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Protective Effects of Laminarin on Cisplatin-induced Ototoxicity in HEI-OC1 Auditory Cells

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Abstract

Cisplatin is an effective chemotherapeutic agent against many common types of cancers. However, one of its most severe and debilitating side effects is ototoxicity. The purpose of this study was to investigate the mechanisms of laminarin protective effects on cisplatin-induced damage in HEI-OC1 auditory cell line. In our research, HEI-OC1 cells were pretreated with laminarin and then exposed to cisplatin. Here, we examined the cell viability, cell apoptosis and the factors associated with apoptotic pathway. Compared with cisplatin injured group, pretreatment with laminarin increased cell viability and decreased the cell apoptosis and necrosis. Furthermore, treatment with laminarin reduced intracellular ROS production, bax mRNA, and cleaved caspase-3, caspase-9, caspase-8 expression, but increased the bcl-2 mRNA level. The protection was ascribed to the radical scavenging activity of laminarin, and the inhibition of both mitochondrial apoptosis pathway and extrinsic apoptotic pathway.

Keywords: Apoptosis pathway; Auditory hair cells; Cisplatin; Laminarin; Oxidant stress

Introduction

Cisplatin is widely used as chemotherapeutic agent against a variety of human neoplasms, including lung, testicular, cervical, and various head and neck cancers [1,2]. However, frequent administration of high doses of cisplatin has severe adverse effects, including ototoxicity, nephrotoxicity, neurotoxicity and bone marrow toxicity [1,3]. The ototoxicity was characterized by irreversible, progressive, bilateral, high-frequency, sensorineural hearing loss and may be accompanied by tinnitus [4]. The ototoxic mechanism of cisplatin has been disclosed to be related to several factors, including mitochondrial dysfunction, increased generation of intracellular ROS [5] and reactive nitrogen species [6], which was the widely accepted mechanism. Moreover, cisplatin can bind with guanine, leading to cell apoptosis via the formation of inter-and intrastrand DNA crosslinks, aberrant genetic transcription and cell cycle arrest [7]. All of these results which decrease the protective factors, such as glutathione and antioxidant enzymes in cochlear tissues, lead to the apoptosis and death of the outer hair cells of Corti [8].

Laminarin was naturally extracted from laminaria japonica, contained mannose (3.27%), arabi-nose (8.61%), glucose (4.23%), galactose (12.12%), fucose (46.93%), widely used in traditional Chinese medicine. It was introduced as a treatment for several diseases, including anticancer [9], immunoregulation [10], anticoagulation [11]. A previous study had suggested that the main compounds of laminarin (fucose and sulphate group) extracted from laminaria japonica were active antioxidants, especially in scavenging superoxide radical and hydroxyl radical. However, whether laminarin has protective effects on cisplatin-induced hair cell damage and the related mechanisms had not been defined. Furthermore, the HEI-OC1 is appropriate cell line for effectively studying signaling pathways, and has shown a good correlation with *in vivo* studies. Moreover, many studies have shown the HEI-OC1 cell line is also a good model for studying cisplatin-induced ototoxicity [12].

Our study aimed to investigate the protective effects of laminarin on cisplatin-induced ototoxicity and the underlying mechanisms in HEI-OC1 cell line.

Materials and Method

Cell culture

The HEI-OC1 cell line was provided by Dr. Federico Kalinec, ULCA, derived from organ of Corti. HEI-OC1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gbico, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) at 33°C under 5% CO_2 in air.

Cell viability assessment

The cisplatin (Sigma, USA) and laminarin (Sigma, USA) were freshly diluted in non-serum culture medium. The HEI-OC1 cells (5000 cells/well in a 96-well plate) were incubated in varying cisplatin concentrations (0-40 μM) for 24 h to determine the half maximal inhibitory concentration (IC50), and the cell viability was measured by MTT assay (Sigma, USA), as described previously [13].

The cells were incubated in varying laminarin concentrations (1-30 µM) for 12 h, then determined the dose-dependent influence of laminarin on cell viability by MTT.

