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ORIGINAL ARTICLE

Molecular Mechanisms of Protective Effect of Resveratrol Against Cisplatinium Induced Ototoxicity

Yuksel Olgun, Zekiye Altun, Safiye Aktas, Pınar Ercetin, Gunay Kirkim, Muge Kiray, Alper Bagriyanik, Candan Ozogul, Bulent Serbetcioglu, Enis Alpin Guneri

Dokuz Eylul University Faculty of Medicine Department of Otorhinolaryngology (YO, GK, BS, EAG)
Institute of Oncology Department of Basic Oncology, Izmir, Turkey (ZA, SA, PE)
Dokuz Eylül University,School of Medicine,Department of Physiology (MK)
Dokuz Eylul University Faculty of Medicine Department of Histology and Embryology (AB)
Gazi University Faculty of Medicine Department of Histology and Embryology (CO)

Objective: Cisplatinium is one of the most widely used anticancer drugs. Side effects such as ototoxicity, nephrotoxicity and neurotoxicity limit to reach high doses. It mainly induces apoptosis in auditory sensory cells. Resveratrol is a polyphenol mostly found in grape skin and seed. It has anti-oxidant, neuroprotective and dose dependent antiapoptotic properties. It was claimed to protect side effects of some chemotherapeutic agents. In this study possible otoprotective effects of resveratrol were studied in House Ear Institute-Organ of Corti 1 cell line.

Materials and Methods: Cells were exposed to cisplatinium, resveratrol and their combination for 24 hours at different doses. Cell viability analysis, apoptosis and expression of 84 apoptosis related genes were determined in pre-optimized doses.

Results: Resveratrol pretreatment of the cells before cisplatinium led to 33% decrease in apoptotic cell count when they were compared with controls. Expression of apototic genes was decreased with cisplatinium and resveratrol combination.

Conclusion: This study revealed that resveratrol in low doses had an in vitro protective effect on cisplatinium ototoxicity mainly working on apoptotic gene expressions, but it has cytotoxic effect in high doses.

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Introduction

Cisplatinium is a highly effective anticancer agent used for both childhood and adult malignancies. It has dose-limiting side effects such as ototoxicity, neurotoxicity and nephrotoxicity. Ototoxicity is characterized by bilateral, usually permanent high frequency hearing loss but can progress to low frequencies. Although ototoxicity is mainly related to sensory hair- cell damage, stria vascularis and spiral ganglion cells are also found to be disrupted. Mechanisms of ototoxicity have been studied both in vitro and in vivo [1-6]. Cisplatinium decreased cell viability and induced apoptosis in HEI-OC1 cells by activating caspase 3, 8, 9 and release of cytochrome C and translocation of apoptosis- inducing factor in HEI-OC1 cells [2, 3, 6].

Resveratrol is a polyphenolic phytoalexin naturally found in spermatophytes such as pines, nuts and wine [7]. In late 1990's, resveratrol has proved to have a wide range of pharmacological effects in a dose dependent manner [7, 8]. Cardiac, renal, and neural protective effects were found to be mainly related to inhibition of apoptosis by declining free oxygen radicals. It also shows anti-inflammatory effects by inhibiting pro-inflammatory mediators [9]. The anticancer potential of resveratrol was

Corresponding address:
Yüksel Olgun
Dokuz Eylul University Faculty of Medicine Department of Otorhinolaryngology
İnciraltı-Izmir 35350 Turkiye
Phone: +905067021434
Email: yuksel.olgun@deu.edu.tr

first published in 1997 by Jang et al. [10,11]. Anticarcinogenic effects are in a dose dependent manner, and also it enhances efficacy of various anti-cancer agents such as etoposide and cisplatiniumium [8].

In vivo studies give very valuable information on hair cell death and survival, but because of the relative low accessibility of drugs to the inner ear, drug screening studies of inner cell lines may be more reliable and sensitive to detect agents that may prevent ototoxicity. Since 1997 many inner ear cell lines have been derived from saccular and cochlear tissues. One of those is House Ear Institute-organ of Corti 1 (HEI-OC1) cell line developed by Kalinec et al. [12] from cochlear cultures of the Immortomouse. This cell line was demonstrated to be susceptible to known ototoxic agents such as gentamycin and cisplatinium. Hence, this cell line has been suggested as a good model for the assessment of cellular and molecular mechanisms involved in drug induced hair-cell toxicity [13, 14].

