EGb 761 shows promise in ameliorating gentamicin-induced ototoxicity, although its preventive effect and possible mechanism remain to be clarified. Therefore, the aims of this study were to assess the protective effects of EGb 761 on hair cell injury, cell apoptosis and ROS and NO production in cultured cochleae treated with gentamicin and to evaluate the ameliorative effect of EGb 761 on the auditory function in gentamicin-treated animals.

2. Materials and methods

2.1. In vitro organotypic cultures of cochleae

The cochlea was dissected from Wistar rats on Postnatal Day 3 and cultured based on the methods of Van de Water and Ruben [37] and Sobkowicz *et al.* [38]. Wistar rats were decapitated on Postnatal Day 3, with their temporal bones removed and tympanic bulla opened. The whole cochlea was carefully dissected out and divided into apical, middle and basal turns in a collagen-coated 35-mm culture dish. The cochlear explants were maintained in Dulbecco's modified Eagle's medium F12 (Gibco, Grand Island, NY, USA) plus 5% fetal bovine serum, 25 mM Hepes buffer and 30 U/ml of penicillin. They were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2, with the culture medium renewed daily. Initially, EGb 761 was added into the medium at a final concentration of 0, 10, 20, 50, 100 or 200 μg/ml for pretreatment. After 12 h, gentamicin was added into the medium at a final concentration of 0, 5, 10, 20 or 50 μM to induce toxicity. In some experiments, five constituents of EGb 761 (kaempferol, quercetin, bilobalide, ginkgolide A and ginkgolide B) were used to compare their effects with EGb 761 against gentamicin-induced cytotoxicity. These compounds were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. In vivo study

Randomly bred male Hartley strain guinea pigs weighing 200–250 g from an in-house breeding company were used for *in vivo* study, housed at 23°C±2°C and 55% ±5% humidity and given a solid diet of GB-1 and water *ad libitum*. For *in vivo* study, general anesthesia was achieved in guinea pigs by intraperitoneal injection of pentobarbital sodium (35 mg/kg). A small postauricular incision was done, and the tympanic bulla was identified. The bulla was then opened to expose the round window area. Gentamicin (40 mg/ml) was injected directly overlaying but not through the round window membrane on the left ear. The right ear was injected with 0.05 ml of saline in the same fashion and served as the control. The rationale to select this dosage is based on the amount of 0.3–1.0 ml (10–80 mg/ml) gentamicin used for humans in clinical practice [39]; thus, one-tenth of the dose was chosen for the guinea pigs [40]. On the other hand, the EGb 761 employed herein was purchased from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany); 120 mg of the proprietary concentrated extract of *G. biloba* EGb 761 equivalent to 6 g of dry leaves standardized to 28.8 mg of ginkgo flavonoid glycosides and 7.2 mg of terpene lactones (3.7 mg of ginkgolides A, B and C and 3.5 mg of bilobalide) and contained less than 0.5 ppm of ginkgolic acids. Gentamicin was obtained from Sigma Pharmaceuticals (St. Louis, MO, USA). Guinea pigs were orally administered EGb 761 with a dosage of 100 mg/kg in liquid form once daily for 2 days of pretreatment. On the third day, in addition to EGb 761 feeding, 50 μl of gentamicin (40 mg/ml) was injected intratympanically overlaying the round window membrane. These animals were subjected to auditory brain stem response (ABR) tests and subsequent evaluation of apoptosis in the cochlea. All animal procedures were approved by the institutional review board of the university and were conducted in accordance with the guidelines for the care and use of laboratory animals by the Animal Research Committee of the National Taiwan University College of Medicine.

2.3. Hair cell staining and counting

The cochlear organotypic cultures from postnatal rats were prepared for hair cell staining and counting. The cochleae were also divided into three turns and were fixed with 4% paraformaldehyde and 1% glutaraldehyde in phosphate-buffered saline (PBS) for 20 min at 20°C and then permeabilized with 5% Triton X-100 in PBS with 10% fetal bovine serum for 30 min. These cochlear explants were stained with a conjugated phalloidin–rhodamine probe (1:100, Texas Red–X phalloidin, Molecular Probes, Invitrogen, Carlsbad, CA, USA) in PBS for 1 h. Phalloidin is a specific marker for cellular F-actin and labels stereociliary arrays and cellular borders in the cuticular plate. The tissues were washed three times with PBS and mounted on glass slides in glycerin containing Fluoromount (Molecular Probes, Invitrogen). Finally, the slides were examined via confocal microscopy (Zeiss LSM 510 Meta, Hamburg, Germany). The excitation wavelength was 561 nm, and the emission wavelength was long-pass-filtered over 575 nm. Hair cells were counted separately along the apical, middle and basal turns in a cochlea. Depending on the whole length of each turn, five to seven areas (each with a length of 100 μm) were randomly selected for hair cell counting by two independent observers. Counting was repeated in at least three cochleae in each experiment group.

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Cochleae isolated from guinea pigs and those from postnatal rats were subjected to apoptosis assay. DNA fragmentation of the apoptotic cells was detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) technique (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI, USA). One week after gentamicin application onto the round window membrane, the cochleae of the guinea pigs were dissected from the temporal bone after fixation by intracardiac perfusion of 4% paraformaldehyde and 2.5% glutaraldehyde in PBS. Cochlear cultures isolated from postnatal rats were also fixed with the same fixatives after treatment. These cochlear explants were incubated in 0.2% Triton X-100 in PBS for 5 min and then washed twice with PBS. Subsequently, the samples were covered with 100 μl of equilibration buffer for 5–10 min at room temperature and then 50 μl of terminal deoxynucleotidyl transferase reaction mix containing fluorescein-12-dUTP and recombinant terminal deoxynucleotidyl transferase enzyme was added for 60-min incubation at 37°C in the dark. Rhodamine–phalloidin probe was used to label hair cells and DAPI to counterstain nuclei of normal or apoptotic cells. Finally, the cochlear explants were mounted and photographed with a confocal microscope. The excitation wavelengths to observe TUNEL stain and DAPI were 488 and 405 nm, respectively, and the emission wavelengths were 505–550 and 420–480 nm, respectively. Deoxyribonuclease I (DNase I) was used as a positive control, which generates strand breaks in the DNA to provide a positive TUNEL reaction.

