

2,3,4',5-Tetrahydroxystilbene-2-O- β -D-glucoside ameliorates gentamicin-induced ototoxicity by modulating autophagy via Sesn2/AMPK/mTOR signaling

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Abstract. Gentamicin is an important aminoglycoside antibiotic used in the treatment of gram-negative bacterial infections, but nephrotoxicity and ototoxicity reduce its utility. The autophagy pathway is involved in damage of auditory hair cells. With the aim of developing new strategies for attenuating gentamicin ototoxicity, the present study investigated the otoprotective mechanism of 2,3,4',5-tetrahydroxystilbene-2-O- β -D-glucoside (THSG) *in vitro* using the mouse cochlear cell line UB/OC-2. MTT assay demonstrated that gentamicin reduced UB/OC-2 cell viability and western blotting showed that gentamicin upregulated autophagy-related proteins, such as Beclin, autophagy related 5 and LC3-II. THSG significantly attenuated gentamicin-induced cytotoxicity, clearly reduced LDH release observed by LDH assay and decreased the expression of autophagy-related proteins. Reverse-transcription-quantitative (RT-q) PCR and western blotting showed that THSG against gentamicin-induced autophagy via suppressing the expression of Sesn2, at both the mRNA and protein level and a possible involvement of AMP-activated protein kinase (AMPK)/mTOR signaling response. Collectively, the present study demonstrated

that THSG decreased gentamicin-induced ototoxicity in UB/OC-2 cochlear cells via the autophagic signaling in regulating Sesn2/AMPK/mTOR pathway. These results suggested that THSG might be a new therapeutic agent with the potential to attenuate gentamicin ototoxicity.

Introduction

Aminoglycosides are a group of antibiotics that includes streptomycin, kanamycin, tobramycin, gentamicin and neomycin. They are used to treat serious gram-negative bacterial infections (1). Gentamicin is a member of the aminoglycoside antibiotics and serves an important role in the treatment of gram-negative organisms. However, the clinical utility and dosage of gentamicin are limited by its well-known side effects, nephrotoxicity, neuropathy and ototoxicity. The ototoxicity of gentamicin, which is cumulative, bilateral and irreversible in the inner ear fluid (2). Patients with gentamicin-induced ototoxicity might suffer from imbalance, dizziness, vertigo, tinnitus, or hearing loss. After parenteral injection, gentamicin is transported into cochlear hair cells via endocytosis or by several aminoglycoside-permeant ion channels (3). Several molecular mechanisms explain how gentamicin may induce ototoxicity, including increased reactive oxygen species (ROS), activated c-Jun N-terminal kinase (JNK), induced caspase signaling cascades and defective mitochondria metabolism (4,5). Therefore, finding ways to relieve cell damage can have therapeutic implications in gentamicin-induced ototoxicity.

The ototoxic effects of gentamicin are mediated by apoptosis, autophagy and the Akt survival pathway (1,6). Among these pathways, autophagy serves an important role in cellular homeostasis under physiological or chemical stress (7). Autophagy appears both pro-survival and pro-death mechanisms and the balance between apoptosis and autophagy determines the fate of injured cells (8,9). Aberrant autophagy

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may cause hair cell loss and influence auditory function (10,11). However, excessive activation of autophagy might also promote cell death via the apoptosis pathway and pathological changes (12). Autophagy induced under severe hypoxia and concomitant to metabolic stress is often associated with cell death (13).

The mammalian target of rapamycin (mTOR) is a key regulator in sensing cellular stress that regulates growth, proliferation, survival and aging (14). The reduction of mTOR may be a potential strategy to prevent age related hearing loss (15). AMP-activated protein kinase (AMPK) is an energy-sensing kinase that could activate autophagy process through the inhibition of mTOR signaling (16,17). *Sesn2*, a member of the oxidative stress pathway, is involved in the regulation of mTOR. *Sesn2* negatively regulates mTOR via AMPK and recombinant activating genes (*Rag*) and thereby attenuates the accumulation of ROS (18). Previous research has shown that *Sesn2* serves a key role in gentamicin-induced hair cell death via modulation of AMPK/mTOR signaling (19).

A main active compound of the traditional Chinese herb plant *Polygonum multiflorum* Thunb is 2,3,4',5-tetrahydroxy stilbene-2-O- β -D-glucoside (THSG) (20). Pharmacological studies have demonstrated that THSG exhibits numerous biological functions in the treatment of atherosclerosis, lipid metabolism, cerebral ischemia, diabetic complications, hair growth problems and a number of other conditions (21-24). THSG is composed of stilbene and glucoside, which contain a number of polar hydroxyl groups in chemical structure and it has been demonstrated to possess strong antioxidant and free radical scavenging activities (Fig. 1) (20). THSG can also activate the AMPK/Nrf2 signaling pathways and increase renal and neural cell survival (23,24).

Previous studies have shown that THSG may be effective in gentamicin-induced ototoxicity (6,25). However, very few studies have attempted to evaluate the functional role of THSG and the molecular mechanism underlying its effects on gentamicin ototoxicity. The current study was conducted to determine whether THSG could attenuate gentamicin-induced autophagy in effort to help develop new strategies in the protection of patients who are vulnerable to gentamicin ototoxicity.

Materials and methods

Chemicals and reagents. Gentamicin (cat. no. 2623184) was purchased from Standard Chemical & Pharmaceutical Co. Ltd. THSG (cat. no. HY-N0652) was obtained from MedChemExpress and dissolved in dimethyl sulfoxide (DMSO) to create a 20 mM stock solution. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; cat. no. 298-93-1) was purchased from VWR International, LLC. Lactate dehydrogenase (LDH) assay kit (cat. no. ENZ-KIT157) and goat anti-rabbit IgG-HRP antibody (cat. no. ADI-SAB-300) was purchased from Enzo Life Sciences. Antibodies against Beclin (cat. no. 3738), autophagy related 5 (ATG5; cat. no. 12994), LC3 (cat. no. 3868), phosphorylated (p)-mTOR (Ser2448; cat. no. 5536), mTOR (cat. no. 2983) and β -actin (cat. no. 4967) were obtained from Cell Signaling Technology, Inc. Antibodies against p-AMPK (Thr172; cat. no. AP0116), AMPK (cat. no. A1229) and *Sesn2* (cat. no. A14220) were

purchased from ABclonal Biotech Co., Ltd. Goat anti-mouse IgG-HRP antibody (cat. no. NEF822001EA) was purchased from PerkinElmer, Inc. Bafilomycin A1 (cat. no. B1793), chloroquine (cat. no. C6628) and monodansylcadaverine (MDC; cat. no. 240141) was purchased from MilliporeSigma.

Cell culture and drug treatment. The mouse cochlear cell line UB/OC-2 was purchased from Ximbo. Cells were cultured in minimal essential medium (MEM)/GlutaMAX (cat. no. 41090036; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; cat. no. SH30066; Hyclone; Cytiva) and 50 U/ml of IFN- γ (cat. no. 485-MI; R&D Systems, Inc.) in a humidified incubator at 33°C with 5% CO₂ (26).