The cells were seeded and incubated overnight. Then they were pretreated with varying doses of laminarin for 12 h, discarded the culture medium, replaced with medium containing 20 µM cisplatin and incubated for 24 h. Medium with vehicle alone was used as the control. MTT assay was used to measure the effects of laminarin on cell viability.

Hoechst 33258 staining

The cells were cultured in the medium with or without laminarin treatment (10 µM) for 12 h, and then replaced with medium with or without cisplatin (20 µM) for 24 h. Medium with vehicle alone was used as the control. The cells were washed twice with phosphate buffered saline, then fixed with 4% paraformaldehyde for 10 min at room temperature. After fixation, the cells were washed twice again with PBS and stained with Hoechst 33258 (Beyotime Company of Biotechnology, China) for 5 min. After washing twice, the cells were observed under confocal fluorescent microscopy (Leica, Germany).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining and flow cytometry

To quantify and verify the percentage of apoptotic and necrotic cells induced by cisplatin with or without laminarin pretreatment, we used Annexin V-FITC/PI double staining and flow cytometry to detect it. The cells were treated as described above, afterwards, we collected the cells and stained them by the Annexin V-FITC/PI apoptosis detection kit (Beyotime Company of Biotechnology, China) following the manufacturer's protocol. Cell distributions were detected by flow cytometry (Beckman Coulter, USA). Ten thousand cells were examined in each sample. The data were analyzed by FlowJo 7.6.1 software.

Measurement of intracellular ROS generation

The generation of ROS was detected by 5-(and 6)-carboxyl-20, 70-dichlorodihydro fluorescein diacetate (DCFDA; Sigma, USA). The cells were seeded and incubated as mentioned previously. Thereafter, the cells were treated with 10 mM DCFDA in serum-free medium for 10 min at 33°C and protected from light. The oxidative burst was measured by flow cytometry with 480 nm excitation wavelength and 530 nm emission wavelengths.

mRNA extraction and quantitative real-time PCR (q-PCR)

Total RNA was extracted from samples by Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocols. With the hexamer primer, 1 mg total mRNA was reverse-transcribed into cDNA by the Revert Aid kit (Fermentas, Canada). Real-time PCR was performed with the Light Cycler Fast Start DNA SYBR Green kit and run in triplicate on Master cycler ep realplex (Eppendorf AG, Germany). The primer sequences used for PCR amplification were as follows: bax F 5'-TTC ATC CAG GAT CGA GCA GG-3'; bax R 5'-CGT CAG CAA TCA TCC TC TGC-3'; bcl-2 F 5'-GCC TTC TTT GAG TTC GGT GG-3'; bcl-2 R 5'-GCT GGG GCC ATA TAG TTC CA-3'; GAPDH F 5'-AGG TCG GTG TGA ACG GAT TTG-3'; and GAPDH R 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'.

Western blotting analysis

The cells were seeded and incubated as previously mentioned. The isolation of proteins was achieved according to the manufacture's protocol (Beyotime, China). The protein concentration was determined using BCA method 20 µg of total protein were mixed with sample buffer containing 2% mercaptoethanol. The samples were heated at 95° for 5 min, and separated by 12% Tris-HCl gels electrophoresis. Gels were then transferred onto polyvinylidene difluoride membranes and blocked with 5% non-fat dry milk for 1 h at room temperature. Next, the membranes were incubated with 3% non-fat dry milk with the primary antibodies of cleaved caspase-3 (17 kDadiluted at 1:600, goat monoclonal, Life Technologies, USA), caspase-9 (35, 37 kDa, diluted at 1:1000, rabbit monoclonal, Life Technologies, USA), caspase-8 (55 kDa, diluted at 1:1000, rabbit polyclonal, Abcam, UK), β-actin (43 kDa, diluted at 1:1000, mouse monoclonal, Santa Cruz, USA), at 4°C overnight. Then the membranes were incubated with the secondary donkey anti-goat, anti-rabbit or anti-mouse IgG antibodies (1:5000, Santa Cruz, USA) for 1 h. The membranes were developed using an ECL detection kit (Santa Cruz, USA) and exposed to X-ray film. The relative expression level was calculated with the Image J software by comparison with β-actin.

