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Rapamycin alleviates cisplatin-induced ototoxicity in vivo

Bin Fang, Hongjun Xiao *

Department of Otorhinolaryngology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

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ABSTRACT

Background: Cisplatin-induced ototoxicity affects a high percentage of new cancer patients worldwide. The detailed mechanism of cisplatin-induced ototoxicity is not completely understood. We investigated whether rapamycin could protect rats from cisplatin-induced ototoxicity.

Methods: Forty-eight male Wistar rats were randomly divided into six groups. Three groups were intraperitoneally (IP) infused with cisplatin at a dose of 16 mg/kg and immediately injected with either dimethylsulfoxide (DMSO), rapamycin, or chloroquine (CQ). The remaining three groups were treated with rapamycin, CQ, or saline alone. The auditory brainstem response (ABR) test was performed to detect the rats' hearing status. Serum was isolated to measure the level of the oxidative marker malondialdehyde (MDA), the basilar membrane was prepared to count the outer hair cell loss, and soft tissue samples extracted from the cochleae were lysed to analyze the microtubule-associated protein light chain 3 (LC3) and Beclin-1.

Results: The rapamycin treatment significantly attenuated cisplatin-induced hearing loss, decreased oxidative stress, and alleviated the hair cell damage that was associated with the upregulation of the LC3-II/GAPDH ratio and increased Beclin-1 expression.

Conclusion: Our results demonstrated that rapamycin has an otoprotective effect; it attenuates cisplatin-induced ototoxicity, probably by attenuating oxidative damage and inducing autophagy.

1. Introduction

Cisplatin is one of the most widely used chemotherapeutic agents for the treatment of neoplastic diseases such as lung cancer, ovarian cancer, and head and neck cancer. However, side effects like nephrotoxicity, ototoxicity, and bone marrow toxicity have restricted its clinical applications. Although the nephrotoxicity has been effectively relieved with hydration and diuretics [1], no routine treatment modality is currently available for cisplatin-induced ototoxicity. The cumulative dose-related ototoxicity is manifested as bilateral, irreversible sensorineural hearing loss and tinnitus. The ototoxicity is more prevalent among children, especially influencing younger ones [2].

Several lines of evidence have suggested that reactive oxygen species (ROS) play an important role in cisplatin-induced ototoxicity models [3–5]. The ROS include superoxide anion radical ($O_2^{\cdot-}$), singlet oxygen (O_2), hydroxyl radical ($^{\cdot}OH$), and perhydroxyl radical ($HO_2^{\cdot}$). ROS are the physiological products of aerobic respiration and serve as signaling molecules in a variety of physiological processes at low levels. However, when the production of ROS by mitochondria exceeds their catabolism, oxidative stress is generated. Exposure to cisplatin causes depletion of glutathione and antioxidant enzymes and increases malondialdehyde levels, which in turn allows calcium influx into cochlear cells, promotes cytotoxicity, and leads to the onset of apoptotic cell death.

Rapamycin is an immunosuppressant drug approved for the prevention of kidney allograft transplant rejection [6]; it is also known as a specific inhibitor of the mammalian target of rapamycin (mTOR). Rapamycin treatment inhibits the mTOR signaling pathway and activates autophagy. Autophagy is a lysosome-mediated degradation process of toxic proteins and unneeded organelles. Basically, autophagy preserves the balance between organelle biogenesis, protein synthesis, and their clearance; it surges in cellular stress conditions, such as starvation, oxidative stress, etc. [7].

Recent studies have demonstrated that rapamycin treatment enhances autophagy and rescues damaged liver and spinal cord organs from oxidative stress [8–10].

However, previous studies have not determined whether rapamycin treatment has an otoprotective role in cisplatin ototoxicity. The purpose of this study was to determine whether rapamycin could protect against cisplatin-induced ototoxicity and to identify

* Corresponding author. Address: Department of Otorhinolaryngology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Road, Wuhan 430022, China. Fax: +86 027 8535 1632.
E-mail address: xhjent@163.com (H. Xiao).

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the possible mechanisms of this process. We speculated that rapamycin treatment can alleviate cisplatin-induced ototoxicity by autophagy.

