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Peanut sprout extract attenuates cisplatin-induced ototoxicity by induction of the Akt/Nrf2-mediated redox pathway

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ABSTRACT

Objective: Cisplatin is commonly used to treat solid tumors. However, permanent hearing loss is a major side effect of cisplatin chemotherapy and often results in dose reduction of the cisplatin chemotherapy. Peanut sprouts show cytoprotective properties owing to their antioxidant activities. This study was designed to investigate the effect of peanut sprout extract (PSE) on cisplatin-induced ototoxicity in an auditory cell line, HEI-OC1 cells.

Methods: Cells were exposed to cisplatin for 24 h, with or without pre-treatment with PSE, cell viability was examined using the MTT assay. Apoptotic cells were identified by double staining with Hoechst 33258 and propidium iodide. Western blot analysis was performed to examine apoptotic proteins including C-PARP and C-caspase, anti-apoptotic protein Bcl-2, and Nrf2 redox system activation. Mitochondrial reactive oxygen species (ROS) were investigated to examine whether PSE could scavenge cisplatin-induced ROS. Real-time PCR analyses were performed to investigate the mRNA levels of antioxidant enzymes including NQO1, HO-1, GPx2, Gclc, and catalase.

Results: The cisplatin-treated group showed reduced cell viability, increased apoptotic properties and markers, and increased ROS levels. PSE pre-treatment before cisplatin exposure significantly increased cell viability and reduced apoptotic properties and ROS production. These effects resulted from the up-regulation of antioxidant genes, including NQO1, HO-1, GPx2, Gclc, and catalase through Akt phosphorylation and Nrf2 activation.

Conclusion: Our results demonstrate that PSE protects from cisplatin-induced cytotoxicity by activating the antioxidant effects via the Akt/Nrf-2 pathway in this auditory cell line, and indicate that PSE may provide novel treatment to prevent cisplatin-induced ototoxicity.

1. Introduction

Cisplatin is commonly used for the management of solid tumors in the testis, ovaries, bladder, and lungs, as well as for head and neck cancers. However, hearing loss caused by its ototoxicity can reduce therapeutic use of cisplatin [1] and limit the efficacy of this anticancer treatment. Although the mechanism of cisplatin-induced ototoxicity is not fully understood, ototoxicity can be caused by an elevated level of reactive oxygen species (ROS); this depletes the intracochlear antioxidants [2] and induces calcium influx within hair cells, resulting in apoptosis [3].

The plant budding process requires active cellular activities and results in extremely high levels of intracellular oxidative stress. Peanut sprouts express higher levels of antioxidants than the original plant, in order to protect from oxidative damage during germination [4]. Peanut sprouts contain relatively high levels of phenolic compounds including resveratrol, protocatechuic acid, gallic acid, and caffeic acid. These compounds have anticancer, anti-inflammatory, and anti-aging effects by eliminating harmful oxidants [5,6]. Peanut sprout extract (PSE) may therefore modulate the oxidative stress induced by cisplatin cytotoxicity. This study aimed to investigate whether PSE had a protective effect against cisplatin-induced ototoxicity in an auditory cell line, thus investigating its potential application as a protective agent against ototoxicity during cisplatin chemotherapy.

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2. Materials and methods

2.1. Cell culture

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line expresses specific markers of cochlear hair cells and supporting cells. Therefore, this cell line is a very useful model for investigating the molecular mechanisms underlying ototoxicity and for screening for otoprotective compounds [7]. HEI-OC1 cells were used in accordance with the guiding principles approved by the ethics committee of Chosun University, and the National Institutes of Health Guide, South Korea for the Care and Use of Laboratory Animals. HEI-OC1 cells were cultured and incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum at 33 °C with 5% CO_2, as described previously [8].

2.2. Cell viability assay

The cultured HEI-OC1 cells were aliquoted in 24-well plates at 3×10^4 cells/well and incubated for 24 h. The peanut sprout extract (PSE) used in this study was kindly provided by Jangsuchae Co., Ltd. (Seoul, South Korea). The cells were pre-treated with PSE at a range of concentrations (0, 50, 100, 200, 300, 400, 500, 600, and 700 μg) for 2 h, and then exposed to cisplatin (30 μM) for 24 h.

The influence of PSE on the effects of cisplatin were also examined in the presence of MK-2206 dihydrochloride (Santa Cruz Biotechnology, CA, USA), as phosphorylated Akt (P-Akt) inhibitor. In this case, the cells were aliquoted in 24-well plates (3×10^4 cells/well), incubated for 24 h, and then pre-treated with or without PSE (300 μg) and MK-2206 (20 or 50 nM) for 2 h prior to incubation with cisplatin (30 μM) for 24 h. Cell viability was evaluated using the MTT (3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and the signal was read at 570 nm in a spectrophotometer (BioTek, VT, USA).

2.3. Hoechst 33258 and propidium iodide (PI) double staining

The nuclei of HEI-OC1 cells were stained with Hoechst 33258 dye (Sigma, Saint Louis, MO, USA) and PI solution (50 μg/mL PI, 50 μg/mL RNase A, and 0.05% Triton X-100 in PBS). The cells were cultured in 6-well plates (3×10^5 cells/well) and pre-incubated with PSE (300 μg) for 2 h prior to incubation with or without cisplatin (30 μM) at 33 °C for 24 h. The cells were then incubated with 2 μg/mL Hoechst 33258 and 1 μg/mL PI for 20 min in the dark. The plates were centrifuged at 1500 rpm for 3 min. After aspirating the supernatant, the cells were fixed in 1 mL of 3.7% formaldehyde for 10 min at room temperature. After fixing, the cells were washed once with PBS and observed under a fluorescence microscope (Olympus IX71, Tokyo, Japan).