Cell viability assay. The MTT method was used to measure cell viability. The cells were seeded into 24-well plates at a density of 4x10⁴ cells/well and treated with the various concentrations of gentamicin for 24 h. The cells were then incubated in culture medium containing 0.2 mg/ml MTT solution for 4 h at 33°C. The formazan crystals were dissolved in DMSO and the absorbance detected at a wavelength of 570 nm using a microplate reader (Infinite 200 PRO Series Multimode Reader; Tecan Group, Ltd.). Cell viability of the control group was considered to be 100% (27).

Lactate dehydrogenase (LDH) release assay. The LDH method was used to measure cellular cytotoxicity. The cells were seeded into 96-well plates at a density of 8x10³ cells/well. The cells were pretreated with THSG (5, 10 and 20 μ M) or an autophagy inhibitor (10 nM bafilomycin A1 or 20 μ M chloroquine) for 6 h and then cotreated with 750 μ M gentamicin for 24 h at 33°C. LDH release was detected using a LDH Cytotoxicity Assay kit according to the manufacturer's instructions. The supernatants were then transferred to another 96-well plate and the absorbance measured at a wavelength of 490 nm using a microplate reader.

Cell morphology. The cells were seeded into 6-well plates at a density of 4x10⁵ cells/well and treated with 750 μ M gentamicin for 6, 12 and 24 h. Cellular morphological changes were then observed using a light microscope at x400 magnification (Olympus BX41 microscope; Olympus Corporation) and then evaluated in 10 randomly selected images per group.

Western blot analysis. Total protein was extracted from the cells and homogenized using protein extraction reagent (cat. no. 78501; Thermo Fisher Scientific, Inc.) containing protease (cat. no. 539134) and phosphates inhibitor (cat. no. 524629; MilliporeSigma). Protein concentration was quantified by BCA reagent (cat. no. 97065; VWR International, LLC). Equal amounts of protein (20 μ g/sample) were subjected to 10 or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis and the separated proteins transferred onto polyvinylidene difluoride membranes. After being blocked with 3% (w/v) bovine serum albumin (BSA; cat. no. A9418; MilliporeSigma) in Tris-buffered saline (TBS; cat. no. 75801; VWR International, LLC) for 1 h at room temperature, the membranes were incubated

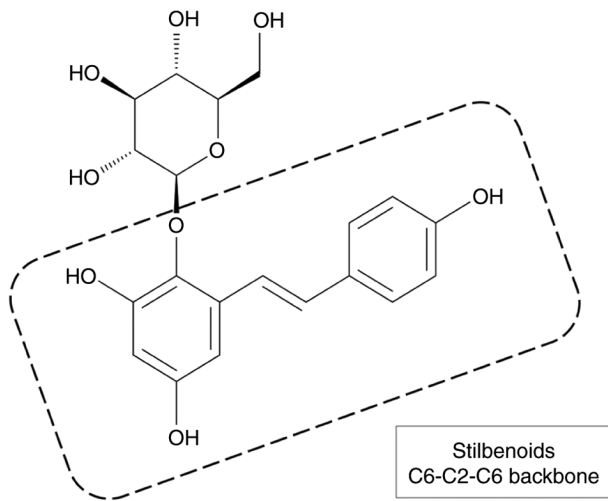


Figure 1. Structure of THSG. THSG is a member of stilbenoids, a group of phenolic compounds containing C6-C2-C6 backbone and which contain a number of polar hydroxyl groups in their chemical structure. THSG, 2,3,4,5-tetrahydroxystilbene-2-O- β -D-glucoside.

overnight at 4°C with primary antibodies diluted 1:1,000. After six washes in TBS containing 0.1% Tween (TBST), the membranes were incubated for 1 h at room temperature with the appropriate secondary antibodies diluted 1:5,000. After six washes in TBST, the target proteins were detected using enhanced chemiluminescence (Bio-Rad Laboratories, Inc.) and imaged using a KETA C Chemi Imaging System (Wealtec Corporation) (28).

Transmission electron microscope (TEM). The cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C and then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer at room temperature for 1 h. The cells were stained with 2% uranyl acetate at room temperature for 30 min, followed by gradient dehydration with ethanol-acetone at room temperature for 15 min each and finally embedded in Spurr's resin at 60°C for 12 h. Serial ultrathin sections (~80 nm) were made with a Ultracut-R ultramicrotome (Leica Microsystems GmbH) and observed with a Hitachi H-7500 transmission electron microscope (Hitachi, Ltd.) at an accelerating voltage of 80 kV (29). Images were captured with AMT XR-16 digital camera system in combination with AMT Capture Engine, v602.600.51 software (Advanced Microscopy Techniques Corporation).

Monodansylcadaverine (MDC) staining. The cells were washed twice with PBS and incubated in 50 μ M MDC dye in culture medium for 20 min at 33°C. After washing twice with PBS, the MDC fluorescence was measured at 335 nm excitation and 420 nm emission. Images were captured using an Olympus BX41 fluorescent microscope at x400 magnification (Olympus Corporation) (27).

Reverse-transcription-quantitative (RT-q) PCR. Total RNA was isolated using a RNeasy Mini kit (cat. no. 74101) and QIAshredder (cat. no. 79654) from Qiagen GmbH according to the manufacturer's recommended protocol. RT was performed according to the protocol supplied with the

SuperScript III Reverse Transcriptase kit (cat. no. 18080; Invitrogen; Thermo Fisher Scientific, Inc.) for qPCR. Gene expression was performed using a SYBR Green PCR kit (cat. no. 208052) and measured with a StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The RT conditions were: 50 min at 50°C for and 20 sec at 70°C, followed by preservation at 4°C. The primers used for *Sesn2* were sense 5'-TAGCCTGCAGCCTCACCTAT-3' and antisense 5'-TATCTGATGCCAAAGACGCA-3'; the primers used for *GAPDH* were sense 5'-GCCAAAAGGGTCATCATC TC-3' and antisense 5'-CACACCCATCACAAACATGG-3'. The qPCR was run with following thermocycling conditions: 10 min at 95°C, followed by 15 sec at 95°C and 1 min at 52°C for 40 cycles. The relative expression level was calculated according to the $2^{-\Delta\Delta C_q}$ method normalized with the internal reference gene to *GAPDH* (25,30,31).

Statistical analysis. Statistical analysis was performed using SPSS Version 22.0 software (IBM Corporation) and data were presented as mean \pm the standard deviation (SD) from at least three independent experiments. The data was used Shapiro-Wilk test to check normality. Differences were determined using one-way analysis of variance (ANOVA) followed by Tukey test or Kruskal-Wallis test followed by Dunn's test for comparing multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Gentamicin decreases viability, increases cytotoxicity and alters cellular morphology in UB/OC-2 cochlear cells. To evaluate the effect of gentamicin on the viability and cytotoxicity of UB/OC-2 cochlear cells, the cells were treated with different concentrations of gentamicin (125, 250, 500, 750 and 1,000 μ M) for 24 h. The MTT assay results showed that gentamicin significantly inhibited cell viability in a concentration-dependent manner and viable cells related to untreated control were observed $52.95 \pm 6.04\%$ at 750 μ M gentamicin (Fig. 2A). Therefore, 750 μ M was chosen in the following experiments for UB/OC-2 cells injury but not 1,000 μ M gentamicin because the estimated IC_{50} was 800 μ M.

