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The beneficial effect of Hangesha-shin-to (TJ-014) in gentamicin-induced hair cell loss in the rat cochlea

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ABSTRACT

Objective: Ototoxic damage caused by aminoglycosides (AG) leads to the loss of cochlear hair cells (HCs). In mammals, mature cochlear HCs are unable to regenerate, and their loss results in permanent hearing deficits. Our objective was to protect the inner ear from damage after an AG challenge. The generation of reactive oxygen species (ROS), one of the earliest events in the process of AG ototoxicity, is considered to play a key role in the initiation of HC death. We examined whether Hangesha-shin-to (TJ-014), a traditional Japanese Kampo medicine considered to be a potent antioxidant, protects HCs from gentamicin (GM)-induced damage.
Methods: Organ of Corti explants removed from postnatal day 3–5 rats were maintained in tissue culture and exposed to 50 μM GM for up to 48 h. The effects of TJ-014 on GM-induced ototoxicity were assessed by HC counts and immunohistochemistry against cleaved caspase-3, 8-hydroxy-2′-deoxyguanosine (8-OHdG), and a probe reacting to mitochondrial function changes.
Results: TJ-014 treatments significantly reduced GM-induced HC loss and immunoreactivities for cleaved caspase-3 and 8-OHdG; these effects were correlated with increasing TJ-014 concentrations. Moreover, TJ-014 protected the mitochondrial membrane potential from GM ototoxicity.
Conclusion: These findings indicate the potential of TJ-014 to prevent GM-induced cochlear damage involving ROS.

1. Introduction

Aminoglycosides (AG) are one of the most commonly prescribed antibiotics worldwide. On the other hand, AGs are well known to be toxic to the inner ear and kidney. However, while nephrotoxicity is often reversible, ototoxicity is irreversible [1].

The generation of reactive oxygen species (ROS), one of the earliest events in the process of AG ototoxicity, is considered to play a key role in the process of hair cell (HC) death by activating c-Jun, p53, and other transcription factors [2–4]. The generation of ROS by AG is thought to be associated with iron-catalyzed oxidations [5]. AG and iron may form redox-active complexes, reducing molecular oxygen to superoxide radicals [5]. Iron chelators [6] and free radical scavengers [7–9] have been widely used for protection against AG-induced hearing loss [10,11]. However, success in the laboratory has not

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translated to the clinic, and cochlear protection-based treatment strategies for AG-induced ototoxicity have been disappointing thus far.

Hangesha-shin-to (TJ-014) is one of the Kampo formulas in Japanese traditional herbal medicine. We ordinarily use TJ-014 for diarrhea, dyspepsia, gastritis, stomatitis, psychoneurosis, and the management of chemotherapy-induced stomatitis. Several mechanisms related to the effects of TJ-014 on these symptoms have been reported [12–18]. Among them, we focused on the effects of baicalein, baicalin, wogonin, and berberine, which are ingredients of TJ-014. These ingredients are considered to have strong antioxidant effects [19,20]. Therefore, we hypothesized that TJ-014 would be a promising therapeutic drug for the treatment of AG-induced ototoxicity. In this study, we examined whether TJ-014 protects HCs from gentamicin (GM)-induced damage by using organ of Corti explants. In addition to HC counts, we also evaluated oxidative stress and some of the apoptotic pathways by immunohistochemistry for cleaved caspase-3, 8-OHdG, and mitochondrion-selective probes.

2. Materials and methods

2.1. Animals

The pregnant Sprague-Dawley (SD) rats (Japan SLC, Hamamatsu, Shizuoka, Japan) used in this study were cared for at the Institute of Laboratory Animals of the National Defense Medical College. The Animal Research Committee of the National Defense Medical College approved all experimental protocols, which were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