2.5. Measurement of ROS in the cochlea

The cell-permeant dye H_2DCF-DA is the reduced and acetylated form of 2′,7′-dichlorofluorescein (DCF). Esterase cleavage of the lipophilic blocking groups yields a charged form of the dye that is much better retained by cells than is the parent compound. ROS oxidizes H_2DCF to the fluorescent compound DCF, which produces bright green fluorescence. We used carboxy-H_2DCF-DA (Invitrogen), the carboxy derivative of H_2DCF-DA, to evaluate the production of ROS in the cochlea. Carboxy-H_2DCF-DA carries additional negative charges that improve its retention compared with noncarboxylated forms. The experiment followed the method of Trayner *et al.* [41] with some modifications. Briefly, cochlear organotypic culture receiving a different treatment was planted in a 96-well microplate (Corning Incorporated, Corning, NY, USA). Each treatment group was composed of eight cochlear explants pooled together from four postnatal rats. After specific treatment in each group, these cochlear explants were washed with PBS and then incubated with 10 mM carboxy-H_2DCF-DA for 30 min at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After 30 min, the extracellular ROS dye was washed away with Dulbecco's modified Eagle's medium F12 and the microplate was then put in a microplate reader (DTX 800 Multimode Detector, Beckman Coulter, Fullerton, CA, USA) to have the fluorescence read. The excitation and emission wavelengths used were 485 and 538 nm, respectively.

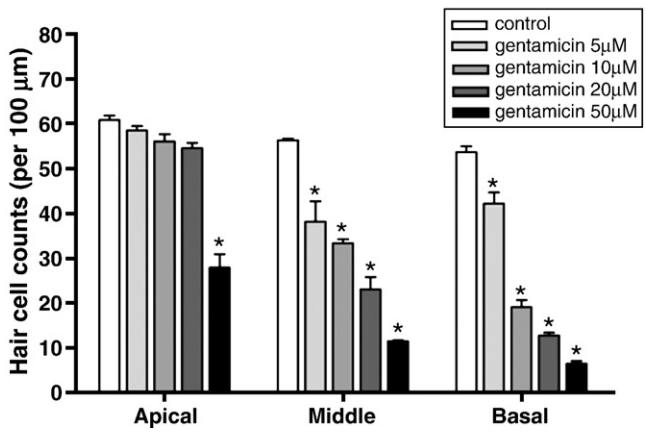

Fig. 1. Dose-dependent cytotoxicity of gentamicin on the hair cells of organotypic cochlear cultures of postnatal rats. The organ of Corti from postnatal 3-day-old rats was incubated in medium containing 0–50 μM gentamicin for 48 h. Labeled with rhodamine–phalloidin, hair cells were counted under a confocal microscope, and their number/100 μm was averaged separately along the apical, middle and basal turns in cochleae. Gentamicin (50 μM) effectively produced global cytotoxicity in the apical, middle and basal turns of the cochleae. Data are presented as the mean±S.D. $^{*}P<.05$, when compared with the respective control group.

2.6. Measurement of NO in the cochlea

Much of the NO in tissue is oxidized to nitrite and nitrate, and the concentrations of these anions have been used as a quantitative measure of NO production. A Total Nitric Oxide Assay Kit (Assay Designs, Ann Arbor, MI, USA) was used to determine total nitrite/nitrate in the cochlea. This involves the conversion of nitrate to nitrite by nitrate reductase, followed by the colorimetric detection of nitrite as a colored azo dye product of the Griess reaction that absorbs visible light at 540 nm. The organotypic cochlear culture of postnatal rats was processed to determine the level of NO. Cochleae from four rats in each group were pooled together to increase detectability of the assay. The cochlear explants were frozen with liquid nitrogen and then pulverized into a powder using a pellet pestle rod with microtube (Kimble/Kontes, Vineland, NJ, USA) and homogenized in ice-cold 50 mM Tris-EDTA buffer (pH 7.0). The homogenate was centrifuged at 10,000*g* for 45 min at 4°C. The supernatant was used to determine the levels of NO metabolites. Nitrate reductase was added to each sample to convert nitrate to nitrite. These samples were incubated with enzyme solution for 30 min at 37°C before Griess reagent was added. Their absorbance at 540 nm was read on a microplate reader.

2.7. ABRs

Two weeks after gentamicin application in the ear, each guinea pig underwent an ABR test to evaluate hearing status. After anesthesia with intraperitoneal pentobarbital sodium, a pair of needle electrodes was placed onto the vertex and the ipsilateral retroauricular region, while a ground electrode was placed on the neck. Click stimuli were delivered through a plastic tube inserted into the ear canal (Smart EP2, Intelligent Hearing Systems, Miami, FL, USA). The repetition rate was 57.7/s, and 1000 sweeps were averaged. The stimulus intensity was from 100 dB SPL initially, followed by 10- to 5-dB step decrements until the absence of waveforms, and the threshold of ABR was thus determined. The mean threshold of ABR was calculated from 10 guinea pigs in each group.