Subsequently, cellular cytotoxicity was evaluated using the LDH method. Gentamicin increased cytotoxicity of UB/OC-2 cells in a concentration-dependent manner (Fig. 2B). To investigate the effect of gentamicin in UB/OC-2 cochlear cells, cell morphology was observed after treatment with 750 μ M gentamicin at various time points (6, 12 and 24 h). As shown in Fig. 2C, the number and area of vacuoles in UB/OC-2 cochlear cells increased after 6 h of 750 μ M gentamicin treatment. Overall, these results suggested gentamicin treatment decreased UB/OC-2 cochlear cell viability, increased cytotoxicity and altered the cell morphological characteristics.

THSG increases the cell viability and suppresses cytotoxicity under the treatment of gentamicin in UB/OC-2 cochlear cells. Following a previous study (25), no cellular toxicity was detectable in THSG-treated UB/OC-2 cochlear cells (≤ 20 μ M). To confirm that the otoprotective effect of THSG, cell viability and cytotoxicity were measured after treatment of gentamicin and THSG for 24 h. The results of MTT assay showed the

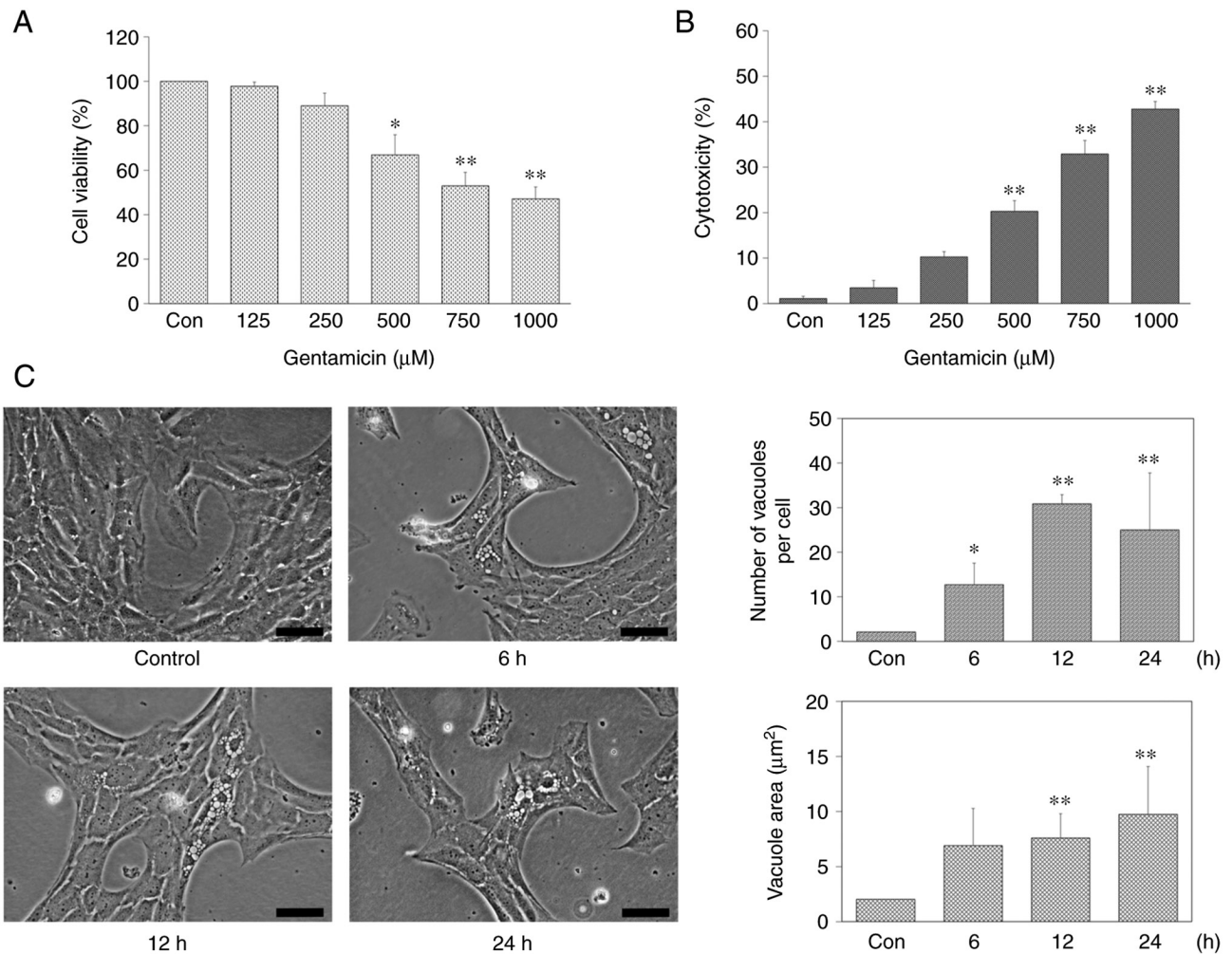


Figure 2. Effects of gentamicin on cell viability, cellular cytotoxicity and morphological changes in UB/OC-2 cochlear cells. The cells were treated with 125, 250, 500, 750 and 1,000 μM gentamicin for 24 h. Following treatment, (A) cell viability was measured using the MTT method and (B) cellular cytotoxicity was evaluated using lactate dehydrogenase method. $n=6$ per group. (C) Morphological changes were observed following treatment with 750 μM gentamicin for 6, 12 and 24 h. Scale bar=50 μm . The number and area of vacuoles per cell were analyzed at least 10 randomly chosen cells. Quantitative data are expressed as mean \pm SD. * $P<0.05$, ** $P<0.01$ vs. the control group.

cell viability was significantly increased in THSG-treated groups compared to the gentamicin-only treated group (Fig. 3A). Furthermore, THSG-treated groups were reduced cytotoxicity compared with the gentamicin-only treated group by measuring LDH release (Fig. 3B). To check ultrastructural changes, UB/OC-2 cochlear cells in the control group, gentamicin group (750 μM), THSG-treated group (pretreated with 20 μM THSG for 6 h before 750 μM gentamicin exposure) and THSG-only group (20 μM) were analyzed by TEM 24 h after treatment. In the THSG-treated group induced fewer vacuole formation compared with in the gentamicin group, but no effect was observed on ultrastructural variable in the THSG-only group (Fig. 3C). These results indicated that THSG could effectively protect UB/OC-2 cochlear cells against gentamicin-induced ototoxicity.

