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# Role of autophagy in cisplatin-induced ototoxicity

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ABSTRACT

*Objective:* Hearing loss is a major side effect of cisplatin chemotherapy. Although cell death in cisplatin-induced ototoxicity is primarily caused by apoptosis, the exact mechanism behind the ototoxic effects of cisplatin is not fully understood. Autophagy is generally known as a pro-survival mechanism that protects cells under starvation or stress conditions. However, recent research has reported that autophagy plays a functional role in cell death also. This study aimed to investigate the role of autophagy in cisplatin-induced ototoxicity in an auditory cell line.
*Methods:* Cultured HEI-OC1 cells were exposed to 30 μM cisplatin for 48 h, and cell viability was tested using MTT assays. To evaluate whether autophagy serves to cell death after cisplatin exposure, western blotting and immunofluorescence staining for LC3-II were performed. Markers of two autophagy-related pathways, mTOR and class III PI3K, were also investigated.
*Results:* The formation of the autophagic protein LC3-II in response to 30 μM cisplatin increased with time. The early upregulation of autophagy exerted cytoprotective activity via the class III PI3K pathway. But later increase in autophagy induced cell death by suppressing the mTOR pathway.
*Conclusion:* Our results prove that autophagy could induce cell death during cisplatin-induced ototoxicity, and modulating the autophagic pathway might be another strategy against cisplatin-induced ototoxicity.


## 1. Introduction

Cisplatin is a highly effective chemotherapeutic agent for the treatment of human solid tumors such as ovarian, testicular, cervical, head and neck, lung, and bladder cancers. However, the ototoxicity induced by cisplatin is an important obstacle in its utility and therapeutic profile [1]. Although the exact mechanism behind the ototoxic effects induced by cisplatin is not fully understood, the increased production of reactive oxygen species, such as superoxide anions, is believed to play a major role in it. This increased oxygen species production results in calcium influx within hair cells, which leads to apoptosis [2,3]. Autophagy is a cellular process involved in some forms of cell death, but not apoptosis [4]. Autophagy primarily has cytoprotective functions via the normal turnover of long-lived proteins and organelles [5], which maintains cells in a healthy state. However, the excessive activation of autophagy might be harmful and which results in cell death under stress conditions [6]. Autophagy might exert harmful effects, including cell death during cisplatin-mediated cytotoxicity. Although knowledge of autophagy has increased, its role in cisplatin-induced ototoxicity has not been revealed.

This study aimed to investigate the role of autophagy in cisplatin-induced ototoxicity in an auditory cell line.

## 2. Materials and methods

### 2.1. Cell culture

The House Ear Institute—Organ of Corti 1 (HEI-OC1) cell line was established from the postnatal organ of Corti of a transgenic Immortomouse. It is extremely sensitive to ototoxic drugs, and has molecular markers that are characteristic of organ of Corti cells [7]. HEI-OC1 cells were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Lonza, Walkersville, MD, USA) at 33 °C in a humidified incubator with 5% $CO_2$.

### 2.2. MTT assay

Cell viability was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays. HEI-OC1 cells were treated with 30 μM of cisplatin and then these were seeded

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at a density of $3 \times 10^4$ cells per well in a 24-well plate and incubated in DMEM containing 10% FBS at 33 °C with 5% $CO_2$. For the MTT assay, 5 mg/mL of MTT solution (Sigma, St Louis, MO, USA) was added to 0.5 mL of cell suspension, and the plates were further incubated for 4 h at 33 °C with 5% $CO_2$. The formazan crystals were centrifuged and the pellets were dissolved by adding 500 μL/well of DMSO. Then the absorption was measured at 570 nm using a spectrophotometer (BioTek, VT, USA). To investigate whether hydroxychloroquine was able to prevent cell death induced by cisplatin, cell viability was determined using MTT assay. Hydroxychloroquine was purchased from Sigma (Saint Louis, MO, USA).

### 2.3. Apoptosis analysis using flow cytometry

Floating cells and trypsin-detached cells after cisplatin exposure were collected and washed twice with ice-cold phosphate-buffered saline (PBS), and then fixed in 70% cold ethanol for 30 min at 4 °C. They were then again washed twice with PBS, resuspended in PI solution (50 μg/mL PI, 50 μg/mL RNase A, and 0.05% Triton X-100 in PBS) for 15 min. The DNA content of these cells was analyzed using PI-fluorescent-activated cell sorting (PI-FACS). At least 10,000 events were analyzed, and the percentage of cells in the sub-G0/G1 population, which was considered to be the apoptotic population, was calculated.