2. Materials and methods

2.1. Animals and grouping

Forty-eight male adult Wistar rats (8 weeks old) were obtained from the Hubei Provincial Academy of Preventive Medicine. The rats were individually maintained in a temperature-controlled room with a 12 h light/dark cycle and had free access to water and food. This study was performed with the approval of the Board of Ethics of Tongji Medical College, Huazhong University of Science and Technology. All of the experimental procedures were conducted in accordance with the rules of our institutional animal care and use committee.

After acclimation for a week, the rats were randomly divided into six groups ($n = 8$ per group). Three groups were intraperitoneally injected with either CQ (60 mg/kg), rapamycin (2 mg/kg), or DMSO (2% DMSO), immediately after being given an intraperitoneal (IP) infusion of cisplatin (16 mg/kg) [3,5] for 30 min. The remaining groups were IP injected with either CQ (60 mg/kg, Group D), rapamycin (2 mg/kg), or saline (equal volume of sodium chloride solution as the 30-min IP infusion).

2.2. Drugs and preparation

The cisplatin, DMSO, CQ, rapamycin, fluorescent dye phalloidin, and the 6-diamidino-2-phenylindole (DAPI) antibodies such as LC3-B and GAPDH were purchased from Sigma–Aldrich (St. Louis, MO, USA). The anti-Beclin-1 was from Life Technology (Shanghai, China). The cisplatin was diluted with sterile physiological saline (pH 7.4) to a final concentration of 1 mg/ml; the rapamycin was dissolved in DMSO as the vehicle.

2.3. Animal procedures

ABR tests were performed before the drug treatment and again at the end of the 72-h experimental period. After the last ABR test, the rats were anaesthetized and a blood sample was collected from the heart and centrifuged for 5 min at 4 °C; the serum was then isolated and colorimetric kits (Jiancheng, Nanjing, China) were used according to the manufacturer's instructions to measure the level of serum oxidative products, specifically MDA. The cochleae were harvested and frozen at −80 °C until the analysis of the cochlear autophagy-related protein was completed. Alternatively, the cochleae were perfused with 4% paraformaldehyde for basilar membrane preparation to evaluate the hair cells damage.

2.4. Auditory brainstem response test

Each rat's hearing status was assessed with an ABR test both pretreatment and 72 h after the administration of the cisplatin, as previously described [3]. Forty-eight rats ($n = 8$ per group) were anaesthetized with a ketamine/chlorpromazine cocktail (30 mg/kg:15 mg/kg, i.p.). The animals were placed on a warming blanket calibrated to maintain body temperature at 37 °C. Rectal temperatures were continuously monitored while the animals were under anesthesia. A subcutaneous active electrode was placed at the vertex and a reference electrode at the tip of nose; the ground electrode was placed over the neck muscle. All of the procedures were conducted in an electrically shielded sound-proof chamber. The auditory stimuli included tone burst stimulus at 8, 16, 24, and 32 kHz using a computer-based signal-averaging system (Tucker-Davis Technology, Alachua, FL, USA). The ABR threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response with a minimum amplitude of 0.5 uV.

2.5. Detection of oxidative products MDA

To investigate the whole oxidative status, the level of the serum oxidative marker MDA was measured after the last ABR test with a colorimetric kit.

2.6. Detection of hair cell damage

To determine the outer hair cell loss after the different treatments, some of the rats ($n = 4$ per group) were transcardially perfused with 4% paraformaldehyde. The oval and round windows of the cochleae were opened and flushed with 4% paraformaldehyde. The cochleae were then fixed in paraformaldehyde overnight, and then decalcified with 10% EDTA (ethylene diamine tetraacetic acid) in 0.1 M PBS (phosphate buffer solution) for a week at 4 °C. The basal turns of the basilar membrane were stripped and then stained with both phalloidin (red fluorescent dye) for 30 min to display the stereocilia and DAPI (blue fluorescent dye) for 5 min to outline the nucleus. A hair cell count was then performed with a confocal scanning laser microscope (Nikon, Japan).