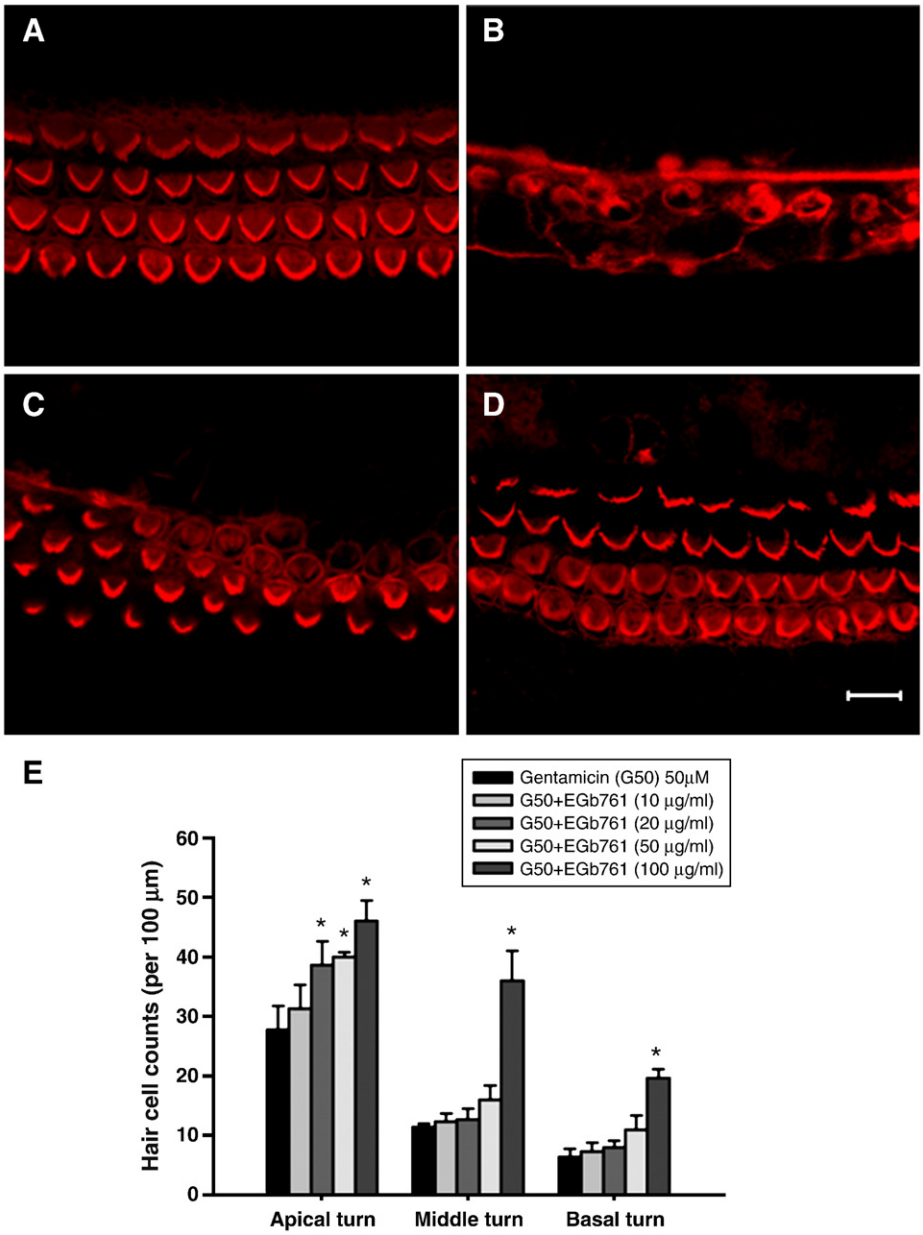

Fig. 2. Effect of EGb 761 on gentamicin-induced cytotoxicity in the cochlear cultures of postnatal rats. Hair cells in the organ of Corti were labeled in red with rhodamine–phalloidin. Confocal microscopic images were taken in the (A) untreated control group, (B) gentamicin (50 μM) for 48 h group, (C) EGb 761 (100 μg/ml) pretreated overnight and then co-treated with gentamicin (50 μM) for 48 h group and (D) EGb 761-only (100 μg/ml) group. Deranged alignment and loss of hair cells in the organ of Corti are shown in (B), but the condition was much improved in (C). The bar chart (E) compares the average hair cell count/100 μm along the apical, middle and basal turns of the cochleae. In each cochlear turn, five to seven areas of 100 μm were selected for hair cell counting and averaging. Results shown are representative of at least three independent experiments. Data are presented as the mean±S.D. *$P<.05$, as compared with the gentamicin group. The scale bar represents 10 μm.

2.8. Statistical analysis

The average hair cell number is represented as the mean±S.D. One-way ANOVA or post hoc test for different interactions within one test was used to analyze statistical significance of the results. The mean ABR thresholds were compared by unpaired two-tailed Student's *t* test. A value of $P<.05$ indicated statistical significance.

3. Results

3.1. In vitro study in rat cochlear cultures: gentamicin-induced hair cell damage

Organotypic cultures of the cochlea isolated from postnatal rats were incubated in the medium with 5–50 μM gentamicin added for 48 h. In the control group, three rows of outer hair cells and one row of inner hair cells kept their shape, with the stereocilia well preserved. In the gentamicin group, viable hair cells were decreased in number (Fig. 1), and they also lost normal contour and alignment (Fig. 2B). The average hair cell count declined significantly in the middle and basal turns despite the gentamicin concentration being varied from 5 to 50 μM. However, for the apical hair cells, a significant difference existed only between the control and 50 μM gentamicin groups (Fig. 1). Because global hair cell loss of the rat cochlea occurred in the 50 μM gentamicin group, the latter was used to evaluate the effect of EGb 761 on gentamicin cytotoxicity.

3.2. In vitro study in rat cochlear cultures: effect of EGb 761 on gentamicin cytotoxicity and apoptosis

Twelve hours before gentamicin treatment, cochlear cultures of postnatal rats were incubated in the medium containing EGb 761 at