Statistical analysis

Results were obtained from triplicate determinations and three independent experiments. Statistical analyses were performed by SPSS 17.0 software package. One-way ANOVA and factorial design were applied and data were expressed as the mean ± SD. P<0.05 was considered as statistically significant.

Results

Effects of laminarin on the viability of HEI-OC1 cells treated with cisplatin

Exposure to cisplatin (0-40 µM) for 24 h decreased the cell viability in a dose-dependent manner (Figure 1A). Cells treated with 20 µM cisplatin exhibited 52% cell viability, therefore, 20 µM cisplatin was used subsequently. When pretreated with 1, 5, 10, 15, 20 or 30 µM laminarin for 12 h, HEI-OC1 cell viability was found to be unaffected within 15 µM, while decreased over 20 µM (Figure 1B). When pretreated with 1, 5, 10, 15, 20 µM laminarin followed by treatment with 20 µM cisplatin for 24 h, HEI-OC1 cell viability was found to be maximally protected by 10 µM laminarin (Figure 1C). Therefore, 10 µM concentration of laminarin was selected to investigate the effects of laminarin pretreatment on cisplatin-induced cell damage. Pretreatment with laminarin (10 µM) alone didn't affect the cell viability. The light microscope showed the consistent results with the results assayed by MTT (Figure 1D).

Effects of laminarin on cisplatin-induced apoptosis and necrosis in HEI-OC1 cells