There are a few in vivo investigations indicating a protective effects of resveratrol against cisplatinium-induced ototoxicity and nephrotoxicity as well doxorubicin-induced cardiotoxicity [4, 15, 16] However, there are no in vitro studies on otoprotective effects of resveratrol against cisplatinium-induced ototoxicity and mechanisms to that effect [17]. The aim of this study is to assess the protective effects of resveratrol in a system that will allow analysis of the molecular mechanisms underlying prevention of cisplatinium cytotoxicity.

Materials and Methods

This study was approved by the Local Ethic Committee for Noninvasive Studies.

Cell Culture

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line was kindly provided by F. Kalinec. It is an immortalized cell line derived from cochlear cultures of Immortomouse [11]. Kalinec et al. [11] suggested that HEI-OC1 cell line was an excellent in vitro system with 10% FBS at 33°C under 10% CO_2 in air. Cells were grown in conditions which had no antibiotics. Cisplatinium (Hexal), Resveratrol (Sigma) and MTT (Sigma) were used in this study. They were freshly prepared prior to all experiments. Cells were exposed to cisplatinium in 10, 20, 40, 100, 200 and 400 µM doses in growth inhibition experiments. Resveratrol was used in 0.1, 1, 10, 50 and 100 µM doses in the experiments. For resveratrol-cisplatinium interaction studies, the cells were pretreated with resveratrol for 60 minutes and then incubated with cisplatinium for 24 hours.

Cell Survival Analysis (MTT)

Cell survival was analyzed using MTT. Cells were briefly dispersed by tyrpsin-EDTA treatment and $1x10^4$ cells/mL, resuspended in mediums and seeded into 96-well culture plates with six replicates. After 24 hours of plating, incubation was continued for another 24 hours in the absence (control) or presence of cisplatinium. At the end of the incubation period, the reaction was terminated by adding 10 µl MTT reagent to each well. The reaction was allowed to proceed for 4 hours at 33°C. The formazan crystals were dissolved by adding the soluble solution. The intensity of the color developed, which is a reflection of a number of live cells, measured at a wavelength of 450 nm by ELISA reader (Thermo, Instruments Inc, USA). All values were compared with the corresponding controls. All assays were performed with 6 replicates.

Apoptosis Analysis (TUNEL)

Apoptotic cell death was monitored with TUNEL assay that detects fragmented DNA in the nucleus during apoptosis (Gen Script TUNEL Apoptosis Detection Kit Cat. No. L00299, for Adherent Cells, FITC-labeled POD). After treatment of cells with agents, cells were smeared on slides by cell scraper. The kit was applied on slides according to the manufacturer's instruction. After fixation and washing, tunel reaction mix containing Equilibration Buffer, FITC-12-dUTP and TdT was applied for 60 minutes at 37°C. Assay was done with an Olympus fluorescence microscope using excitation wave 450-500 nm and emission wave 515-565 nm (green). Five thousand cells per condition were evaluated and scored as a percentage of apoptosis per all condition. DAPI was the background staining.

Transmisson Electron Microscopy

Cells from flasks of four groups (HEI-OC1 cells without any drug, with cisplatinium (20 µM), resveratrol (0.1 µM), and with cisplatinium+resveratrol) that were collected by cell scraper were immediately fixed in 2%

paraformaldehyde plus glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and stored at +4 Cº for minimum 24 hours. After centrifugation at 2000G, each cell pellet was mixed with 0.5 cc melted 2% agar-agar (40Cº) on a slide. After cooling, the solidified agar with cells were cut to small pieces (2x2 mm). The pieces in each eppendorf were postfixed with osmium tetraoxide in a 0.1 M sodium phosphate buffer for 90 minutes at room temperature and subsequently contrasted with 0.5% uranyl acetate in 70% acetone and 1% phosphotungstic acid overnight at +4 Cº. The pieces were processed by routine method and embedded in araldit. After control by semi thin sections, ultrathin (<0.1 µm) sections were mounted on grids and analyzed with a transmission electron microscope.