2.7. Immunoblot analysis of autophagy-related proteins

An immunoblot analysis of the soft tissues extracted from the cochleae was used to determine the expression levels of autophagy-related proteins (LC3 and Beclin-1) in the cochleae. Twenty-four rats were decapitated after the last ABR test and the soft tissues of one side of the cochleae from each rat were dissected and homogenized in an RIPA lysis buffer (Beyotime, China) supplemented with PMSF (phenylmethanesulfonyl fluoride) on ice for 30 min. Then, the debris was removed by centrifugation and the total protein concentration was determined using a bicinchoninic acid kit (Beyotime, China). The resulting supernatants were separated with SDS–PAGE in 12% gel and transferred to PVDF membranes. The membrane was blocked with casein and probed with the appropriate dilution of antibodies against LC3 (diluted 1:500), Beclin-1 (diluted 1:500), or GAPDH (diluted 1:3000), followed by horseradish peroxidase-coupled detection.

2.8. Statistical analysis

All of the data were presented as mean ± SEM and statistical analysis was performed with SPSS 16.0 software; the differences between groups were separately compared by analysis of variance (ANOVA) and $P < 0.05$ was considered the criterion for statistical significance.

3. Results

3.1. Hearing loss was alleviated by rapamycin treatment

The changes in the ABR threshold for all of the frequencies were compared before and three days after the cisplatin or saline injections. The results are shown in Fig. 1. Significant hearing loss was noted in the cisplatin + DMSO group at 8, 16, 24, and 32 kHz compared to the saline injection group ($P < 0.05$, $n = 8$ per group). An injection of rapamycin after exposure to cisplatin alleviated tone burst ABR with average threshold shifts of 30.76 ± 2.87 dB (decibel) at 8 kHz, 36.50 ± 3.47 dB at 16 kHz, 38.85 ± 2.64 dB at 24 kHz, and 64.81 ± 2.79 dB at 32 kHz. However, no significant change was seen in rats treated with cisplatin + CQ or in rats treated with

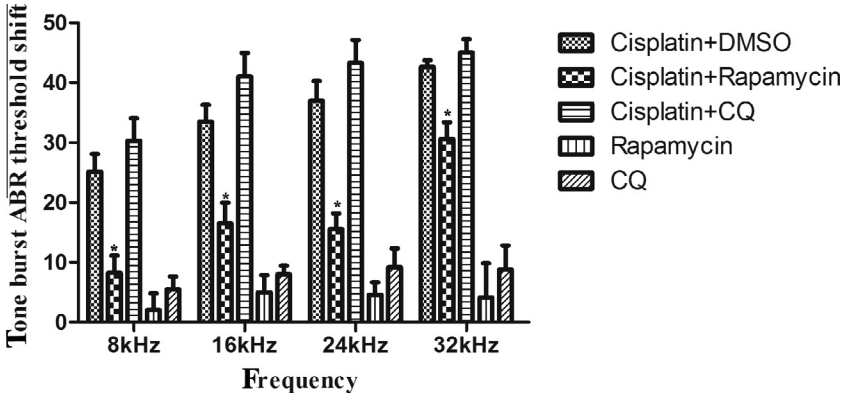

Fig. 1. Rapamycin treatment attenuated the cisplatin-induced tone burst auditory brainstem response (ABR). The ABR threshold shift was based on the difference between pre-treatment and post-treatment results. Compared to the saline group, the ABR threshold in the cisplatin + DMSO group increased obviously at all four frequencies; administration of rapamycin immediately after cisplatin exposure decreased the ABR shifts at all of the frequencies. No significant threshold shifts were observed in the groups receiving either the rapamycin or CQ alone. Data are expressed as the mean ± SEM of eight rats from each group (*$P < 0.05$ vs. cisplatin + DMSO group).

cisplatin + DMSO ($P > 0.05$, $n = 8$ per group). These results demonstrated that cisplatin causes hearing loss in rats, but this loss can be attenuated by rapamycin (Fig. 1).