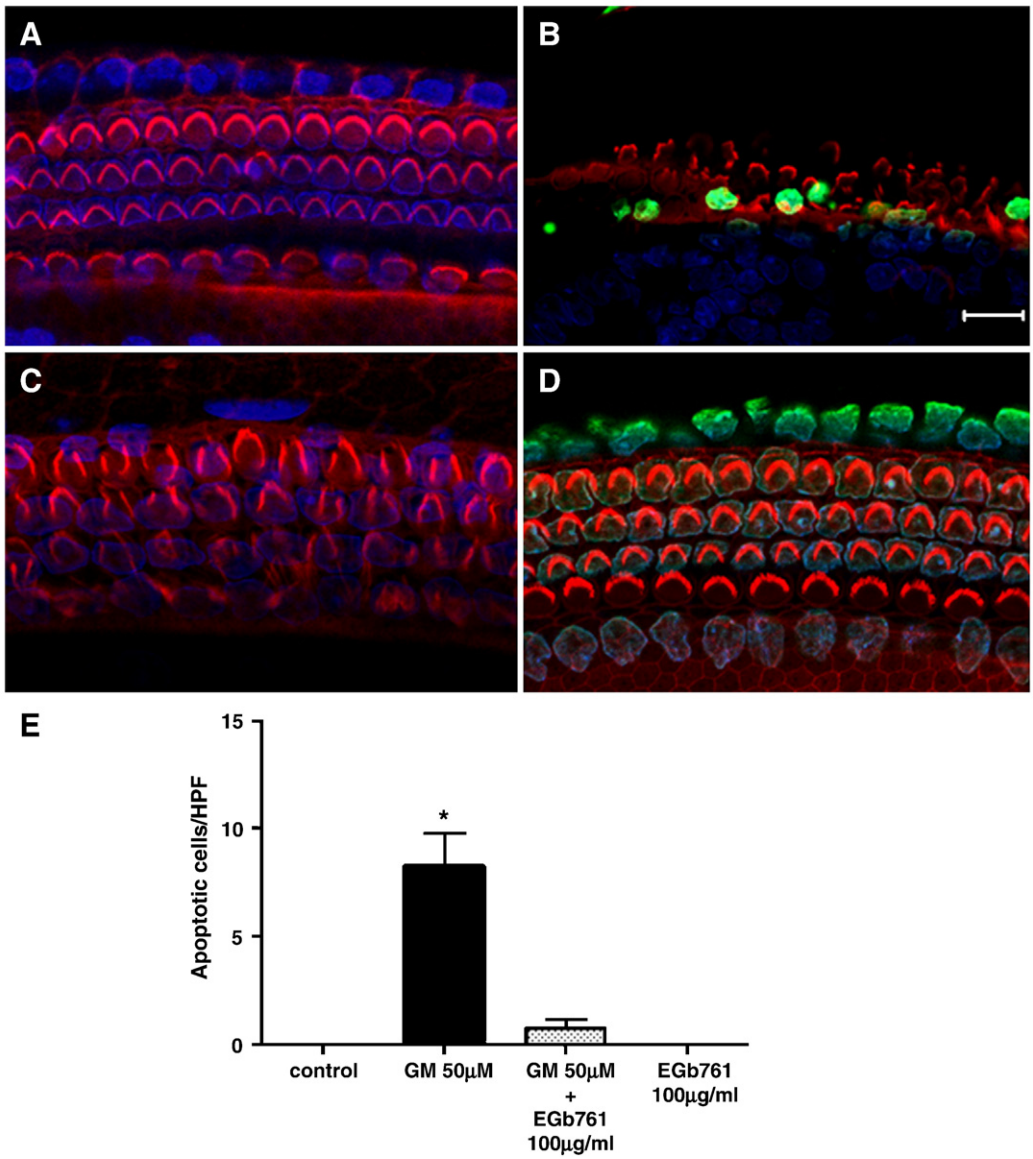

Fig. 3. Effect of EGb 761 on gentamicin-induced apoptosis in the cochlear cultures of postnatal rats. Apoptotic cells in the organ of Corti were evaluated using TUNEL assay. Hair cells were labeled in red with rhodamine–phalloidin. Nuclei were counterstained in blue with DAPI. TUNEL-positive cells were labeled in green and should also be positive for DAPI. Confocal microscopic images of the organ of Corti represent the following groups: (A) untreated control, (B) gentamicin (50 μM) for 30 h, (C) EGb 761 (100 μg/ml) pretreated overnight and then co-treated with gentamicin (50 μM) for 30 h and (D) DNase I-treated sample that served as a positive control. TUNEL-positive cells are shown scattered in the organ of Corti in (B) as green dots with co-localization of blue DAPI stain. In (A) and (C), such cells are hardly seen. The bar chart in (E) compares the average number of apoptotic cells in five high-power fields (630×) of the organ of Corti. The number was much higher in the gentamicin-only group. $^{*}P<.001$, as compared with the other groups. GM indicates gentamicin. Results shown are representative of three independent experiments. The scale bar represents 10 μm.

concentrations of 10–100 μg/ml. Viable phalloidin-labeled hair cells were counted after 48 h of gentamicin (50 μM) treatment. Compared with untreated controls (Fig. 2A), gentamicin severely distorted the anatomy of the organ of Corti in the 50 μM gentamicin group. Nonviable hair cells were deformed, deranged and not even recognizable (Fig. 2B). EGb 761 was not toxic to the cochlea (Fig. 2D) and reduced gentamicin-induced cytotoxicity (Fig. 2C); the alignment of cells in the organ of Corti was somewhat irregular, but hair cells retained their shape and stereocilia (Fig. 2C). The preventive effect was more prominent in the apical turn in a dose-dependent manner (20–100 μg/ml) (Fig. 2E). In the middle and basal turns, 100 μg/ml of EGb 761 effectively provided protection against gentamicin (Fig. 2E). Apoptosis was evaluated by TUNEL assay, which stained DNA fragments in the apoptotic cells. TUNEL-positive (apoptotic) cells were found in the cochlear culture after 30 h of gentamicin (50 μM) incubation (Fig. 3B). Pretreatment with EGb 761 (100 μg/ml) could significantly reduce the number of TUNEL-positive cells in the gentamicin-treated cochlea (Fig. 3C). In the control (Fig. 3A) and EGb 761-only (data not shown) groups, apoptotic cells were not observed. DNase I-treated cochleae served as positive controls of TUNEL staining (Fig. 3D).

3.3. In vitro study in rat cochlear cultures: EGb 761 reduced gentamicin-induced ROS and NO production

ROS production between the different treatment groups was compared by reading the fluorescence of DCF. The fluorescence was assessed every 9 min consecutively for 3 h, and the value is presented as fluorescence intensity. Evaluation of ROS levels started after 1 h of gentamicin incubation. Gentamicin (50 μM) elevated ROS levels significantly (Fig. 4). Administration of 100 μg/ml of EGb 761 effectively reduced ROS production to about the same level as that in the control cochleae. EGb 761 alone decreased ROS levels even further. The ROS level in each group was consistently raised during the whole test period as time passed, except for the EGb 761-only group (Fig. 4). A similar trend was also observed in NO production. Gentamicin (50 μM) significantly increased NO levels in the cochleae. Administration of EGb 761 reduced NO level to the same level as that in the untreated cochleae (Fig. 5).