RNA Isolation and Apoptosis Related Gene Expression Analysis by Real Time PCR

After HEI-OC1 Cell line was cultured in 125cm^2 large flasks and the agents and their combinations were applied for 24 hours in pre-optimized doses (0.1µm for resveratrol and 20µm for cisplatinium),, cells were collected by cell scraper not to cause RNA damage by enzyme digestion. RNA isolation was performed using an RNA extraction kit. Mouse apoptosis Real-Time PCR Array kit (SA Biosciences PAMM-012 A) was used for evaluation of apoptosis related gene expressions. After cDNA converting, expression of 84 genes of Mouse apoptosis related gene array was determined by Real Time PCR. PCR Array (84 genes, 5 housekeeping gene, 1 genomic DNA control, 3 reverse transcriptase control, 3 positive PCR control) was studied on 96 well PCR plate for each condition (Table 1). Total RNA extraction was performed according to manufacturer's instruction (High Pure RNA Isolation Kit (Roche Cat No. 11 828 665 001). Complementary DNA synthesis was done by RT2 First Strand Kit (QIAGEN Cat No. 330401). For gene expression analysis, Real time PCR on ABI PRISM 7000 Sequence Detection System was applied on custom arrayed plates. SABiosciences's PAMM-012A array included Master Mix, primary for each gene or housekeeping genes matched on array code. Only cDNA and SYBR Green was loaded on PCR 96 well plates. The protocol was loaded as one cycle of 10 minutes at 95C°, followed by 45 cycles of 15 seconds 95C° and 1 minute at 65C° each. SYBR Green Florescence was the detection method. Gene analysis was done one time for each condition.

Cp values on excel file listed according to the A1-H12 array codes were uploaded on http://www.sabiosciences.com/pcr/arrayanalysis.php, online free array analysis system. Fold changes of each condition compared with control HEI-OC1 cells were

Table 1. Changes in expression of genes that were highly expressed with cisplatinium treatment after resveratrol and cisplatinium and resveratrol combination. ↑: increase, ↓: decrease, ↔: stable

	Gene symbol	Cisplatinium	Resveratrol	Resveratrol+cisplatinium
Apoptotic	caspase 8	↑	↓	↓
	Bid,	↑	↔	↔
	Trp 53inp1,	↑	↓	↔
	Card6,	↑	↔	↓
	Cd40,	↑	↓	↓
	Cideb	↑	↔	↔
	Cd70.	↑	↓	↓
Anti-apoptotic	Bcl212,	↑	↔	↔
	Bcl10,	↑	↔	↓
	Pak7,	↑	↓	↓
	Naip1,	↑	↓	↓
	Akt1,	↑	↓	↔
	Bnip 31	↑	↔	↔
	Bcl2,	↑	↔	↔
	Bcl211	↑	↔	↔
	Bax	↑	↓	↔

calculated. Genes that showed >5- fold up- or downregulation were taken into consideration for expression changes. P values were not assessed for geneexpression because the analysis was not performed triplicate or more.

Statistical Analysis

All statistical analyses were performed using the SPSS 15.0 software program. All results were expressed as means ± SEM. Continuous variables were compared with the Mann–Whitney rank sum test. All treatment experiments were repeated at least three times to generate statistically relevant data ($P < 0.05$ was considered statistically significant).

Results

Cell Culture and Survival Analysis according to dose range of Cisplatinium and/or Resveratrol

Cells were exposed to cisplatinium in 10, 20, 40, 100, 200 and 400 µM doses in growth inhibition experiments. Resveratrol was used in 0.1, 1, 10, 50 and 100 µM doses in the experiments. In resveratrol-cisplatinium combination, the cells were pretreated with resveratrol for 60 minutes and then incubated with cisplatinium for 24 hours. Cisplatinium inhibited cell growth in a time and dose dependent manner in HEI-OC1 cells as shown in Figure 1. Cisplatinium inhibited 50% cell growth at 20 µM dose for 24 hours incubation in the cells. Nonproliferative resveratol dose at 0.1, 1 and 10 µM concentration was determined from cell viability experiments. 50 and 100 µM doses of resveratrol inhibited cell growth at 24 hours incubation period. In resveratrol-cisplatinium combination group, resveratrol at 0.1 µm dose was selected for protective dose of the experiment. After 60 minutes pretreatment with 0.1 µm resveratrol, auditory cells were protected from 20 µm cisplatinium induced cytotoxicity as shown in Figure 2.

