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Protective effect of silymarin against cisplatin-induced ototoxicity

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ABSTRACT

Objectives: Silymarin is a plant extract with strong antioxidant properties in addition to anti-inflammatory and anticarcinogenic actions. The aim of this study was to investigate the potential preventive effect of silymarin on cisplatin ototoxicity in an auditory cell line, HEI-OC1 cells.
Methods: Cultured HEI-OC1 cells were exposed to cisplatin (30 μM) with or without pre-treatment with silymarin (50 μM). Cell viability was evaluated using MTT assay. Hoechst 33258 staining was used to identify cells undergoing apoptosis. Western blot analysis was done to evaluate whether silymarin inhibits cisplatin-induced caspase and PARP activation. Cell-cycle analysis was done by flow cytometry to investigate whether silymarin is capable of protecting cisplatin-induced cell cycle arrest.
Results: Cell viability significantly increased in cells pretreated with silymarin compared with cells exposed to cisplatin alone. Pre-treatment of silymarin appeared to protect against cisplatin-induced apoptotic features on Hoechst 33258 staining. Cisplatin increased cleaved caspase-3 and PARP on Western blot analysis. However, pre-treatment with silymarin inhibited the expression of cleaved caspase-3 and PARP. Silymarin did attenuate cell cycle arrest and apoptosis in HEI-OC1 cells.
Conclusions: Our results demonstrate that silymarin treatment inhibited cisplatin-induced cytotoxicity in the auditory cell line, HEI-OC1. Silymarin may be a potential candidate drug to eliminate cisplatin induced ototoxicity.

Introduction

Cisplatin is one of the most prescribed drugs for the treatment of human solid tumors such as ovarian, testicular, cervical, head and neck, lung, and bladder cancer. However, ototoxicity-induced by cisplatin has been shown to be an important obstacle for its utility and therapeutic profile [1,2]. Though the exact mechanisms of ototoxic effects by cisplatin is not widely revealed, reactive oxygen species (ROS) such as superoxide anion are believed to play a major role. Increased ROS results in depletion of the cochlear antioxidant enzymes [3,4]. This can lead to calcium influx within hair cells, leading to apoptosis [5]. Ototoxicity of cisplatin can be reduced by various antioxidants by counteracting this response [6].

Silymarin is a lipophilic extract from the seeds of the milk thistle (*Silybum marianum*) and composed of three isomers of flavonolignans (silybin, silydianin, and silychristin), and two flavonoids (tamoxifen and quercetin) [7,8]. Silymarin has been used in the treatment of liver diseases such as cirrhosis, viral hepatitis due to its hepatoprotective effect. The effect is mediated by scavenging of free radicals, decreasing formation of ROS and inhibition of fatty acid peroxidation. Another mechanism involves anti-apoptotic actions and anti-inflammatory actions [9,10]. Silymarin has no significant adverse reactions in human studies and has been reported to be safe in animal models [11]. In addition, silymarin has been shown to exert anti-neoplastic effects in a variety of cancer models [12,13]. Anti-oxidant and anti-apoptotic properties of silymarin may also have protective role against cisplatin-induced ototoxicity.

In this study, we aimed to investigate the effect of silymarin on cisplatin-induced ototoxicity in an auditory cell line and establish potential application for prevention of ototoxicity after cisplatin chemotherapy.

Materials and methods

Cell culture

The establishment of House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line was derived from postnatal organ of Corti of a transgenic immortomouse. The auditory cell line is extremely sensitive to ototoxic drug and express molecular markers, which

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are characteristic of organ of Corti cells [14]. HEI-OC1 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, NY, USA) supplemented with 10% fetal bovine serum (FBS; Lonza Walkersvile, MD, USA) at 33 °C in a humidified incubator with 5% CO_2.

MTT assay

Cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. HEI-OC1 cells were seeded at 3×10^4 cells per well of a 24-well plate and were incubated in DMEM with 10% FBS at 33 °C with 5% CO_2. Cells were divided into four groups: control, silymarin, cisplatin, silymarin plus cisplatin. Silymarin was purchased from Sigma (Saint Louis, MO, USA). Control group, silymarin, and cisplatin treated groups were incubated for 48 h. A 30 μM concentration of cisplatin for 48 h was used as adequate concentration to study cisplatin-induced cytotoxicity on the HEI-OC1 cells [15]. In order to investigate the effects of silymarin on cisplatin ototoxicity, the cells were pretreated with silymarin (50 μM) for 1 h, and exposed to cisplatin (30 μM) for 48 h. For the MTT assay, 5 mg/ml of MTT solution (Sigma, Saint 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 dissolved by the addition of 500 μL/well of DMSO. Absorption was measured using a spectrophotometer (BioTek, VT, USA) at 570 nm.