The apoptosis of HEI-OC1 cells was determined by observing the nuclear morphology using Hoechst 33258 staining (Figure 2). The nuclei of normal cells were roundness with homogeneous intensity. Laminarin (10 µM) alone had no effect on the gross morphology of the HEI-OC1 cells compared with that of the controls. However, cisplatin (20 µM) alone lead to the characteristic apoptotic morphology of heterogeneous intensity, condensation and fragmentation of the nuclei.
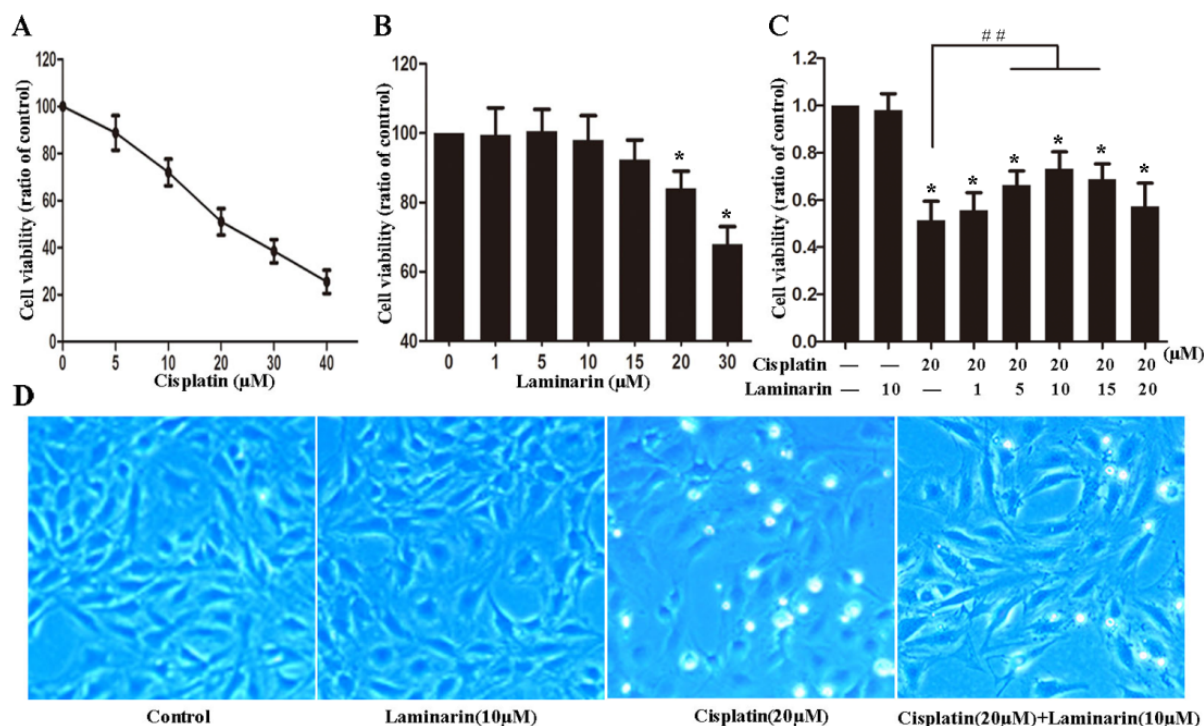
Figure 1: Effects of Laminarin on cisplatin-mediated decrease in viability of HEI-OC1 cells. (A) The cell viability after cisplatin (0-40 μM) treatment for 24 h. The 20 μM cisplatin decreased the cell viability by IC50; (B) The cell viability after laminarin (0-30 μM) treating for 12 h; (C) The protective effects of varying laminarin concentrations (1-30 μM) on the viability of HEI-OC1 cells following 20 μM cisplatin treatment; (D) Cell survival observed by **microscope** (×200), Scale bar: 50 μm (*vs the control; *p<0.05; ##p<0.01).
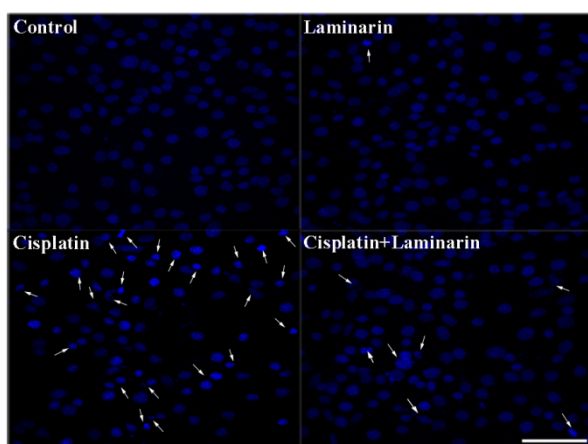
Figure 2: Effects of laminarin on apoptosis of cisplatin-treated HEI-OC1 cells. Cells were pretreated or absent with laminarin (10 μM) for 12 h, followed by cisplatin (20 μM) treatment for 24 h. Cell apoptosis was stained by Hoechst 33258 and observed under the fluorescence microscope (×400), the arrows represent the apoptotic cells (Scale bar: 40 μm).

Pretreatment with 10 μM laminarin diminished the numbers of apoptotic cells.

To quantify and verify the number of apoptotic cells, we used flow cytometry. Annexin V-FITC/PI double staining was used to analyze the percentage of apoptotic cells treated with cisplatin in the absence or presence of laminarin (Figure 3). The number of cells was counted as late apoptotic and necrotic cells shown in the upper right quadrant (Q_2) and early apoptotic cells as shown in the lower right quadrant (Q_3) of the histograms. The cisplatin plus laminarin significantly decreased the number of apoptotic cells and necrotic cells compared with cisplatin only (P<0.05).

Those results showed that cisplatin promoted apoptotic and necrotic cells, which was significantly inhibited by laminarin pretreatment.