### 2.4. Western blotting

The proteins of surviving cells and dead cells after cisplatin exposure were investigated by western blotting. The cells were washed with PBS and lysed at 0 °C for 30 min in lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 50 mM glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM $Na_3VO_4$, and 5 mM NaF). The protein content of the samples was measured using a Bio-Rad dye binding microassay (Bio-Rad, Hercules, CA, USA), and the samples were heated at 98 °C for 5 min in Laemmli sample buffer and then subjected to SDS–PAGE. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 2 h in 5% skimmed milk in TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) at room temperature, and then incubated overnight at 4 °C with the following primary antibodies at appropriate dilutions: β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, USA), LC3 (NanoTools, Teningen, Germany), P-S6 (Cell Signaling Technology), and P-Beclin-1 (Santa Cruz Biotechnology). Unbound antibodies were removed by washing four times with TBST for 15 min. The membranes were then incubated with the appropriate secondary antibodies (1:4000; Santa Cruz Biotechnology) in blocking buffer for 2 h, and washed again. The protein bands were detected using the WEST-ZOL plus western blot detection system (iNtRON Biotechnology, Sungnam, South Korea), and signals were acquired using an image analyzer (Kodak Image Station 4000MM, Kodak, NY, USA).

### 2.5. Detecting LC3 using flow cytometry

To investigate total LC3-II of surviving cells and dead cells after cisplatin exposure, flow cytometry was performed. Floating cells and trypsin-detached cells were collected and washed twice with ice-cold PBS. They were then fixed in methanol for 10 min, washed twice with 1% bovine serum albumin (BSA), and incubated in 1% BSA for 2 h. These cells were incubated overnight with anti-LC3B antibodies (1:100 dilution, Cell Signaling Technology) diluted in PBS containing 0.1% BSA. After washing in 1% BSA, the cells were incubated for 2 h with Alexa Fluor 488 chicken anti-rabbit IgG (1:200) diluted in PBS containing 1% BSA. Cells were then washed twice, and at least 10,000 events were analyzed using flow cytometry. The FL1 channel was used to assess the fluorescence intensity. The fluorescence intensity of unchallenged cells was set to a geometric mean of $\sim 10^1$ relative fluorescence units by adjusting the photodiode gain.

### 2.6. Immunofluorescence staining

Autophagosomes of surviving cells after cisplatin exposure were examined using LC3 immunofluorescence staining. Cells growing on slides were washed in PBS and fixed in methanol for 10 min. Slides were then washed in PBS and incubated in PBS containing 1% BSA for 2 h. The attached cells were incubated overnight with anti-LC3B antibodies (1:100 dilution, Cell Signaling Technology) diluted in PBS containing 0.1% BSA. After washing in PBS, the cells were incubated for 2 h with Alexa Fluor 594 chicken anti-rabbit IgG (1:200) diluted in PBS containing 1% BSA. The cells were then washed and imaged using a Zeiss confocal microscope.

### 2.7. Statistical analysis

The results were analyzed statistically using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-tests were used for pairs of data, and one-sample *t*-tests were used for comparison of means. A $p$ value $< 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Viability of HEI-OC1 cells after cisplatin injury

To investigate cisplatin-induced cell death, the viability of HEI-OC1 cell cultures was tested using MTT assays. Fig. 1 shows the viability of HEI-OC1 cells. When the cells were exposed to 30 μM cisplatin, the initiation of cell death was seen after 16 h of cisplatin treatment. The cell viability at 16 h, 24 h, 32 h, and 48 h was $78.9 \pm 1.6\%$, $57.1 \pm 5.4\%$, $35.4 \pm 2.5\%$, and $7.7 \pm 5.3\%$, respectively. The death rate of HEI-OC1 cells increased with time after cisplatin treatment (Fig. 1).

### 3.2. The ratio of apoptosis after cisplatin injury

To investigate the apoptotic cell death induced by cisplatin injury, the cellular DNA content was analyzed. Cells with sub-G0/G1

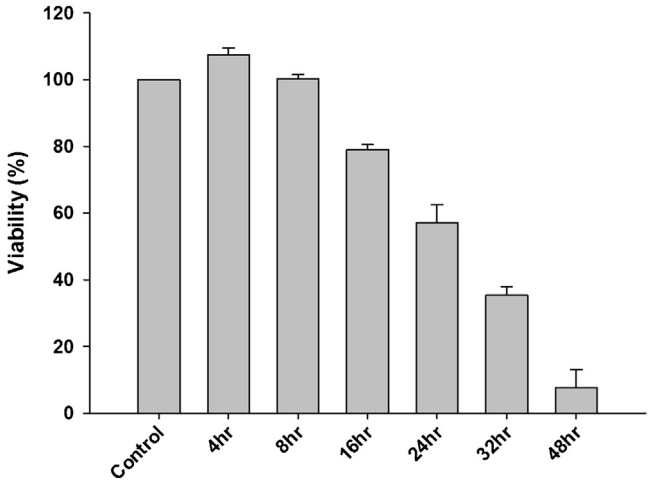


**Fig. 1.** Cell viability was determined using MTT assays. Cell death increased with time after exposure to 30 μM cisplatin. The initiation of cell death was seen after 16 h of cisplatin treatment.