Gentamicin induces autophagy in UB/OC-2 cochlear cells. Based on morphological and ultrastructural attribute, autophagy may be involved in gentamicin-induced ototoxicity. Autophagy-related proteins Beclin, ATG5 and LC3-II were estimated the autophagic levels by western blot analysis. As

shown in Fig. 4A, the protein expression of Beclin, ATG5 and LC3-II significantly increased in the gentamicin group compared with the control group. Protein expression was analyzed after treatment of the cells with 125, 250, 500, 750 and 1,000 μM gentamicin for 24 h (Fig. 4B). The expression of Beclin, ATG5 and LC3-II each increased as gentamicin concentration was increased. Accordingly, gentamicin might induce autophagy in a time- and concentration-dependent manner in UB/OC-2 cochlear cells. Next, to clarify whether the effects were actually due to autophagy or just proteosomal protein degradation following gentamicin toxicity, autophagic flux was analyzed in gentamicin-treated UB/OC-2 cochlear cells by lysosomal degradation inhibitors (bafilomycin A1 or chloroquine). Bafilomycin A1 is a V-ATPase inhibitor to prevent lysosome acidification and block autophagosome-lysosome fusion (32). Chloroquine is a lysosomotropic agent that can inhibit autophagic degradation in the lysosomes by altering the lysosomal pH (32). As shown in Fig. 5A, a marked increase in LC3-II accumulation was found in gentamicin-treated UB/OC-2 cochlear cells with 10 nM bafilomycin A1 or 20 μM chloroquine; however, the protein levels of Beclin and ATG5

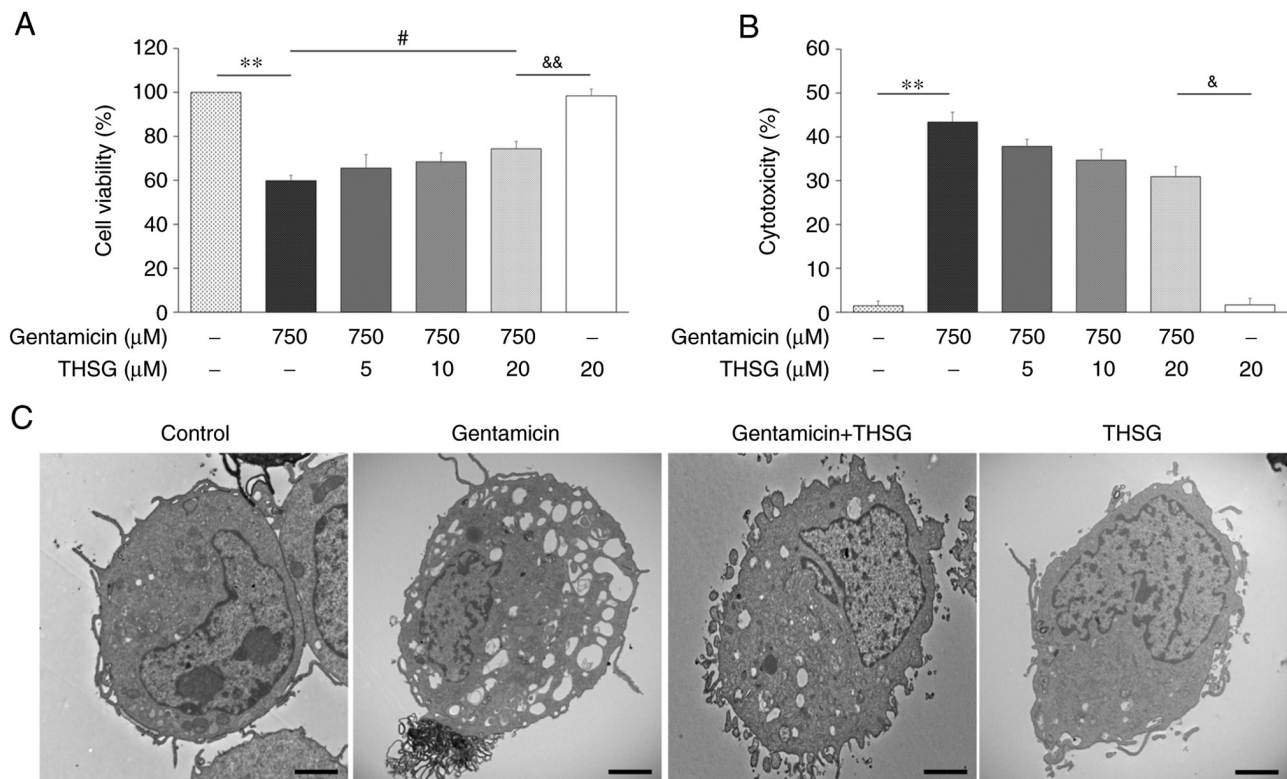


Figure 3. Effects of THSG on cell viability and cytotoxicity under the treatment of gentamicin in UB/OC-2 cochlear cells. The cells were pretreated in the presence of indicated concentrations of THSG (5, 10, or 20 μM) for 6 h and then cotreated with or without 750 μM gentamicin for 24 h. (A) Cell viability was analyzed by MTT assay. n=6 per group. (B) Cellular cytotoxicity was evaluated using lactate dehydrogenase method. n=6 per group. Quantitative data are expressed as mean \pm SD. **P<0.01 vs. the control group; #P<0.05 vs. the gentamicin group; &P<0.05, &&P<0.01 vs. the 20 μM THSG group. (C) Representative transmission electron microscopy images of UB/OC-2 cochlear cells in four groups: control; 750 μM gentamicin treatment for 24 h; 20 μM THSG pretreatment for 6 h and then cotreated with 750 μM gentamicin for 24 h; and 20 μM THSG after 24 h. Scale bar=2 μm . THSG, 2,3,4',5-tetrahydroxystilbene-2-O- β -D-glucoside.

were not significantly altered. Impaired autophagic degradation led to increased LDH release (Fig. 5B). Disruption of autophagic flux by bafilomycin A1 or chloroquine may slightly enhance gentamicin-induced cytotoxicity in UB/OC-2 cochlear cells.

THSG decreases gentamicin-induced autophagy in UB/OC-2 cochlear cells. The effect of THSG on gentamicin-induced autophagy was explored through the determination of autophagy related protein expression. Protein expression of Beclin, ATG5 and LC3-II were evaluated by western blot analysis in cells pretreated with 5, 10 and 20 μM THSG for 6 h and then cotreated with 750 μM gentamicin for 24 h. Levels of all three proteins were decreased in the THSG-treated groups compared to that in the gentamicin-only treated group, especially in the 20 μM THSG-treated group (Fig. 6A). To analyze the effect of THSG on gentamicin-induced autophagy, the cells were stained with MDC dye to detect autophagic vacuoles. In addition, the number of MDC-labeled vacuoles was reduced in the THSG-treated groups compared with the gentamicin-only treated group (Fig. 6B). Taken together, these results showed that THSG decreased gentamicin-induced autophagy in UB/OC-2 cochlear cells.