Effects of laminarin on ROS generation

We measured the intracellular levels of ROS generated by HEI-OC1 cells in response to cisplatin and laminarin using the fluorescent probe DCFH-DA (Figure 4). Figure 4A showed that HEI-OC1 cells treated with 20 μM cisplatin significantly increased fluorescence intensity in comparison to the control group. Pretreatment with 10 μM laminarin for 12 h markedly reduced the cisplatin-induced generation of ROS in HEI-OC1 cells (Figure 4A). The mean probe intensity in comparison to that of the control group is shown in Figure 4B. Treatment with 20 μM cisplatin markedly increased the fluorescence intensity, whereas pretreatment with laminarin resulted in a decrease in the fluorescence intensity compared with that of cisplatin alone, and treatment with laminarin alone did not alter the fluorescence.
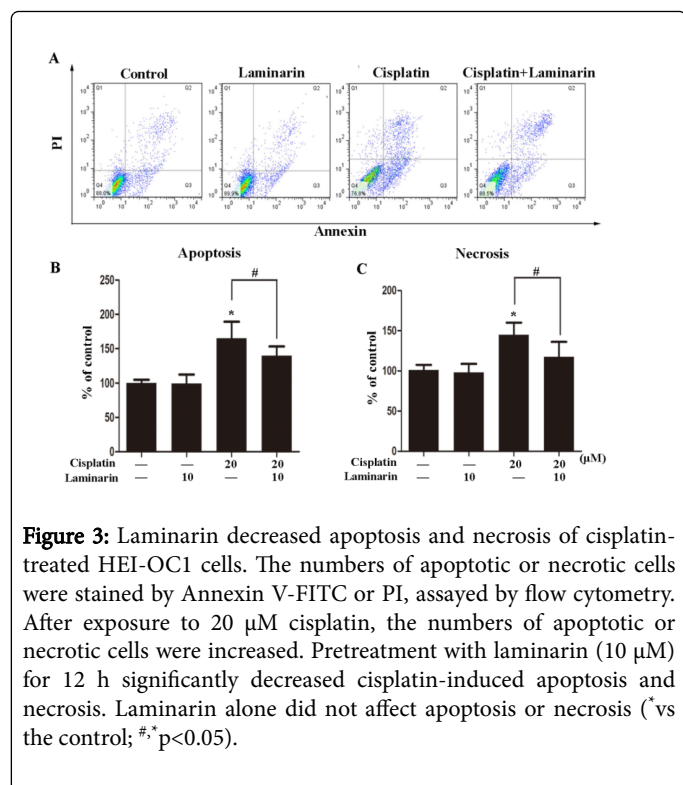
Figure 3: Laminarin decreased apoptosis and necrosis of cisplatin-treated HEI-OC1 cells. The numbers of apoptotic or necrotic cells were stained by Annexin V-FITC or PI, assayed by flow cytometry. After exposure to 20 μM cisplatin, the numbers of apoptotic or necrotic cells were increased. Pretreatment with laminarin (10 μM) for 12 h significantly decreased cisplatin-induced apoptosis and necrosis. Laminarin alone did not affect apoptosis or necrosis (*vs the control; #,*$p<0.05$).
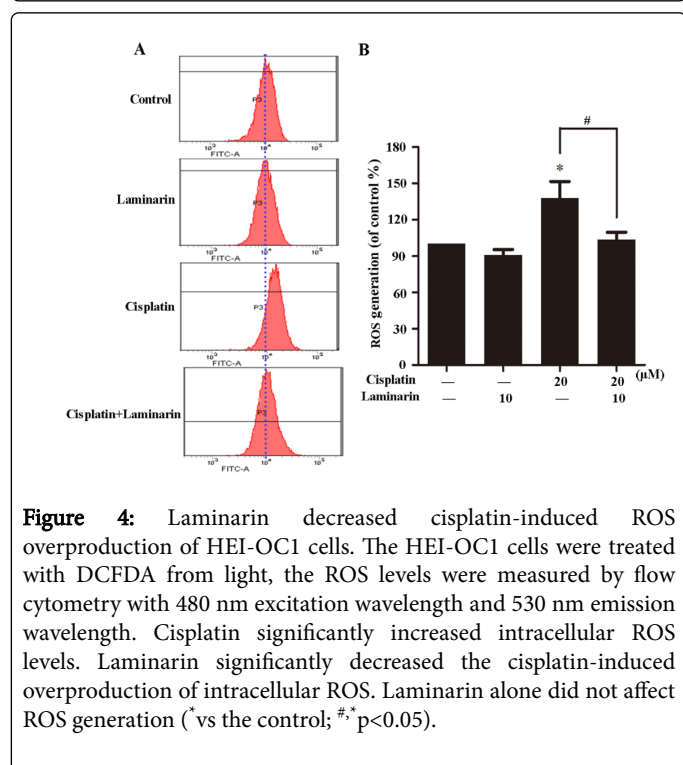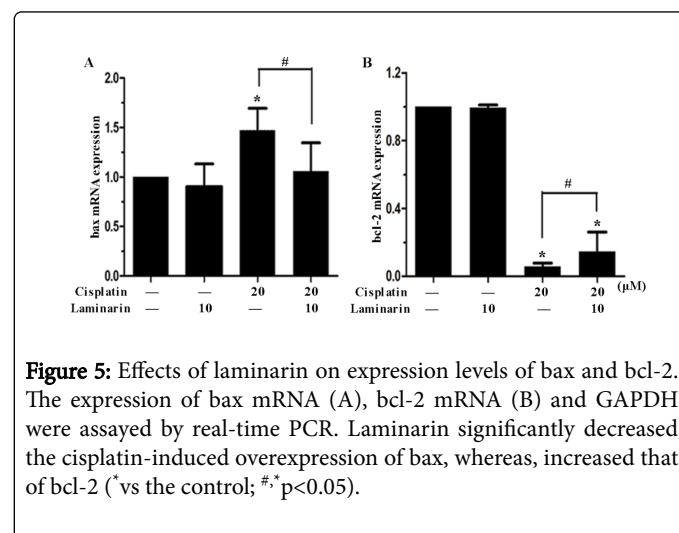