Apoptosis in Number

Cisplatinium at 20µm dose caused 18% apoptotic cell death in HEI-OC1 cells. Cisplatinium-induced apoptoticcell death was decreased (12%) by pretreatment with resveratrol. Apoptotic cell percentage for the HEI-OC1 control group cells is 3%, while 9% for the resveratrol group.

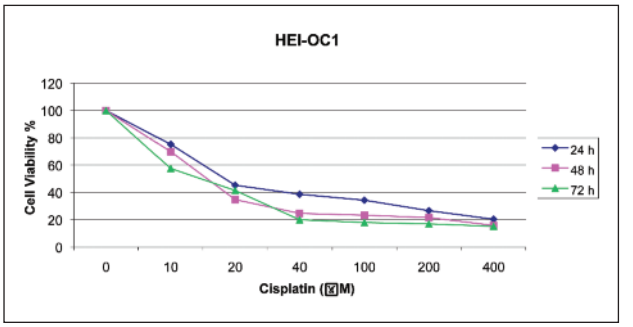

Figure 1. Cell growth curve of House Ear Institute-Organ of Corti 1 (HEI-OC1) cultured cells with cisplatinium. Cells were exposed to cisplatinium in 10, 20, 40, 100, 200 and 400 µM doses in growth inhibition experiments. Cisplatinium decreased cell viability in both dose and time dependent manner in HEI-OC1 cells.

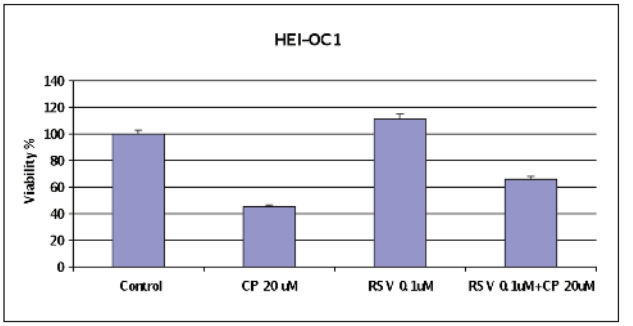

Figure 2. The effect of resveratrol on cisplatinium induced cytotoxicity in HEI-OC1 cells. In resveratrol-cisplatinium combination, the cells were pretreated with resveratrol for 60 minutes and then incubated with cisplatinium for 24 hours. Resveratrol at 0.1 µM dose decreased the cisplatinium induced cell death in resveratrol-cisplatinium combination. Values represent the mean ± SEM

Electron Microscopic Findings

In normal HEI-OC1 cells and in cells treated only with resveratrol there were no changes and the cells were intact. In the cisplatinium group, many of the cells showed apoptotic, necrotic or aponecrotic changes. In the combination-treatment group, the number of apoptotic, necrotic and aponecrotic cells were decreased. The cells collected from cell culture showed microvillus-like cytoplasmic extensions but the typical W figure that are specific for hair cells seen in tissue ultrathin sections were not observed. This finding might be a result of two dimensional configuration of organ of Corti cells in tissue culture. In the cisplatinium group, the microvilli were disrupted and lower in number compared with control group; and in this group large amounts of cellular