3.2. Rapamycin treatment reduced the level of oxidative products

The concentration of MDA in serum is shown in Fig. 2. Compared with the saline group (2.31 ± 0.19 mmol/L), cisplatin elevated the level of MDA (5.42 ± 0.27 mmol/L) in serum ($P < 0.05$, $n = 8$ per group). However, the administration of rapamycin immediately after the administration of cisplatin (4.54 ± 0.15 mmol/L) decreased the level of MDA in the serum ($P < 0.05$, $n = 8$ per group). There were no differences between the group of cisplatin-challenged rats treated with CQ and the group of cisplatin-challenged rats treated with DMSO. These results demonstrated that rapamycin alleviates cisplatin-induced oxidative status (Fig. 2).

3.3. Hair cell loss was alleviated by rapamycin

Because basal turn outer hair cells (OHCs) are the site most vulnerable to cisplatin administration [11], the basal turn cells were separated and stained to identify the hair cell loss in the different treatment groups. Cisplatin induced more OHC loss than any other treatment ($P < 0.05$, $n = 4$ per group). However, the administration of rapamycin immediately after the administration of cisplatin reduced outer hair cell loss ($P < 0.05$, $n = 4$ per group). Cisplatin-induced damage of outer hair cells was alleviated by the administration of rapamycin in cisplatin-challenged rats. These results suggested that cisplatin causes hair cell damage in rats, but this damage can be reversed by rapamycin (Fig. 3).

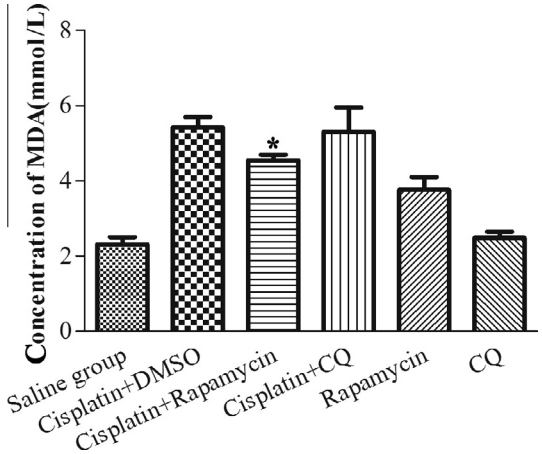

Fig. 2. Rapamycin treatment decreased cisplatin-induced oxidative stress in serum. The levels of MDA in serum were measured at 3 days after cisplatin or saline administration. The values are expressed as means ± SEM ($n = 8$ per group, *$P < 0.05$ vs. cisplatin + DMSO group).

3.4. The detection of autophagy-related proteins

The expression levels of LC3-II and Beclin 1 proteins were significantly increased in the rapamycin-treated rats and the vehicle-treated rats compared to the saline group. CQ treatment decreased the expression level of LC3-II or Beclin 1 compared to the vehicle groups (Fig. 4).

4. Discussion

In this study we found that rapamycin treatment alleviated cisplatin-induced ototoxicity in rats. Rapamycin treatment improved the 8, 16, 24, and 32 kHz tone burst ABR threshold shift in

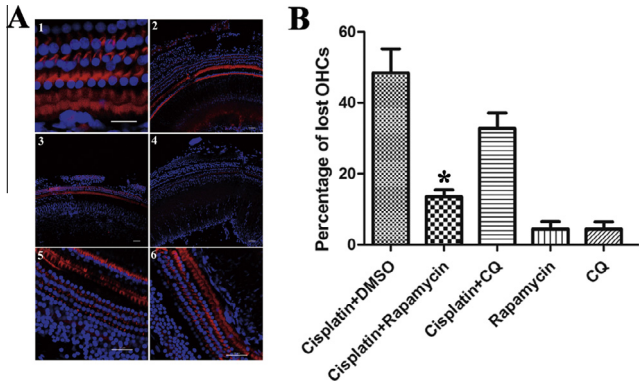

Fig. 3. In Part A, rapamycin treatment attenuated the cisplatin-induced damage of hair cells. The hair cells were stained for phalloidin and DAPI at 3 days after cisplatin or saline administration. The representative micrographs of the basal turns are as follows: (1) saline group; (2) cisplatin + DMSO group; (3) cisplatin + rapamycin group; (4) cisplatin + CQ group; (5) rapamycin group; and (6) CQ group. Graph 1 shows the amplified natural image of the OHCs. The OHCs are distributed smoothly with cilium. Cisplatin treatment destroyed the sequences and caused hair cell losses. Rapamycin treatment reversed the cisplatin-induced OHC damage (scale bar: 20 μm). In Part B, hair cell loss was evaluated at each cochlear turn at 3 days after cisplatin administration. The values are expressed as means ± SEM ($n = 4$ per group, *$P < 0.05$ vs. cisplatin + DMSO group).