2.4. Western blotting

The cells were harvested by centrifugation at 2000 rpm for 3 min and the media were removed, followed by washing with 1 mL PBS. The cells were suspended 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 Na3VO4, and 5 m-M NaF) and incubated for 10 min on ice. The lysed samples were sonicated (Sonics & Materials Inc., CT, USA) for 2 s each and centrifuged at 15,000 rpm for 15 min to produce the protein extract. These extracts were separated by 10–15% SDS-PAGE and then transferred to PVDF membranes (Millipore Corp, MA, USA). The membranes were blocked in TBS-T (20 mM Tris-HCl, 137 mM NaCl, pH 7.5, and 0.1% Tween-20) containing 5% skim milk at room temperature for 1 h. After washing with TBS-T, the membranes were incubated with primary antibodies overnight at 4 °C and then washed with TBS-T. The primary antibodies employed were raised against cleaved caspase-3 (1:100), cleaved PARP (1:500), Bcl-2 (1:200), P-Akt (Ser 473) (1:2000), Akt (1:1000), nuclear factor erythroid 2-related factor 2 (Nrf2; 1:500) (Cell Signaling Technology, Danvers, USA), NAD(P)H: quinone oxidoreductase 1 (NQO1; 1:1000) (Calbiochem, Darmstadt, Germany), and β-actin (1:3000) (Santa Cruz Biotechnology). The membranes were incubated at 4 °C overnight with each antibody diluted in TBS-T. The membranes were then washed three times with TBS-T for 10 min, prior to incubating with secondary antibodies for 2 h. The secondary antibodies were sheep anti- mouse (1:3000) and donkey anti-rabbit (1:3000) (Jackson Immunoresearch, PA, USA). The protein bands were visualized using a western blot detection system (iNtRON biotechnology, Daejeon, Korea) and developed by an image analyzer (LAS-3000 imaging system, Fujifilm, Tokyo, Japan).

2.5. Measurement of mitochondrial reactive oxygen species (ROS) levels

Mitochondrial superoxide in live cells was detected using the MitoSOX™ Red mitochondrial superoxide indicator (M36008, Invitrogen Molecular Probes, Eugene, OR). Cells were cultured in 6-well plates (3×10^5 cells/well) and incubated with PSE in the presence or absence of cisplatin at 33 °C for 24 h. The plates were then centrifuged at 1000 rpm for 5 min and the medium was removed. The cells were incubated with 5 μM MitoSOX™ reagent working solution in HBSS/Ca/Mg buffer (5 mM MitoSOX™ reagent stock solution in dimethyl sulfoxide) at 37 °C for 10 min, protected from light. The cells were washed gently three times with HBSS/Ca/Mg buffer and the fluorescence intensity of the cells was detected by flow cytometry (FACScalibur; BD Biosciences, San Diego, CA, USA). ROS levels were expressed as the mean fluorescence intensity of 10^5 cells/sample, calculated by Cell Quest software (BD Biosciences).

2.6. Real-time PCR analysis

Total cellular RNA from HEI-OC1 cells was extracted using Trizol (Invitrogen Corp., NY, USA) and quantified by UV spectroscopy at 260 nm. The RNA quality was evaluated using the 260/280 ratio (>1.8–2.0). Total RNA was reverse transcribed into cDNA using the M-MLV cDNA Synthesis kit (Enzynomics, Daejeon, Korea), and then amplified using TOPreal™ qPCR 2× PreMIX (SYBER Green with high ROX) (Enzynomics, Daejeon, Korea) and primers for NQO1, heme oxygenase 1 (HO-1), glutathione peroxidase 2 (GPx2), glutamate-cysteine ligase (Gclc), catalase. and GAPDH (internal control). The primers for these genes were as follows: NQO1, 5′-TTC TGT GGC TTC CAG GTC TT-3′ (forward) and 5′-AGG CTG CTT GGA GCA AAA TA-3′ (reverse); HO-1, 5′-CTT TCA GAA GGG TCA GGT GTC-3′ (forward) and 5′-TGC TTG TTT CGC TCT ATC TCC-3′ (reverse); Gclc, 5′-TGG CCA CTA TCT GCC CAA TT-3′ (forward) and 5′-GTC TGA CAC GTA GCC TCG GTA A-3′ (reverse); Gpx2, 5′-CAG CTT CCA GAC CAT CAA CA-3′ (forward) and 5′-CAC TGA GCC CTG AGG AAG AC-3′ (reverse); catalase, 5′-AAA TGC TTC AGG GCC GCC TT -3′ (forward) and 5′-GTA GGG ACA GTT CAC AGG TA-3′ (reverse); and GAPDH, 5′-GTA TTG GGC GCC TGG TCA CC-3′ (forward) and 5′-CGC TCC TGG AAG ATG GTG ATG G-3′ (reverse). The PCR parameters were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 20 s. The mRNA levels were expressed as the relative copy number of each target mRNA to GAPDH for each sample, and the cycle threshold (Ct) of the control group was normalized to 1.