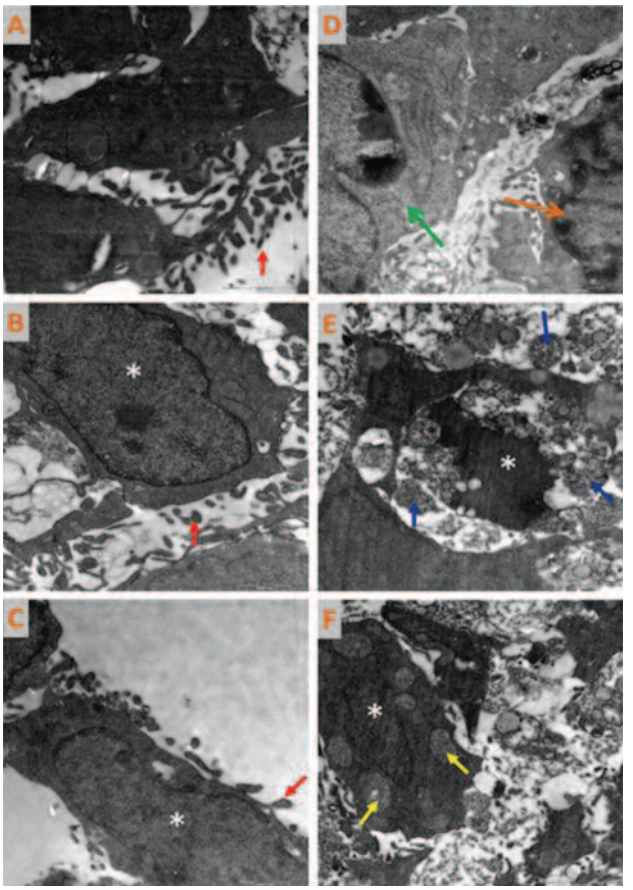

Figure 3. A: Control, B: Resveratrol, C: Cisplatinium+Resveratrol, D, E, F: Cisplatinium group. A, B: Normal appearance of hair cells. Red arrows; microvilli-like cytoplasmic expensions, Star; nucleus. C: Hair cell in normal appearance that has microvilli-like expensions (red arrow) like the control group. (*). D: Hair cell containing large amounts of cytoplasmic debris in pericellular area. Green arrow; apoptotic cell, Orange arrow; aponecrotic cell E: necrotic hair cell containing large amounts of cytoplasmic debris around (blue arrows) (*). F: Hair cell with irregular nucleus containing mitochondrial swelling and large amounts of cytoplasmic debris observed around the cell (yellow arrows) (*).

debris in pericellular area and mitochondrial swelling were seen. In the resveratrol only group, there were no changes in microvilli (Figure 3).

Apoptosis Related Gene Expression Analysis

Cisplatinium, resveratrol and resveratrol-cisplatinium induced apoptotic gene expressions were determined with 84 custom array genes of Mouse apoptosis (SA, Biosciences) (Figure 4).

Highly expressed apoptotic genes with cisplatinium treatment were caspase 8, Bid, Trp 53inp1, Card6, Cd40, Cideb and Cd70. Anti- apoptotic gene expressions of Bcl212, Bcl10, Pak7, Naip1, Akt1, Bnip 31, Bcl2, Bcl211 and Bax were also induced with cisplatinium. Apoptotic genes of caspase 8, Trp53inp1, Cd40, Cd70 and antiapoptotic genes of Pak7, Naip1, Akt1 and Bax expressions were decreased with resveratrol treatment. Expression of apoptotic genes such as caspase 8, Card6, Cd40, Cd70 and also expression of anti-apoptotic genes Bcl10, Pak7 and Naip1 were decreased with cisplatinium and resveratrol combination (Table 1). Other highly expressed apoptotic and anti-apoptotic genes were not distinctly affected with the same combination.

Discussion

Cisplatinium is a highly potent antineoplastic agent but side ffects can limit its use at high doses. One of the most important side effects is ototoxicity [1]. Ototoxicity is mainly related to hair cell, spiral ganglion cell and stria vascularis damage. Although the exact mechanism of ototoxicity is not clearly known, free radical induced