THSG decreases gentamicin-induced autophagy via modulation of *Sesn2*/AMPK/ mTOR signaling. *Sesn2* serves a major role in suppression of oxidative stress and the regulation

of AMPK/mTOR signaling, which is crucial for autophagy induction (19). In the current study, UB/OC-2 cochlear cells were pretreated with 5, 10 and 20 μM THSG for 6 h and then cotreated with 750 μM gentamicin for 16 h. The mRNA levels of *Sesn2* in the cells decreased as the concentration of THSG was increased (Fig. 7A). In addition, the protein level of *Sesn2* was measured after pretreated with 5, 10 and 20 μM THSG for 6 h and then cotreated with 750 μM gentamicin for 24 h. The protein expression level of *Sesn2* was diminished in the THSG-treated groups compared to the gentamicin-only group (Fig. 7B). The results showed that pretreatment with THSG produced a significant inhibition of this gentamicin-induced effect on the mRNA and protein levels of *Sesn2*.

The cells were pretreated with 5, 10 and 20 μM THSG for 6 h and then cotreated with 750 μM gentamicin for 24 h. The following experiments were conducted to show whether THSG regulates *Sesn2* downstream effectors, AMPK and downstream mTOR. Although AMPK levels in UB/OC-2 cochlear cells increased as THSG concentrations were increased, levels of the active form of the enzyme, p-AMPK, inversely decreased (Fig. 7C). On the other hand, mTOR levels decreased relative to increased THSG concentrations, but again levels of the active form, p-mTOR, inversely increased (Fig. 7D). Taken together, the results suggested that THSG could decrease *Sesn2* expression at both the mRNA and protein level and thereby reduce autophagy in the UB/OC-2 cochlear cells in regulating AMPK/ mTOR signaling response.

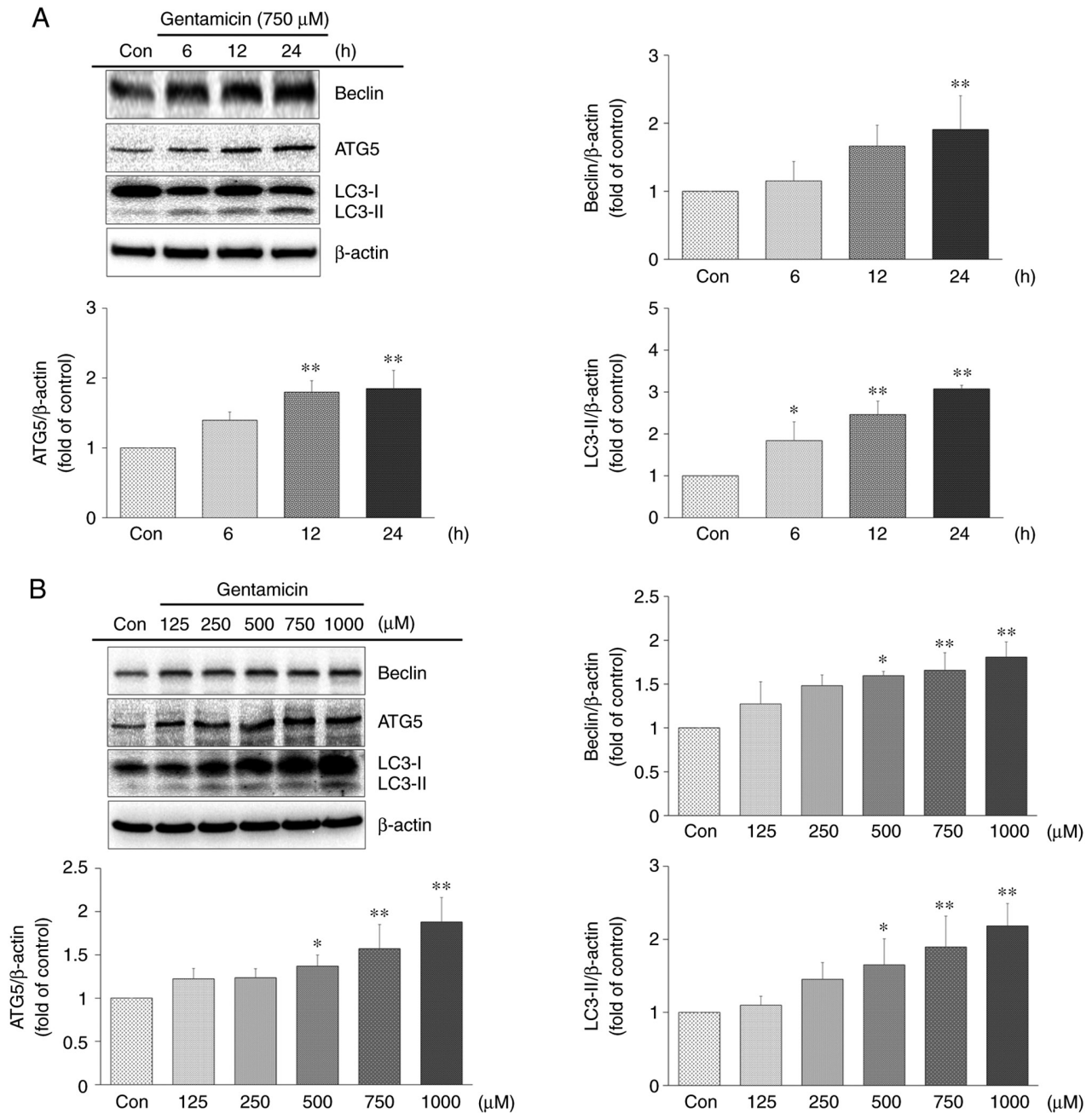


Figure 4. Effects of gentamicin on levels of autophagy related proteins in UB/OC-2 cochlear cells. (A) The cells were treated with 750 μ M gentamicin for 6, 12 and 24 h and analyzed by western blotting for protein expression of Beclin, ATG5 and LC3-II. $n=3$ per group. (B) The cells were treated with 125, 250, 500, 750 and 1,000 μ M gentamicin for 24 h and analyzed by western blotting to evaluate protein expression of Beclin, ATG5 and LC3-II. β -actin was used as a loading control. $n=3$ per group. Quantitative data are expressed as mean \pm SD. * $P<0.05$, ** $P<0.01$ vs. the control group. ATG5, autophagy related 5.

Discussion

Cochleototoxicity is generally observed with the use of amikacin, kanamycin and neomycin, whereas the use of streptomycin and gentamicin are associated with vestibulotoxicity (1). Some patients are more vulnerable to aminoglycoside ototoxicity, including the elderly, those with renal insufficiency, diuretic users and those with gene polymorphisms (33). Gentamicin ototoxicity is cumulative and dose dependent (4,34). Consistent with previous reports, data in the current study showed that UB/OC-2 cochlear cells exhibit reduced viability and increased cytotoxicity as gentamicin concentrations increase (Fig. 2).