2.2. Hangesha-shin-to (TJ-014)

TJ-014, a dried powder extract prepared by Tsumura & Co. (Tokyo, Japan), and manufactured from a mixture of *Pinellia tuber* (relative quantity = 5.0), *Scutellaria root* (2.5), *Glycyrrhiza* (2.5), *processed ginger* (2.5), *Ginseng* (2.5), *Jujube* (2.5), and *Coptis Rhizome* (1.0), and mixture was dissolved in the initial medium described below before use. The yield of extract was 24% for TJ-014. Lot number of provided TJ-014 is '2100014010'. The quality variation among batches is minimized by using only Japanese pharmacopeias, and the specific amount of baicalin, glycyrrhizic acid, and berberine; the presence of the spots of active ingredients of the seven herbal components are ascertained in every batch. Detailed chemical profile of Hange-sha-shin-to extract is described in Japanese pharmacopeia 16th edition supplement I: 2508–2511 (http://www.pmda.go.jp/files/000152883.pdf).

TJ-014 is ordinarily used for diarrhea, dyspepsia, gastritis, stomatitis, and psychoneurosis. Furthermore, we typically use TJ-014 for the management of chemotherapy-induced stomatitis. In fact, its efficacy is widely recognized [12].

TJ-014 solution was prepared prior to organ culture. Firstly, TJ-014 extract was dissolved into the initial medium described below to a 1 mg/mL concentration using a magnetic stirrer in a sterile environment at 37 °C for 1 h. Secondly, the TJ-014 solution was filtered by using a DISMIC®-13CP disposable syringe filter unit (Toyo Roshi, Tokyo, Japan) and gradually diluted to obtain 1, 10, or 100 μg/mL concentrations for the initial medium.

2.3. Cochlear explant culture

Postnatal day 3–5 (P3–5) SD rats were deeply anesthetized with isoflurane and decapitated. The basal turn of the organ of Corti was dissected within 10 min and cultured by following previously reported methods [21,22]. Control explants were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 25 mM HEPES, and 30 U/mL penicillin, and were cultured in an incubator at 37 °C with 5% CO_2 and 95% humidity. GM explants were maintained in the above-described initial medium plus 50 μM of GM. TJ-014 explants were maintained in the initial medium plus 50 μM of GM with 0, 1, 10, or 100 μg/mL of TJ-014. These five groups were cultured for up to 48 h. At least 10 cultures were used for each condition tested.

2.4. Cell counts and immunohistochemistry

At the end of the culturing procedures, the explants were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min and permeabilized with 5% Triton X-100 (Sigma, St. Louis, MO, USA) in PBS with 10% FBS for 10 min. The specimens were stained with rhodamine phalloidin probe (1:100, rhodamine phalloidin, Invitrogen, Eugene, OR, USA) at room temperature for 45 min. Phalloidin is a specific marker for cellular F-actin and labels the stereociliary arrays and cuticular plates of HCs.

Caspase-3 activation was detected using cleaved caspase-3 antibody (1:100, Cleaved Caspase-3 D175 Rabbit Antibody, Cell Signaling Technology, Beverly, MA, USA). Specimens were then counterstained with Alexa 488 (1:200 Alexa Fluor® 488 Goat Anti-Rabbit IgG, Invitrogen).

To evaluate oxidative stress, we used anti-8-OHdG monoclonal antibody (1:10 JaICA, Hukuroi, Shizuoka, Japan). 8-OHdG is one of the predominant methods of inducing free radical DNA lesions and used as an indicator of free radical formation. Specimens were then counterstained with Alexa 488 (1:200 Alexa Fluor® 488 Goat Anti-Mouse IgG, Invitrogen). After rinsing in PBS, the cochlear explants were mounted on slides with an antifade mounting medium (VECTASHIELD with DAPI; Vector Laboratories, Burlingame, CA, USA). DAPI labeling was used to identify condensed HC nuclei.

To evaluate the mitochondrial membrane potential, we used mitochondrion-selective probes (MitoTracker® Red CMXRos, Molecular Probes®, Eugene, OR, USA) at a concentration of 200 nM and observed the mitochondrial membrane potential in live cells. After the cochlear explant culture procedure, these explants were washed three times in the initial medium and bathed in 200 nM MitoTracker® for 30 min. Subsequently, explants were washed three times in the initial medium and observed through a confocal microscope. All procedures to