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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☆

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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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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.

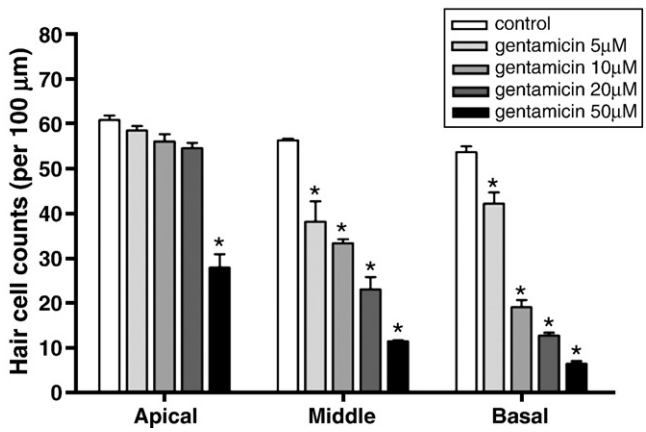

Fig. 1. Dose-dependent cytotoxicity of gentamicin on the hair cells of organotypic cochlear cultures of postnatal rats. The organ of Corti from postnatal 3-day-old rats was incubated in medium containing 0–50 μM gentamicin for 48 h. Labeled with rhodamine–phalloidin, hair cells were counted under a confocal 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.