3.4. In vivo study in guinea pigs: auditory function and hair cell damage in groups with gentamicin and/or EGb 761

Auditory function of the animals was evaluated by determining the ABR threshold. One week after gentamicin treatment, the averaged ABR threshold of gentamicin-treated ears was significantly higher than that of saline-treated control ears (Fig. 6B and E). With oral administration of EGb 761 (100 mg/kg per day) to the gentamicin-treated guinea pigs, the ABR threshold was reduced to a level not different from that in the control and EGb 761-only groups (Fig. 6A, C and D). Meanwhile, hair cell damage was evident in the cochleae of gentamicin-treated ears compared with the saline controls (Fig. 7B). However, if the animals were treated with daily EGb 761 at 100 mg/kg, hair cell damage was no longer seen in the cochleae (Fig. 7C), similarly as observed in the control and EGb 761-only groups. Likewise, the TUNEL-positive (apoptotic) cells in the organ of Corti were markedly increased in the gentamicin-treated group (Fig. 7B) but were hardly observed in either the controls or the EGb 761-fed guinea pigs with gentamicin treatment (Fig. 7A and C). DNase I-treated cochleae served as positive controls of TUNEL staining (Fig. 7D).

3.5. Effects of active constituents of EGb 761 on gentamicin cochleotoxicity in rat cochlear cultures

The effects of kaempferol, quercetin, bilobalide, ginkgolide A and ginkgolide B on gentamicin cochleotoxicity were evaluated as compared

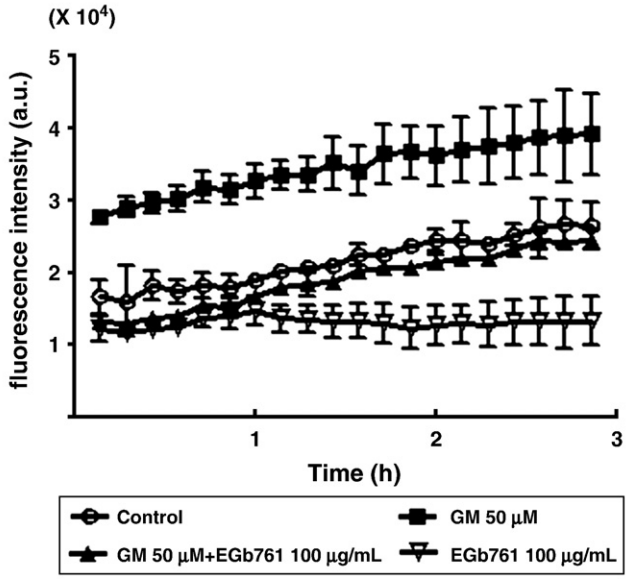

Fig. 4. Effect of EGb 761 on gentamicin-induced ROS levels in the cochlear cultures of postnatal rats. Fluorescence of carboxy-H_2DCF-DA (ROS indicator) from cochlear explants was read every 9 min consecutively for 3 h. Change of fluorescence intensity (a.u.) with time was plotted as a line chart. Four groups were compared: untreated control, gentamicin (50 μM) for 1 h, EGb 761 (100 μg/ml) overnight plus gentamicin (50 μM) for 1 h and EGb 761 only. The ROS level in the gentamicin-only group was significantly elevated compared with the other groups ($P<.001$). The ROS level in the EGb 761 group with or without gentamicin was not different from that in the control group statistically ($P>.05$). Data are presented as the mean±S.D. Results shown are representative of three independent experiments.

with EGb 761. Twelve hours before gentamicin treatment, cochlear cultures of postnatal rats were incubated in medium containing kaempferol, quercetin, bilobalide, ginkgolide A and ginkgolide B at concentrations of 0.3–100 μg/ml. Viable phalloidin-labeled hair cells were counted after 48 h of gentamicin (50 μM) treatment. Quercetin, bilobalide, ginkgolide A and ginkgolide B, but not kaempferol, significantly prevented gentamicin-induced hair cell damage (Fig. 8).

4. Discussion

Hair cells are the most vulnerable element in the cochlea to gentamicin toxicity and were thus chosen to be an indicator of

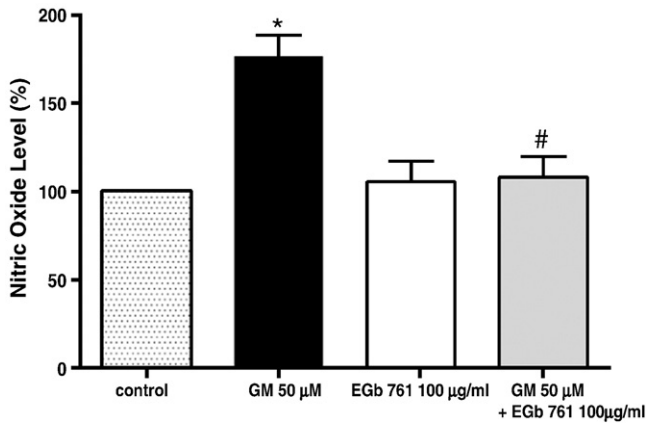

Fig. 5. Effect of EGb 761 on gentamicin-induced NO production in the cochlear cultures of postnatal rats. NO production in cochlear culture incubated with gentamicin (50 μM) for 3 h in the presence or absence of EGb 761 (100 μg/ml) was quantified and plotted as a bar chart. $^{*}P<.05$, as compared with the control group. $^{\#}P<.05$, as compared with the gentamicin group. Data are presented as the mean±S.D.