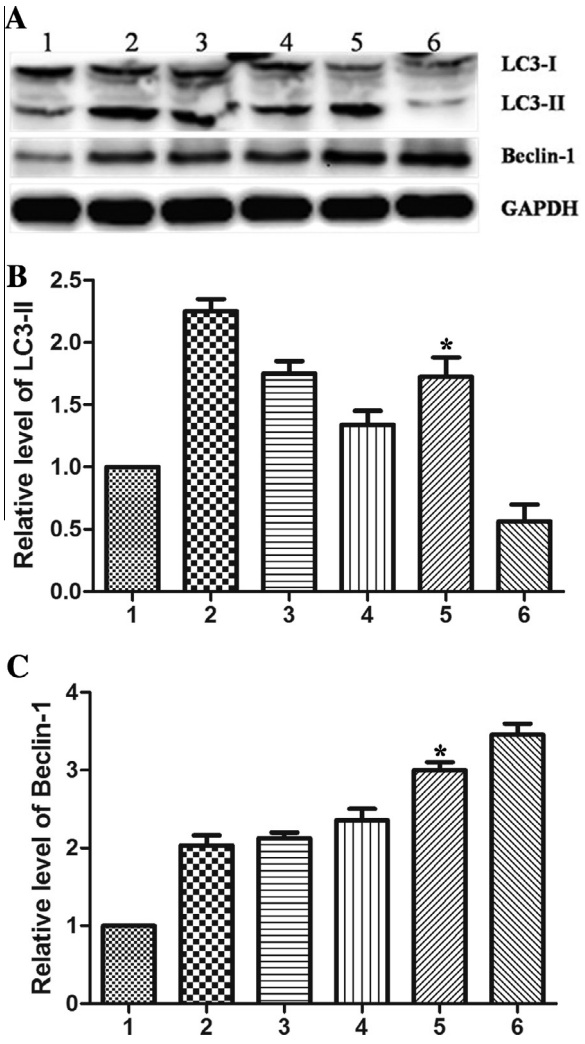

Fig. 4. Rapamycin treatment increased the expression of LC3-II and Beclin-1. A representative immunoblot analysis showed the expression levels of LC3 and Beclin-1 in the different groups. From 1 to 6, the order of the samples is (1) saline group; (2) rapamycin group; (3) CQ group; (4) cisplatin + DMSO group; (5) cisplatin + rapamycin group; and (6) cisplatin + CQ group. The results showed that the levels of LC3-II and Beclin-1 in the rapamycin-treated rats were significantly higher than in the cisplatin + DMSO rats ($^{*}P < 0.05$ vs. cisplatin + DMSO group).

cisplatin-challenged rats. Furthermore, rapamycin treatment decreased the level of oxidative products and preserved hair cells in cisplatin-challenged rats compared to rats in groups injected with the vehicle only. In addition, rapamycin treatment promoted the expression of autophagy-related proteins LC3-II and Beclin-1. These results indicate that rapamycin treatment, at least in part, has an otoprotective effect in alleviating cisplatin-induced ototoxicity by promotion of autophagy. Unfortunately, our results failed to show that CQ treatment enhances cisplatin-induced hearing loss or increases MDA in serum. In addition, CQ treatment did not exacerbate outer hair cell loss in cochleae.

MDA is the reaction product of free radical-induced lipid peroxidation and the MDA level correlates with the degree of free radical-induced oxidative damage. We found that the level of serum MDA was increased by cisplatin, yet decreased by rapamycin. This result suggests that reducing oxidative stress may contribute to the protective effect of rapamycin treatment, which is consistent with previous studies [5].