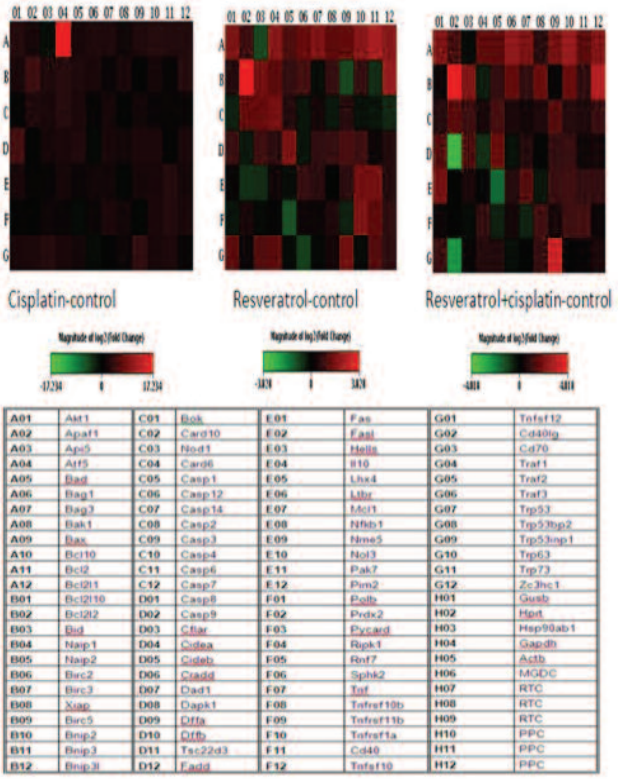

Figure 4. Heatmaps of apoptosis related genes array comparing three groups with control HEI-OC1 cells. Gene symbols of each gene from A1-G12 are given in Table 1. More green color means lower expression; more red color means higher expression

apoptosis of those cells has been suggested [18] and may be responsible.

Antiapoptotic agents have been studied both in vivo and in vitro against cisplatinium induced ototoxicity. Many endogenous and exogenous substances have been used in different studies for; protection against cisplatinium ototoxicity. Among those apocynin [3], Rehmannia glutinosa [6], Korean Red Ginseng [19], cannebionoid receptor-2 agonist [13] and purple bamboo salt [14, 20] were studied on HEI-OC1 cells. Yu et al. [6] showed that steamed root of Rehmania glutinosa had a protective effect against cisplatinium cytotoxicity in HEI-OC1 cells via scavenging radical activities especially in superoxide radicals and reduction of lipid peroxidation. Im et al.[19] showed that Korean Red Ginseng could play both an antiapoptotic and antioxidative role on cisplatinium induced ototoxicity in the same cell line. So et al. [21] demonstrated that sensory hair cell damage caused by cisplatinium was related to pro-inflammatory cytokines. Jeong et al. [14] suggested that cisplatinium causes activation of caspase 3, 8 and 9; release of cytochrome C and translocation of apoptosis-inducing factor in HEI-OC1 cells. Both OC-k3 and HEI-OC1 cell line studies showed that cisplatinium can induce apoptosis due to p53 and caspase independently. Similar to our study, cisplatinium decreased cell viability and induced apoptosis in HEI-OC1 cells [14, 22].

The anticancer elements and other broad spectrum health benefits of resveratrol are mediated through several cell signaling pathways included in inflammation, proliferation, apoptosis and/or angiogenesis. However, some investigators claim that the anticancer effects are mainly related to antiapoptotic effects of RSV [11]. This contradictory statement may be attributed to dose dependant behavior of RSV. It also has a sensitizing effect on chemotherapeutic resistant cells [8]. The chemosensitization of tumor cells by resveratrol is shown to occur by increasing p53, Fas/CD95, P21, caspase 6 activity and by inhibiting c-Flip, p glycoprotein, MRP-1, bcl-2, Bcl-xL, survivin, Cyclin D1, NF-κB activity. Nicolini et al. [23] investigated the antiapoptotic potential of resveratrol and its effect on paclitaxel in SH-SY5Y neuroblastoma cell line. They found that resveratrol abrogated paclitaxel induced apoptosis by inhibiting activation of caspase 7 and PARP cleavage and inducing S phase cell cycle arrest. The neuroblastoma cells were prevented from entering the mitosis phase.