The ototoxicity of aminoglycosides is attributed to the production of excessive ROS (1). Stilbenoids, a group of phenolic compounds containing C6-C2-C6 backbone, have antioxidant activities by reacting with ROS, inducing antioxidant enzymes (such as catalase, glutathione peroxidase, heme oxygenase and superoxide dismutase) and activating Nrf2-antioxidant response element system (Fig. 1) (35,36). Previous studies have reported that stilbene-glycosides possess biological activities underlying the antioxidant, anti-inflammatory and anti-apoptotic effects, which is due to partial deglycosylation by the intestine and/or liver (37,38). In a previous study, THSG appears to be a good antioxidant with its free radical scavenging activity being comparable to ascorbic acid (25). Furthermore,

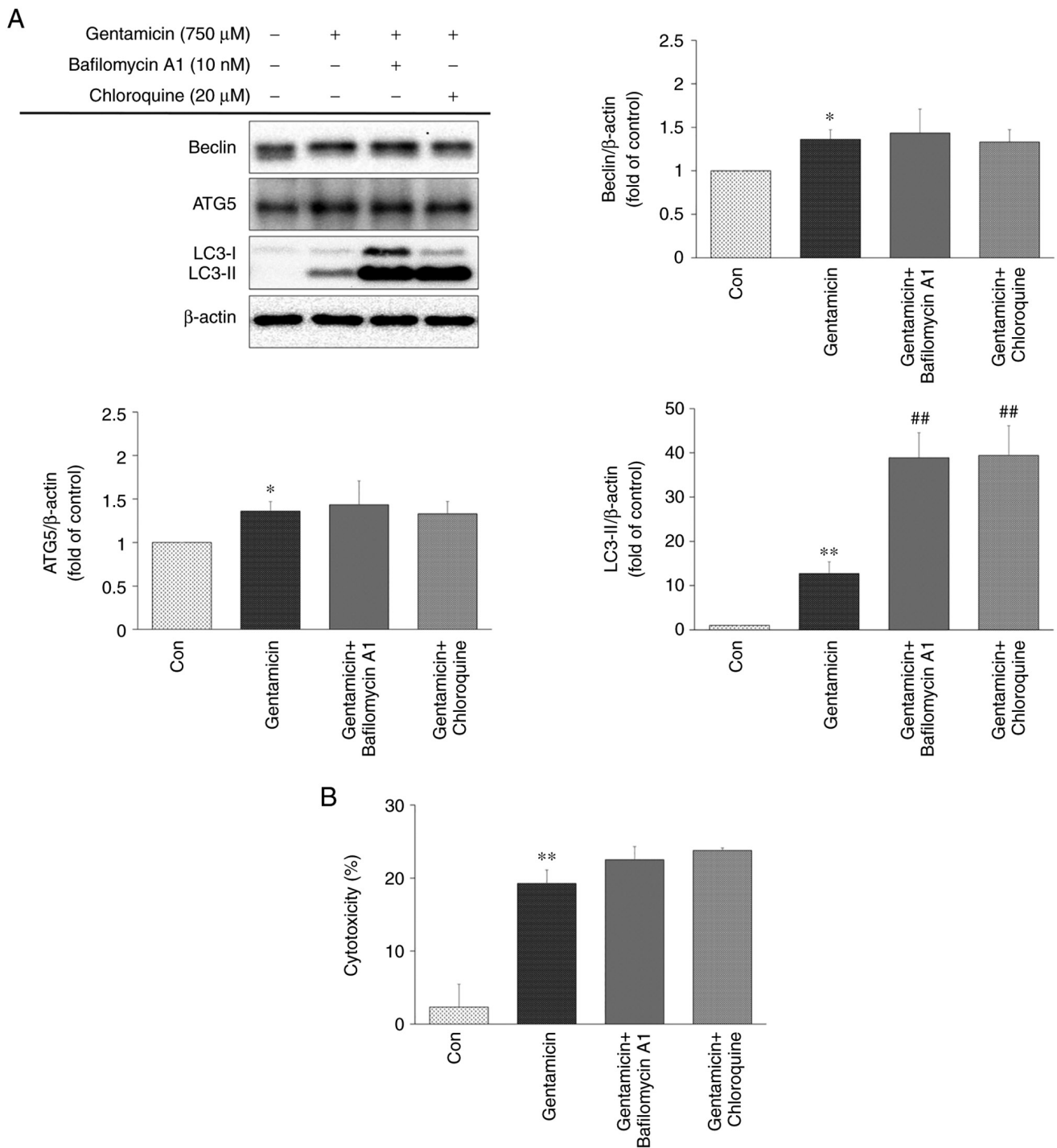


Figure 5. Effects of lysosomal degradation inhibitors on gentamicin-induced autophagy in UB/OC-2 cochlear cells. The cells were pretreated with 10 nM bafilomycin A1 or 20 μ M chloroquine for 6 h and then cotreated with 750 μ M gentamicin for 24 h. (A) The expression of Beclin, ATG5 and LC3-II were analyzed by western blotting. β -actin was used as a loading control. n=3 per group. (B) Cellular cytotoxicity was evaluated using lactate dehydrogenase method. n=6 per group. Quantitative data are expressed as mean \pm SD. *P<0.05, **P<0.01 vs. the control group; ##P<0.01 vs. the gentamicin group. ATG5, autophagy related 5.

THSG is able to promote several antioxidant pathways, such as Nrf2-Keap1 and AMPK/Nrf2 (23,24). THSG also inhibits apoptosis in gentamicin-induced cell damage (6). Data in the present study showed that THSG significantly protected against gentamicin-induced ototoxicity by the MTT assay and LDH method (Fig. 3A and B).

Autophagy serves a protective role in a number of situations of cochlear hair cell stress or injury, such as drug-induced ototoxicity, noise-induced hair cell injury and

aging (39-41). However, excessive activation of autophagy may cause cell damage or death (8,9). Meclofenamic acid may inhibit excessive autophagy and protect hair HEI-OC1 cells from cisplatin-induced cell death (12). In the current study, gentamicin induced vacuole formation in UB/OC-2 cochlear cells, which were comparable to autophagosomes. The data showed that if the exposure time or concentration of gentamicin was increased, the level of autophagy proteins Beclin, ATG5 and LC3-II increased (Fig. 4). Disruption of