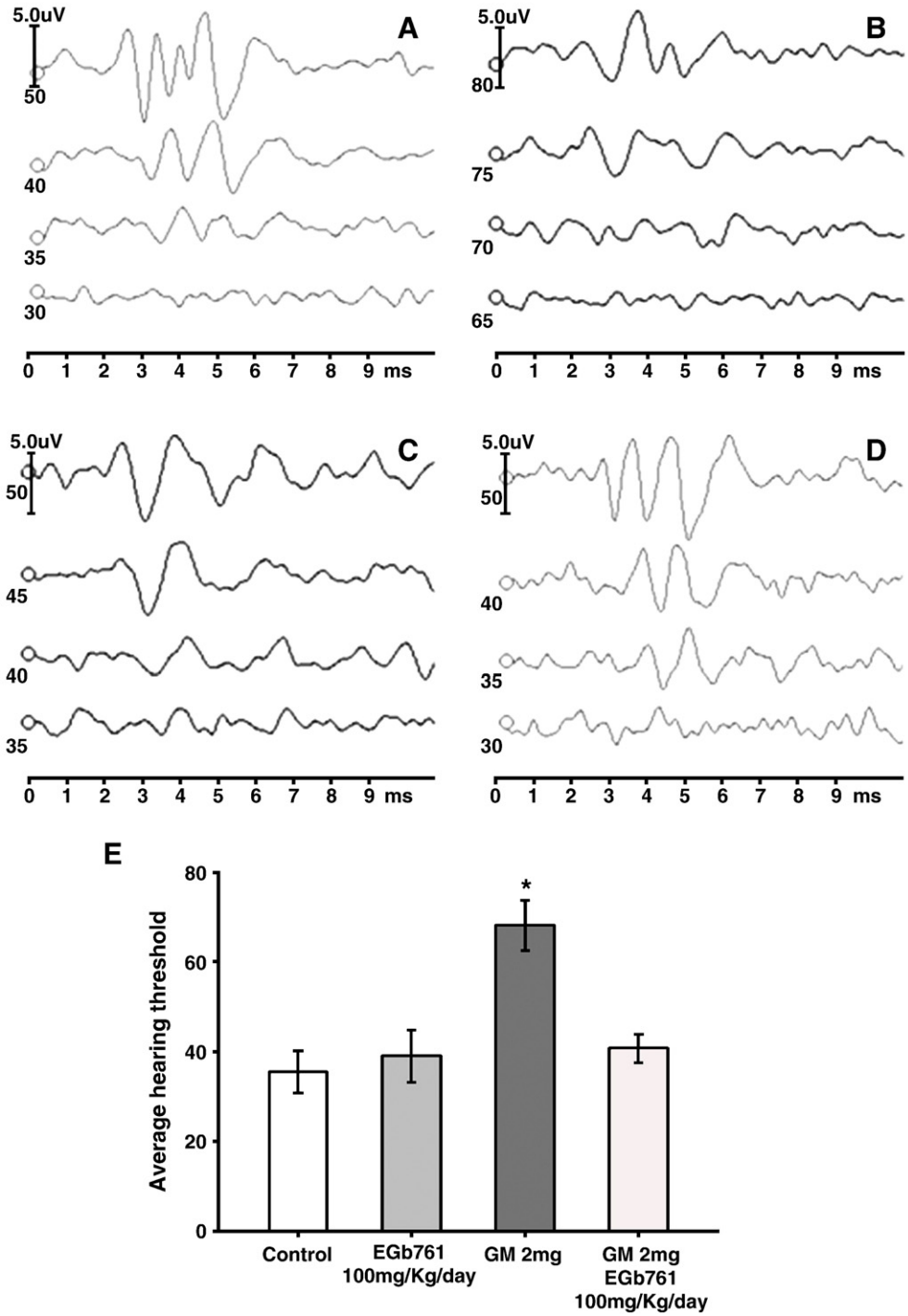

Fig. 6. ABRs recorded from guinea pigs. Two weeks after intratympanic application of gentamicin, ABR was recorded. The control ear underwent saline injection. Oral EGb 761 (100 mg/kg per day) feeding was begun 2 days prior to gentamicin treatment and continued for another week afterwards. The waveforms of click-induced ABR by different stimulus intensities are shown in the following groups: (A) saline control, (B) gentamicin (2 mg) only, (C) gentamicin plus EGb 761 and (D) EGb 761 only. The bar chart in (E) shows the average hearing threshold in different groups. Average hearing threshold was calculated from ABR recordings of 10 ears in each group. The hearing threshold was significantly higher in the gentamicin group compared with the other groups. No significant difference in hearing threshold was found between the control and EGb 761-treated groups. Data are presented as the mean±S.D. $^{*}P<.005$, as compared with the other groups.

gentamicin cytotoxicity in this study. In this work, at a low concentration of gentamicin (5 μM), significant hair cell loss was already observed in the middle and basal turns, while most hair cells of the apical turn remained intact. When the gentamicin concentration was elevated to 50 μM, all cochlear turns showed a significant decrease in hair cell counts. Thus, in this study, gentamicin at a concentration of 50 μM, which was adopted to produce global toxicity of the cochleae, was used to evaluate the ameliorated effect of EGb 761 on gentamicin ototoxicity. On the other hand, apical hair cells apparently tolerated a higher concentration of gentamicin, while the hair cells in the basal turn were more susceptible to noxious damage than those in the apical turn. Likewise, clinical patients with drug-induced ototoxicity very often present with early high-frequency sensorineural