Figure 4: Laminarin decreased cisplatin-induced ROS overproduction of HEI-OC1 cells. The HEI-OC1 cells were treated with DCFDA from light, the ROS levels were measured by flow cytometry with 480 nm excitation wavelength and 530 nm emission wavelength. Cisplatin significantly increased intracellular ROS levels. Laminarin significantly decreased the cisplatin-induced overproduction of intracellular ROS. Laminarin alone did not affect ROS generation (*vs the control; #,*$p<0.05$).
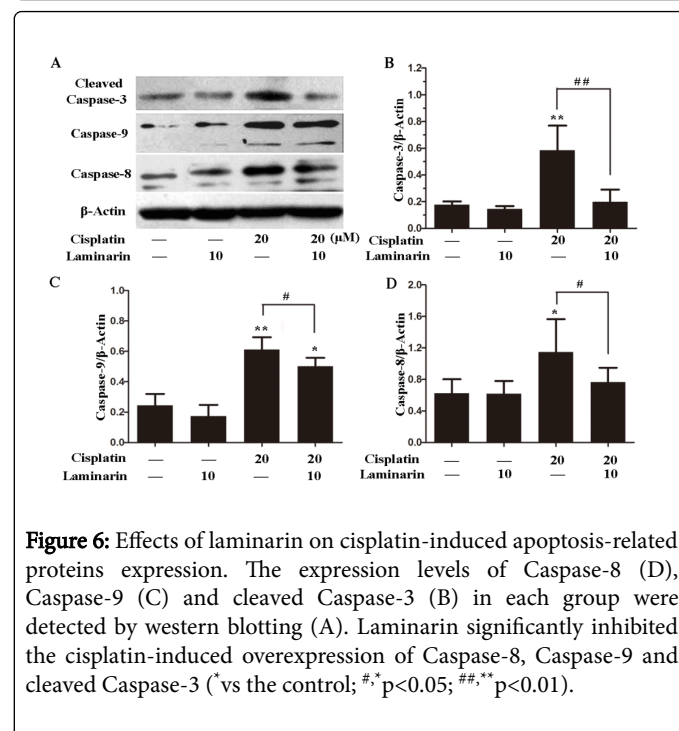
Effects of laminarin on the apoptosis pathways

RT-PCR analysis indicated decreased bcl-2 mRNA and increased bax mRNA levels 24 h after cisplatin treatment. However, pretreatment with laminarin for 12 h resulted in a significant decrease in the bax mRNA expression (Figure 5A) and increase in the bcl-2 mRNA

expression (Figure 5B) in comparison to treatment with cisplatin alone.