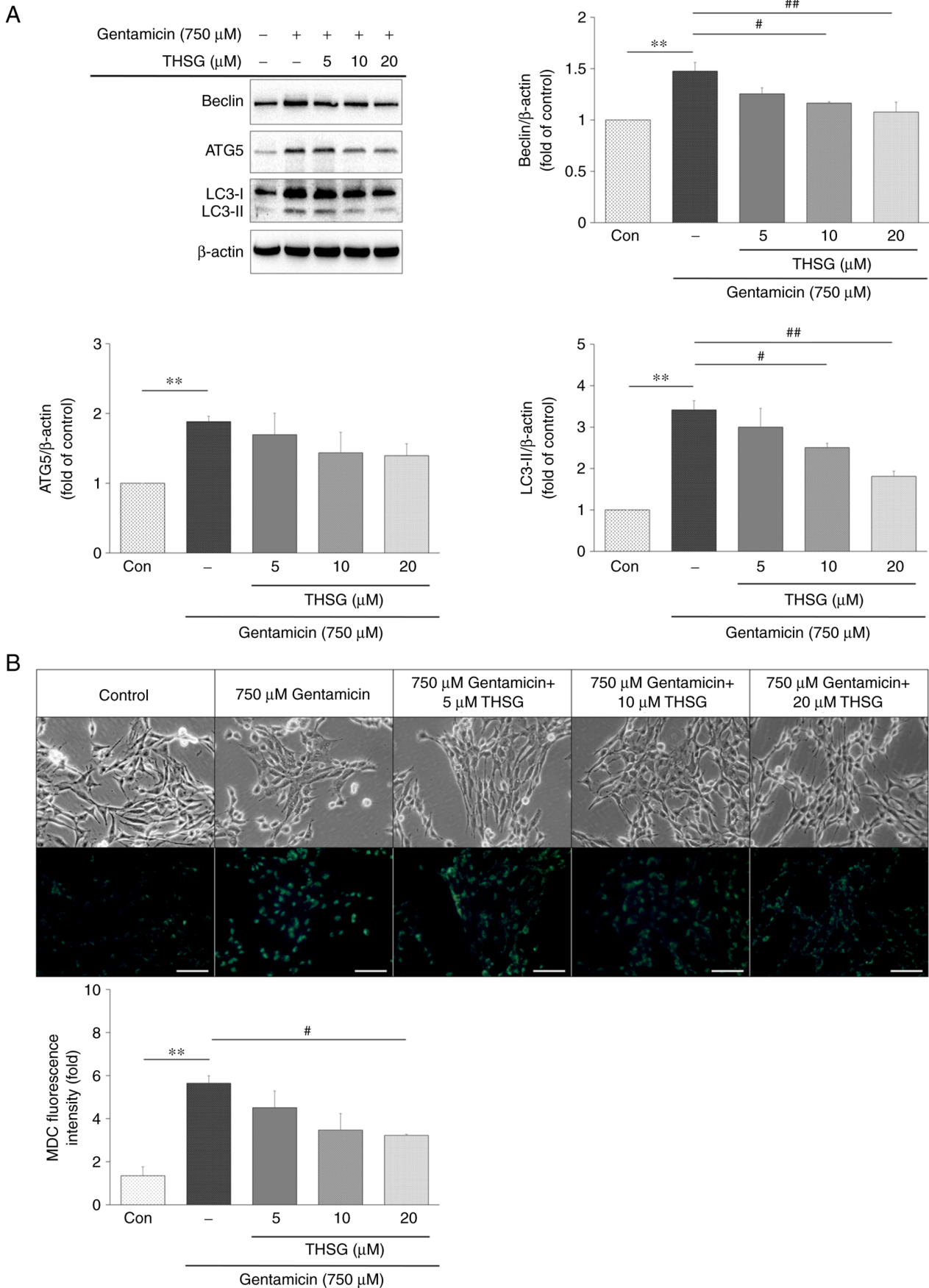


Figure 6. Effects of THSG on gentamicin-induced autophagy in UB/OC-2 cochlear cells. The cells were pretreated with 5, 10 and 20 μ M THSG for 6 h and then cotreated with 750 μ M gentamicin for 24 h. (A) Protein expression of Beclin, ATG5 and LC3-II were detected by western blotting. β -actin was used as a loading control. $n=3$ per group. (B) Autophagic vacuoles were labeled using MDC dye. Scale bar=100 μ m. $n=6$ per group. Quantitative data are expressed as mean \pm SD. ** $P<0.01$ vs. the control group; # $P<0.05$, ## $P<0.01$ vs. the gentamicin group. THSG, 2,3,4',5-tetrahydroxystilbene-2-O- β -D-glucoside; ATG5, autophagy related 5; MDC, monodansylcadaverine.