Hoechst 33258 staining

Apoptotic cell death was determined by evaluating the nuclear morphology using Hoechst 33258 staining. Cells were incubated with 10 μg/mL of the Hoechst 33258 (Sigma, Saint Louis, MO, USA) for 30 min. Membrane-permeable Hoechst 33258 was a blue fluorescent dye and stained the cell nucleus. After washing twice with phosphate buffered saline (PBS), the cells were detached by trypsinization and fixed with 4% paraformaldehyde for 10 min at room temperature (RT). The cells were placed over the slides and mounted with glycerol after drying with air. The cells were observed under a fluorescence microscope (DM5000, Leica, Wetzlar, Germany).

Western blot analysis

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 contents were measured using a Bio-Rad dye binding microassay (Bio-rad, Hercules, CA, USA), and heated at 98 °C for 5 min in Laemmli sample buffer and were subjected to SDS-PAGE on gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked for 2 h in 5% skim milk with TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) at RT and then incubated overnight at 4 °C with primary antibodies at appropriate dilutions; actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase 3 and PARP (Cell Signaling Technology, Danvers, MA, USA). Unbound antibody was removed by four washes for 15 min with TBST. The membranes were then incubated with the appropriate secondary antibodies (1:4000; Santa Cruz Biotechnology, Inc.) in blocking buffer for 2 h, and washed again. The protein bands were detected using the Super Signal West Pico chemiluminescence kit (Thermo Scientific, Waltham, MA, USA) and signals were acquired by image analyzer (LAS-3000 Imaging System, FujiFilm, Tokyo, Japan).

Propidium iodide staining

The floating and trypsin-detached HEI-OC 1 cells were collected and washed once with ice-cold PBS, followed by fixing in 70% cold ethanol for 30 min at 4 °C. The cells were then stained in PBS and propidium iodide (50 μg/ml), RNase A (50 μg/ml), and 0.05% Triton X-100 for 45 min at RT. The DNA content of the HEI-OC1 cells was analyzed by fluorescent-activated cell sorting (FACSort, Becton Dickinson). At least 10,000 events were analyzed, and the percentage of cells in sub-G1 population was calculated. Aggregates of cell debris at the origin of histogram were excluded from the sub-G1 cells.

Statistical analysis

Statistical analysis of the results was performed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The Student's *t*-test was used for pairs of data. A $p < 0.01$ was considered statistically significant.

Results

MTT assay

To investigate whether silymarin was able to prevent apoptosis induced by cisplatin, cell viability in HEI-OC1 cultures was determined by MTT assay. HEI-OC1 cells were treated with various concentrations of silymarin and with 30 μM of cisplatin for 48 h, the cell viability of HEI-OC1 cells was maximally protected at 50 μM of silymarin. Silymarin in concentrations over 100 μM had cytotoxic effects on the HEI-OC1 cells. Therefore, a 50 μM concentration of silymarin was used as the optimal experimental concentration for this study (Fig. 1). Fig. 2 shows cell viability of HEI-OC1 cells. When the cells were exposed with 30 μM of cisplatin for 48 h, the cell viability was 23 ± 0.9%. The cell viability of the HEI-OC1 cells was not affected by a 50 μM concentration of silymarin and it was 98 ± 2.8%. After pre-treatment with 50 μM of silymarin for 1 h, the cells were exposed with 30 μM of cisplatin for 48 h. The cell viability was 70 ± 6.8% (Fig. 2). Pretreatment of HEI-OC1 cells with silymarin significantly prevented the loss of cell viability induced by cisplatin treatment.

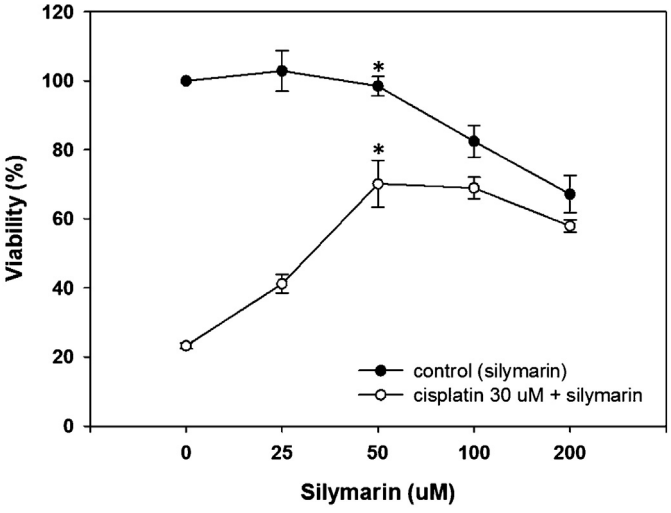

Fig. 1. The effect of silymarin on cisplatin-induced cell viability in HEI-OC1 cells. Cell viability using MTT assay was not significantly affected until a 50 μM concentration silymarin was reached. However, silymarin in concentrations over 100 μM had cytotoxic effects. The maximal protective effect against cisplatin cytotoxicity was observed at a concentration of 50 μM of silymarin on the HEI-OC1 cells. * Optimal experimental concentration of silymarin.