Simultaneously, we examined the effects of laminarin on cleaved caspase-3, caspase-8 and caspase-9 activation using western blot analysis (Figure 6). Exposure of cells to 20 μM cisplatin increased the expression of cleaved caspase-3, caspase-8 and caspase-9. Pretreatment of cells with laminarin for 12 h prior to cisplatin treatment induced a marked reduction in cleaved caspase-3, caspase-8 and caspase-9 ($P<0.05$), in comparison to the cisplatin-treated group.

Figure 5: Effects of laminarin on expression levels of bax and bcl-2. The expression of bax mRNA (A), bcl-2 mRNA (B) and GAPDH were assayed by real-time PCR. Laminarin significantly decreased the cisplatin-induced overexpression of bax, whereas, increased that of bcl-2 (*vs the control; #,*$p<0.05$).

Figure 6: Effects of laminarin on cisplatin-induced apoptosis-related proteins expression. The expression levels of Caspase-8 (D), Caspase-9 (C) and cleaved Caspase-3 (B) in each group were detected by western blotting (A). Laminarin significantly inhibited the cisplatin-induced overexpression of Caspase-8, Caspase-9 and cleaved Caspase-3 (*vs the control; #,*$p<0.05$; ##,**$p<0.01$).

Discussion

In the present study, we demonstrated that laminarin increased the viability of HEI-OC1 auditory cells, which was related to its radical scavenging activity and inhibition of cell apoptosis.

The overexpression of ROS caused by cisplatin was recognized as a central mechanism of its ototoxicity [14], which can overwhelm the antioxidant defense mechanisms in the cochlea, leading to mitochondrial release of cytochrome c, activating the apoptosis pathway, inducing out hair cells death. Therefore, antioxidants could be a desirable method for protection against cisplatin-induced hearing loss [15]. Laminarin is a tropical plant traditionally used in Chinese medicine, which has been proven to inhibit sepsis-induced oxidative stress, lipid peroxidation in rats by inhibiting caspase-3 activity and enhancing SOD, CAT and GSH-Px activities [16]. Our results showed the laminarin decreased the cisplatin-induced ROS production, indicating its antioxidant activity.

We evaluated the caspases pathways, which are the key markers of apoptosis, and have been proven to be key factors in cisplatin-induced apoptosis of hair cells [2]. To address the apoptosis, we focused on bcl-2, bax, caspase-3, caspase-8 and caspase-9. In this study, caspase-3, an ultimate mediator in apoptosis [4], was down-regulated when the cells were pretreated with laminarin, indicating that the preventive effect of laminarin is related to the apoptosis pathway. For the apoptotic pathway, it contains mitochondrial apoptotic pathway and extrinsic apoptotic pathway. Previous findings have indicated that Bax/Bcl-2 and caspase-9 in mitochondrial pathways have a critical role in cisplatin-induced apoptosis of auditory cells [17,18]. Our results showed that laminarin reduced the levels of bax mRNA and caspase-9, elevated the levels of bcl-2 mRNA, which indicated laminarin protect cells from cisplatin-induced auditory cell damage through inhibiting the mitochondrial apoptotic pathway. For the extrinsic apoptotic pathway, caspase-8 was thought to be the primary mediator, which can cause cellular destruction by apoptosis without mitochondria participation [19]. Therefore, we assayed caspase-8 expression, which showed a decrease when pretreated with laminarin in cell. These results demonstrated that the preventive effect of laminarin is also related to the inhibition of extrinsic apoptotic pathway.

Despite the protective effect of laminarin against cisplatin-induced ototoxicity, its clinical application in patients remains inconclusive. Additionally, it must be noted that high doses of laminarin have a cytotoxic effect instead of the beneficial effects. Future studies will concentrate on animal models to confirm the underlying protective mechanisms and to establish an efficacious laminarin dose before any clinical recommendations.

In conclusion, we have observed that low doses of laminarin could provide protective effects against cisplatin-induced ototoxicity. Moreover, the preventive effect of laminarin is related to its radical scavenging effect and inhibition of both mitochondrial apoptotic pathway and extrinsic apoptotic pathway.

Acknowledgment

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