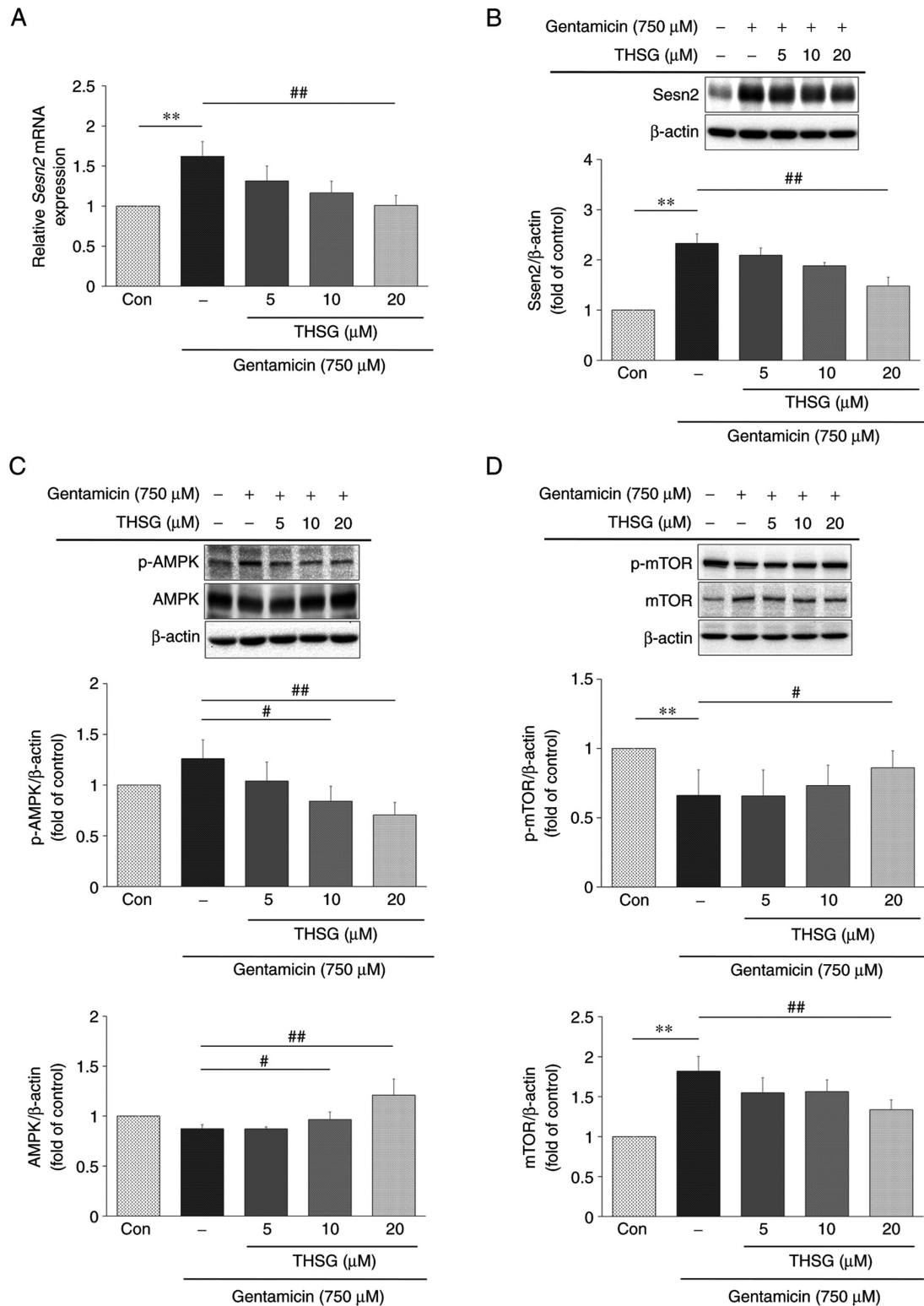


Figure 7. Effects of THSG on Sesn2, AMPK and mTOR levels in UB/OC-2 cochlear cells following gentamicin treatment. (A) UB/OC-2 cochlear cells were pretreated with 5, 10 and 20 μ M THSG for 6 h and then cotreated with 750 μ M gentamicin for 16 h. The expression levels of *Sesn2* mRNA were evaluated using reverse-transcription-quantitative PCR. *GAPDH* was used as an internal control. n=3 per group. UB/OC-2 cochlear cells were pretreated with 5, 10 and 20 μ M THSG for 6 h and then cotreated with 750 μ M gentamicin for 24 h. Protein expression of Sesn2 (B), p-AMPK and AMPK (C), p-mTOR and mTOR (D) were evaluated by western blotting. β -actin was used as a loading control. n=3 per group. Quantitative data are expressed as mean \pm SD. **P<0.01 compared with the control group; #P<0.05, ##P<0.01 vs. the gentamicin group. THSG, 2,3,4',5-tetrahydroxystilbene-2-O- β -D-glucoside; AMPK, AMP-activated protein kinase; p-, phosphorylated.

autophagic flux by bafilomycin A1 or chloroquine augmented the level of LC3-II and slightly promoted gentamicin-induced cytotoxicity (Fig. 5). This suggested that autophagy did not

serve a major role in cell survival in the present study. No further experiments on the synergy between THSG and the autophagy inhibitors were performed and this was the

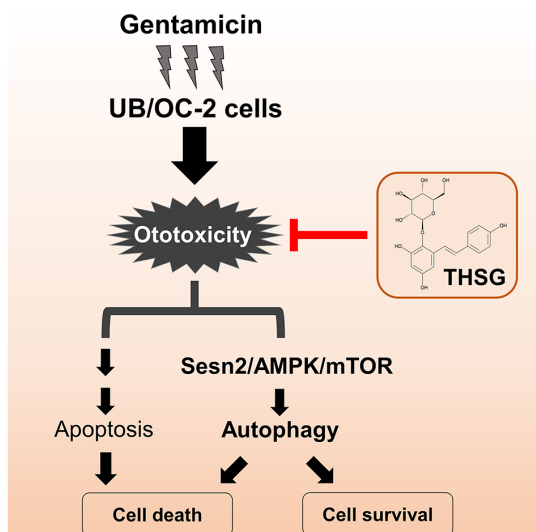


Figure 8. A proposed model of the potential mechanisms involved in the protective effect of THSG against gentamicin-induced ototoxicity. THSG, 2,3,4,5-tetrahydroxystilbene-2-O- β -D-glucoside; AMPK, AMP-activated protein kinase.

limitation of the present study. Furthermore, THSG reduced the expression of gentamicin-induced autophagy related proteins and autophagic vacuoles (Fig. 6). In view of the above, the protection effects of THSG might be initiated in front of autophagy process.

Autophagy is especially regulated by the AMPK/mTOR pathway (17). Sesn2 is a conserved antioxidant protein that reduces ROS (42). In the current study, when UB/OC-2 cochlear cells were cotreated with gentamicin and THSG, the expression of Sesn2 decreased at both the protein and mRNA levels and thus decreased autophagy. It is possible that THSG reduced ROS in the gentamicin-treated cells and Sesn2 expression decreased consequently. Unlike in the cochlear of mice where the expression of Sesn2 is unchanged and ultimately downregulated in gentamicin-treated explants (19), the data showed that Sesn2 levels increased both on the mRNA and protein level in gentamicin-treated UB/OC-2 cochlear cells (Fig. 7A and B). However, there are still some mechanisms of interaction between Sesn2 and gentamicin that should be further evaluated. When cells are in a stress situation or when ROS increases, AMPK is activated to its phosphorylated form, which then suppresses mTOR, a suppresser of autophagy and autophagy consequently increases in the cells. Sesn2 activates the AMPK/mTOR pathway and enhances autophagy (19,42). When mTOR activity is inhibited by rapamycin, hair cell survival increases following gentamicin exposure (19). Gentamicin increased the phosphorylation of AMPK, which then reduced phosphorylation of mTOR. However, THSG was able to reverse the effect of gentamicin by decreasing phosphorylation of AMPK and increasing phosphorylation of mTOR, thereby reducing autophagy (Fig. 7C and D). The Sesn2/AMPK/mTOR pathway might have acted as a possible stress-relieving mechanism and is involved autophagy induced by gentamicin.

According to the results of the present study combined with a previous study (6), gentamicin could induce cell toxicity followed by the programmed cell death pathways, autophagy and apoptosis. Evidence showed THSG decreased not only

gentamicin-induced apoptosis but also stress-inducible protein Sesn2 and thereby reduced gentamicin-induced autophagy by regulating AMPK/mTOR signaling. Under gentamicin treatment, THSG might modulate the UB/OC-2 cochlear cells toward autophagic survival but not autophagic cell death or apoptosis (Fig. 8).

Gentamicin can not only induce ototoxicity, but also damage other organs. Further studies about protection effects of THSG in other organs such as kidney or nerves will be conducted. In addition, THSG has idiosyncratic hepatotoxicity that involves the effect of THSG on cytochrome P450 (CYP) enzyme activity (43). There were only few studies on CYP and cochlear cells. Maybe studies about the effect of THSG in cochlear cell CYP activity should be induced in the future.

In summary, the results of the present study showed that THSG significantly suppressed gentamicin-induced ototoxicity and thus modulated autophagy via the Sesn2/AMPK/mTOR signaling pathway in UB/OC-2 cochlear cells. Therefore, THSG may serve as a protective agent against gentamicin-induced ototoxicity. The pharmacological effects of THSG *in vivo* in animal model should be explored in future research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YHW and HYL were responsible for the study design and implementation, data analysis and manuscript preparation. YHW, HYL and JNL confirm the authenticity of all the raw data. JNL and CCL interpreted the data and wrote the manuscript. YHW, HYL and JNL were responsible for data collection and statistical analysis. GFT, CFH, CJH and HPW served an important role in study design and guidance and were responsible for the revision of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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