The morphological research revealed that the damage in cisplatin-induced ototoxicity includes the reduction of outer hair cells and the distortion and twisting of the stereocilia. In addition, the spiral ganglion cells and lateral wall (stria vascularis and spiral ligament) were also involved. This loss typically affects the first row of outer hair cells (OHC) in the cochleae [5]. In our study, we found that cisplatin treatment resulted in typical hair cell loss, but rapamycin treatment decreased hair cell loss, demonstrating that rapamycin treatment attenuates cisplatin-induced hair cell loss.

Previous studies using cisplatin-induced ototoxicity models have suggested that oxidative stress and free radicals are key pathogenic factors [11], and some antioxidative molecules have been applied to treat the disease. However, it should be noted that antioxidants are a kind of matchmaker that try to match unpaired electrons, but if they do not make a proper pair, antioxidants can themselves become incomplete and behave as free radicals. There is an equilibrium between the antioxidants and the oxygen free radicals and this equilibrium can be broken if antioxidant levels exceed those of the reactive species [11]. Dündar et al. [12] proposed the term antioxidative stress for such disequilibrium. Furthermore, although systemic administration of antioxidants has been shown to have an otoprotective role in cisplatin-induced ototoxicity models, these agents may compromise the anti-cancer efficacy of cisplatin [13]. Given the deficiency of antioxidative therapies, we speculate that the induction of autophagy may be a new method to deal with the disease.

In addition to the induction of autophagy, rapamycin influences a variety of essential cellular processes. Considering its various pharmacological actions, one may questions whether its ability to alleviate cisplatin-induced ototoxicity is due to its specific effect on autophagy or through other pathways such as immunosuppression effect. Therefore, an immunoblot analysis was performed to detect the level of LC3-II and Beclin-1.

Detecting LC3 conversion (LC3-I to LC3-II) using the immunoblot technique has become an important approach to monitor autophagy activities [14]. As the amount of LC3-II is clearly correlated with the number of autophagosomes, comparison of the amount of LC3-II among samples is an accurate method for detecting the trends in autophagic activities.

Beclin 1 is essential for the formation of autophagosomes, and acts as a promoter of autophagy. Some studies have demonstrated that increased expression of Beclin 1 activates the autophagic activity in response to the neural tissue damage of brain [15]. In this study, the level of Beclin-1 expression increased in the rats treated with either cisplatin or rapamycin. Therefore, autophagy was activated in the cochleae after cisplatin exposure.

To our knowledge, this is the first study to demonstrate the otoprotective effects of rapamycin in cisplatin-induced ototoxicity rat models. Julien Menardo et al. [16] have shown that the modulation of autophagy in spiral ganglion cells offers promising perspectives for the prevention of neural presbycusis. Marino et al. [17] have shown that autophagy plays an essential role in inner ear development and equilibrioception. Recent studies have also suggested that ROS are a key factor in aging and presbycusis [18]. Our results show that rapamycin is a potential disease-modifying treatment for sensorineural hearing loss diseases.

We confirmed previous findings that CQ treatment inhibited autophagic activities [8]. However, the impairment of autophagy did not exacerbate cisplatin-induced ototoxicity, induce more severe oxidative damage, or cause more outer hair loss, as expected. There are two possible reasons for these findings. First, the mechanism of cisplatin-induced cytotoxic includes more than oxidative stress; it may include endoplasmic reticulum stress, lysosomal toxicity, genotoxic stress, etc. [19]. Therefore, alleviating oxidative stress alone cannot reverse all of the cisplatin-induced ototoxicity. Second, mitochondria are not as abundant in cochleae as in organs such as the kidney or the liver.

Several considerations should be taken into account before the clinical use of rapamycin in sensorineural hearing loss disease can be realized: the duration of administration, timing of administration, and side effects. Moreover, the dosage and frequency of administration of rapamycin should also be considered. Further studies should emphasize the relationship between oxidative stress and autophagy [9,20].

In summary, our study demonstrated that rapamycin treatment alleviated cisplatin-induced ototoxicity, probably by decreasing oxidative stress and promoting autophagy.

Acknowledgments

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