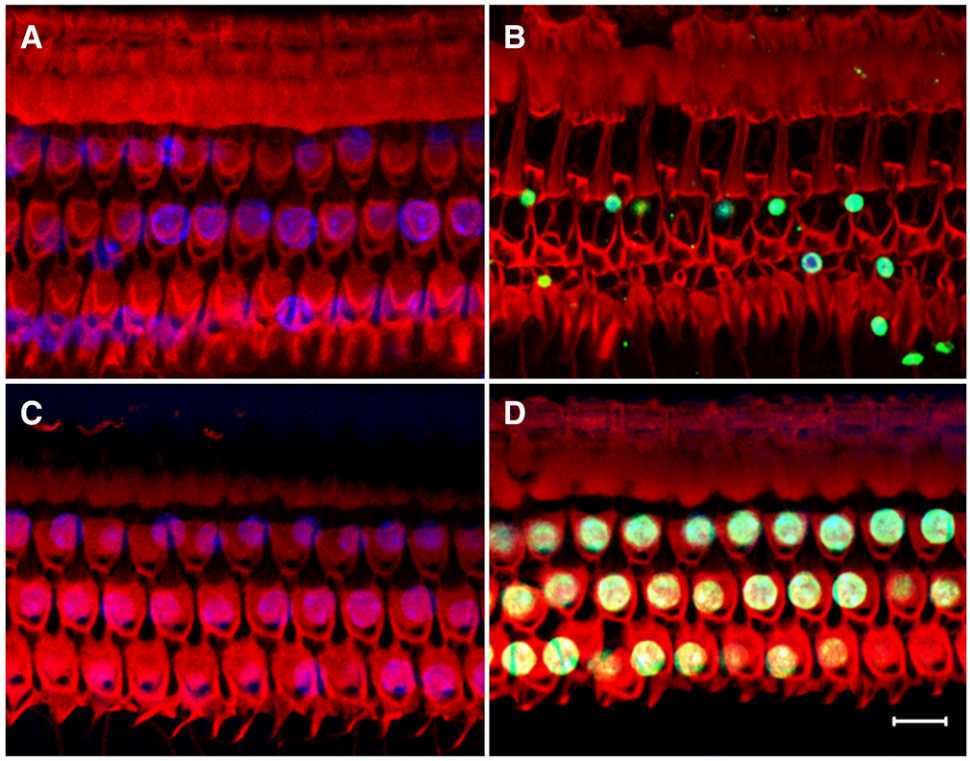

Fig. 7. Effects of EGb 761 on gentamicin-induced apoptosis and hair cell damage in the cochleae from guinea pigs. Animals underwent intratympanic application of gentamicin (2 mg). Oral EGb 761 (100 mg/kg per day) feeding was begun 2 days prior to gentamicin treatment and continued for another week afterwards. Saline-injected ears served as controls. One week later, these animals were sacrificed and their cochleae were dissected from the temporal bone. Apoptotic cells in the organ of Corti were evaluated using TUNEL assay. Hair cells were labeled in red with rhodamine–phalloidin. Nuclei were counterstained in blue with DAPI. TUNEL-positive cells were labeled in green and should also be positive for DAPI. Confocal microscopic images of the organ of Corti represent the following groups: (A) saline-injected control, (B) gentamicin (2 mg) only, (C) gentamicin (2 mg) plus EGb 761 (100 mg/kg per day) and (D) DNase I-treated sample that served as a positive control. In (B), loss of hair cells was evident in the organ of Corti. Cell bodies and nuclei of hair cells disappeared, and no stereocilia were found. TUNEL-positive cells are shown scattered in the organ of Corti as green dots with co-localization of blue DAPI stain. In (A) and (C), hair cells retained a normal shape and no TUNEL-positive cells were seen. Results shown are representative of three independent experiments. The scale bar represents 10 μm.

hearing loss. Is variation of toxicity in each cochlear turn attributable to the concentration gradient of the drug from base to apex? The present study found otherwise, since each turn was exposed to the same concentration of gentamicin in an organotypic cochlear culture system. Moreover, Sha *et al.* [42] have reported that basal outer hair cells display a lower level of glutathione and died earlier than apical ones in an untreated cochlear culture of guinea pigs. Thus, the intrinsic difference of the hair cells in various cochlear regions should be responsible for their dissimilar tolerance to gentamicin insult.

Overproduction of ROS was suggested to be an initial step to trigger apoptotic pathways and cause cell death in aminoglycoside-induced ototoxicity. The methods of blocking ROS in the cochlea under *in vitro* or *in vivo* aminoglycoside exposure have been shown in many studies [3,43,44]. NO is also involved in gentamicin ototoxicity. Peroxynitrite, formed by NO together with superoxide, is more reactive and toxic and plays an important role in gentamicin-induced ototoxicity [5]. Recent *in vivo* studies have shown that gentamicin is capable of inducing up-regulation of iNOS [45] and endothelial NO synthase [46] expression in the cochlea of guinea pigs. Heinrich *et al.* [1] discovered the correlation between hearing threshold shift and NO production in the cochlea and concluded that increased NO contributed to gentamicin-induced hearing loss. In this work, we found that increased levels of ROS and NO production were evident after gentamicin treatment in the cochlear cultures and that EGb 761 could effectively reduce ROS and NO production to a level near or even lower than that in the untreated cochleae. Therefore, these findings suggest that EGb 761 possesses the ability to antagonize gentamicin-induced ototoxicity possibly via inhibiting ROS and NO formation.

Our *in vivo* study on guinea pigs has demonstrated that EGb 761 prevents apoptosis and hair cell loss in the cochlea and consequently protects against gentamicin-induced impaired auditory function. EGb has also been suggested to improve blood flow or microcirculation [47]. Didier *et al.* [19] have reported that vasodilatation of the cochlear vessels in the spiral lamina is observed in guinea pigs after 6 weeks of treatment with EGb 761. Therefore, it is possible that, in addition to reduction of gentamicin-induced oxidative stress, the hemodynamic modulatory effect of EGb 761 may ameliorate the microenvironment of hair cells and improve hearing status under gentamicin exposure. Moreover, the incomplete recovery of hair cell loss in the *in vitro* cochlear cultures might be attributed to the prolonged exposure of tissue in a high concentration of gentamicin, unlike the transiently increased concentration in the cochlear perilymph in the gentamicin-treated animals. These findings imply that the strategy on the limitation of ROS and NO production may not be sufficient to prevent gentamicin ototoxicity.