Althought RSV is a potent antiapoptotic agent it is not widely investigated against ototoxicity. In one in vivo study it was found to protect from ototoxic effects of gentamycin [24]. Also recently reported were in vivo studies which demonstrated the protective effect of intraperitoneal- administered resveratrol against cisplatinium induced ototoxicity [4, 25, 26]. However there is not any in vitro study which has allowed the investigation of the mechanisms underlying the protective effects of RSV on cisplatinium ototoxicity.

Our study evaluated the protective effects of resveratrol against cisplatinium ototoxicity on HEI-OC1cells. As the most prominent protection is at the 0.1 μM concentration of RSV, we investigated molecular mechanism of protection at this dose. In higher doses, (50- and 100mM) RSV has been found to have cytotoxic effects on HEI-OC1 cell lines. Similar dual effects of RSV is found in studies on neuronal and neuroblastoma cells [27, 28]. This dose dependent dual effect of RSV needs to be investigated further in both in vitro and in vivo studies.

We studied 0-400 μM doses of cisplatinium on HEI-OC1 cells, but we determined the 50% cell proliferation inhibitor dose of cisplation that was 20 μM at 24 hours incubation period as shown in Figure 1. 20 μM cisplatinium dose inhibited cell growth by 50% so we had enough living cells to study molecular mechanism of cisplatinium ototoxicity and resveratrol otoprotection. If we had used higher doses of cisplatinium, we would not have enough cells alive to investigate these mechanisms. Also in many previous studies investigating cisplatinium ototoxicity 20 or even 10 μM doses of cisplatinium was used as an inhibitor concentration of hair cells [3, 6, 13, 14].

In our study highly expressed apoptotic genes with cisplatinium treatment were appropriately caspase 8, Bid, Trp 53inp1, Card6, Cd40, Cideb and Cd70. Our findings are consistent with Jeong et al. [13] who showed that cisplatinium induced activation of caspase 3, 8 and 9 in cochlear cell death. Anti- apoptotic gene expressions of Bcl212, Bcl10, Pak7, Naip1, Akt1, Bnip 31, Bcl2, Bcl211 and Bax were also induced with cisplatinium

treatment of the HEI-OC1 cells in our analysis. We speculate that this reflected an upregulation of the endogenous system to prevent cisplantin-induced cell death. Wang et al. [20] showed that cisplatinium caused important signaling events in redistribution of Bax and release of cytochrome C in HEI-OC1 cells. In contrast, Rybak et al. [17] showed that cisplatinium can induce apoptosis due to p53 and caspase independently in both OC-k3 and HEI-OC1 cell line studies.

In this study, we demonstrated that expression of apoptotic genes such as caspase 8, Card6, Cd40, Cd70 and also the expression of anti-apoptotic genes Bcl10, Pak7 and Naip1 are decreased with resveratrol pretreatment of subsequently cisplatinium-treated HEI-OC1 cells. Protective mechanisms of resveratrol against cisplatinium-induced apoptosis was mainly due to inactivation of extrinsic pathway of apoptosis (caspase 8 and cd40). Furthermore, apoptotic genes of caspase 8, Trp53inp1, Cd40, Cd70 and antiapoptotic genes of Pak7, Naip1, Akt1 and Bax expressions were decreased with resveratrol treatment. Other highly expressed apoptotic and antiapoptotic genes were not distinctly affected with the same combination. Transmission electron microscopic findings supported otoprotective effects of resveratrol at 0.1 μM dose, and showed that resveratrol protected cells from the effects of cisplatinium by decreasing both apoptosis and necrosis.

Our next step will be assessment of molecular mechanisms of protective effects of orally administeredresveratrol against cisplatinium- induced ototoxicity in vivo using experimental laboratory rat animal models. We plan to administer resveratrol orally to animals, because we think that it is clinically more feasible .

Conclusion

This in vitro study revealed that resveratrol may have a protective effect against cisplatinium ototoxicity. It has protective effect in low doses and toxic effect in high doses. Molecular mechanisms of this protection is based on upregulation of antiapoptotic genes. Given this significant protection and the dose dependent nature of this in vitro otoprotective effect, further in vivo studies, with different routes of RSV administration, seem appropriate to translate this basic finding to useful clinical application.

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Conflict of interest: The authors do not have any financial relationship with the organization that sponsored the research.

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