Hyun *et al.* [48] have demonstrated that 6-hydroxykynurenic acid and other flavonoids obtained from yellow leaves of *G. biloba* evidenced a marked scavenging activity on authentic ONOO–. It has also been suggested that kaempferol and quercetin, two components of EGb 761, effectively induce caspase-3-dependent apoptosis of oral cavity cancer cells and can be considered as possible antioral cavity cancer agents [49]. In this study, we tested the effects of five constituents of EGb 761 on gentamicin-induced cochleotoxicity in cochlear culture. As suggested in previous reports [50–52] and in

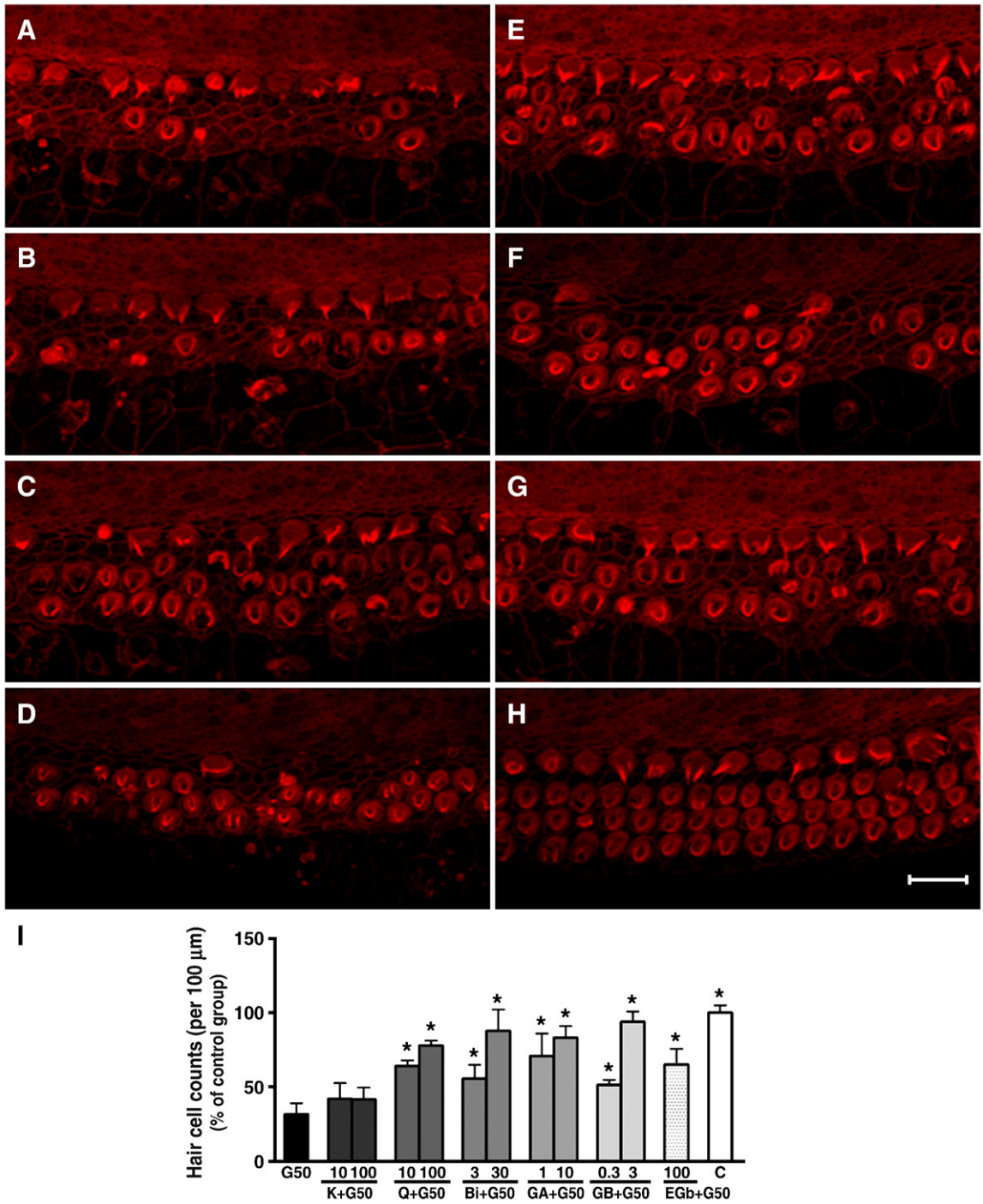

Fig. 8. Cytoprotection of constituents of EGb 761 against gentamicin in rat cochlear culture. Confocal microscopic images of hair cells stained with rhodamine–phalloidin in cochleae receiving different treatments as follows: (A) 50 μM gentamicin only for 48 h; (B–G) pretreatments of kaempferol (B), quercetin (C), bilobalide (D), ginkgolide A (E), ginkgolide B (F) and EGb 761 (G) overnight before co-treatment of gentamicin (50 μM) for 48 h; and (H) control group without treatment. The residual hair cell number in each group compared with the control group is drawn as a bar figure (I). G50 indicates 50 μM gentamicin; K, kaempferol; Q, quercetin; Bi, bilobalide; GA, ginkgolide A; GB, ginkgolide B. Data are presented as the mean±S.D. (n=4). $^{*}P$<.05, as compared with the gentamicin-only group. The scale bar represents 10 μm.

the EGb 761 datasheet of Dr. Willmar Schwabe Pharmaceuticals, the average levels of kaempferol, quercetin, bilobalide, ginkgolide A and ginkgolide B in EGb were around 10%, 10%, 3%, 1% and 0.3%, respectively. Consequently, the concentrations of these constituents in EGb 761 (100 μg/ml) became 10, 10, 3, 1 and 0.3 μg/ml, respectively. Among them, quercetin, bilobalide, ginkgolide A and ginkgolide B were all able to rescue hair cells against gentamicin. Kaempferol, however, did not show such protection even at high concentration. The results suggest that the two major types of constituents (terpene trilactones and flavonoid glycosides) in EGb 761 are responsible for the protective effect of EGb 716 against gentamicin-induced cochleotoxicity.

In conclusion, EGb 761 possesses a protective effect against gentamicin ototoxicity in the cochlea. EGb 761 attenuates gentamicin cytotoxicity via reducing the formation of ROS and NO, subsequently inhibiting apoptosis of cochlear hair cells. Consequently, EGb 761 can

preserve more cochlear hair cells and improve hearing status in gentamicin-treated guinea pigs. Our findings suggest that EGb as a dietary supplement/healthy food may possess the potential to prevent gentamicin ototoxicity.

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