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Original Article

Acetyl-L-Carnitine protects HEI-OC1 auditory cells from radiation and cisplatin induced toxicity

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Abstract: Concomitant applications of CDDP and RT have synergistic effects in oncology. CDDP and RT can cause ototoxicity through oxidative stress. Nrf2 is a transcription factor that has antioxidant properties. The aim of this study is to evaluate the protective effect of ALC via Nrf2 and target genes against CDDP and RT induced ototoxicity in The House Ear Research Institute-Organ of Corti I cells (HEI-OC1). HEI-OC1 cells were exposed to a single dose of 5 Gray RT. After incubation for 72 h with or without ALC, CDDP, and their combinations, ototoxicity of HEI-OC1 cells were examined through analyzing apoptosis, oxidative stress and Nrf2 and related gene expressions by TUNEL, flow cytometry, HPLC and quantitative real-time PCR. ALC protected from RT, CDDP and CDDP-RT induced cell death by inhibiting apoptosis and oxidative stress. ALC-CDDP-RT treatment increased the expressions of Quinone oxidoreductase1 (NQO1), SODIII, Heme Oxygenase (HO-1) and Glutathione-S Transferase (GST). In conclusion, Nuclear factor erythroid 2-related factor 2 (Nrf2) and oxidative stress might play important roles in cisplatin (CDDP), radiotherapy (RT) and CDDP-RT induced ototoxicity. Therefore, targeting Nrf2 by Aceytl-L-Carnitine (ALC) might be potential therapeutic strategies.

Keywords: HEI-OC1, ototoxicity, radiotherapy, cisplatin, Nrf2, Aceytl-L-Carnitine

Introduction

Cisplatin (CDDP) is a platinum-based chemotherapeutic agent widely used in therapy of solid tumors that has some adverse effects such as ototoxicity, neurotoxicity and nephrotoxicity. CDDP and RT have synergistic effects for cancer therapy but they may also enhance their side effects when they are applied together such as ototoxicity. CDDP and RT related ototoxicity and sensorineural hearing loss can decrease especially the quality of life of pediatric cancer patients.

CDDP and RT exert their effects through DNA direct or indirectly which can result in single or double strand breaks and oxidative stress [1-3]. Reactive oxygen species (ROS) is a signaling molecule in cells but excess amounts of ROS can damage and leads to apoptosis in the cochlear structures. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a nuclear transcription factor that has some antioxidant and anti-inflammatory features in many cell types including auditory cells [3-5]. Activated Nrf2 induces the transcription of many cytoprotective genes that encode antioxidant genes and detoxifying enzymes.

Aceytl-L-Carnitine (ALC) is an antioxidant agent that offers some protection against CDDP related toxicities such as neurotoxicity and ototoxicity both *in vitro* and *in vivo* [5, 6]. Our previous work showed that ALC can modulate CDDP induced oxidative stress in different ways such as reducing glutathione levels and lipid peroxidation, while apoptotic effects of CDDP are not interfered with ALC treatment in neuroblastoma [6]. ALC also has a protective effect against cisplatin induced ototoxicity in auditory cells, with induction of anti-apoptotic gene expression and attenuating levels of pro-inflammatory cyto-

kines [1]. However, there has been no study about the Nrf2 related molecular mechanism of ALC against CDDP, RT and CDDP-RT induced ototoxicity. L-carnitine has a protective role against radiation induced injury in another tissues such as the testis and ileal mucosa [2, 8]. ALC prevented radiation-induced sickness by reducing oxidative lipid peroxidation levels [9]. Only one *in vivo* study has demonstrated the amelioration of total cranial radiation-induced cochlear degeneration in guinea pigs by L-carnitine [10].

The aim of this study was designed to examine the role of ALC against CDDP, RT and CDDP-RT induced ototoxicity and clarify the molecular mechanisms of Nrf2 and target genes in The House Ear Research Institute-Organ of Corti I (HEI-OC1) cells. Moreover, the possible mechanisms of the underlying this otoprotective effect were investigated via ROS, as well as changes in mitochondrial membrane potential, lipid peroxidation and apoptosis.

Material and methods

The House Ear Research Institute-Organ of Corti I cell line (HEI-OC1) was kindly obtained from F. Kalinec. This auditory cell line is a useful model for ototoxicity studies [11]. Cells were maintained in high-glucose DMEM containing 10% FBS at 33°C [1]. Cisplatin (Hexal, Sandoz/Novartis/Bangladesh) and Acetyl-L-carnitine (Sigma-Aldrich Co, St. Louis, MO, USA) were freshly prepared before each experiment. Cells were incubated with CDDP, RT and ALC and combinations (ALC-RT, ALC-CDDP, ALC-RT-CDDP) by 24, 48 and 72 hours (h). Control cells only were treated with the medium. In ALC group, cells were treated with only 50 μM ALC at 24, 48 and 72 h incubations in all experiments. After 24 h plating, cells were pretreated with 50 μM ALC or 50 μM CDDP or 50 μm ALC-50 μm CDDP for 30 min before and then RT 5 Gray (5 Gy) was applied on ALC, ALC-RT, ALC-CDDP, ALC-CDDP-RT groups. ALC and CDDP were left in the culture mediums during the experiments. Cells (1×10^4/wells in 96-well microtiter well plates) were exposed to a 5 Gy single fraction dose of irradiation using 6MV-X power in a Linear accelerator (Primus, Siemens, USA) at Dokuz Eylul University Radiation Oncology Department. Cells were kept at room temperature when they were treated with RT about 20 min and then they were incubated at 33°C. After RT treatment, cells were further cultured at 24, 48 and 72 h. At the end of the incubation periods, the viability of cells was evaluated by using a WST-1 assay kit (Roche Applied Science, Mannheim, Germany) which is a colorimetric method. WST-1 analysis was performed as previously described [1]. All values were compared to the corresponding controls. Mean of triplicate experiments for each dose was used to calculate 50% cell viability inhibitor doses of CDDP, RT and CDDP-RT combinations. ALC dosage was chosen at 50 μM from a previous study and treatment of 50 μM ALC similarly protected the cells from CDDP and RT induced cell death at 72 h [1]. Then all experiments and measurements were performed at 72 h after RT treatment.

Cells were exposed to RT, CDDP, ALC and combinations for 72 h, and then apoptotic cell death was monitored using the TdT-mediated dUTP nick end labelling (TUNEL) assay to detect fragmented DNA in apoptotic nuclei (GenScript TUNEL Apoptosis Detection Kit, for adherent cells, FITC-labelled POD, USA, Piscataway, NJ) as previously described [1]. A total of 5000 cells/condition were evaluated by fluorescence microscope and scored as per cent of apoptosis per total cells.

The effects of RT, CDDP, RT-CDDP and ALC on intracellular reactive oxygen species levels and mitochondrial membrane potential ($\Delta\Psi m$) in HEI-OC1 cells were evaluated by using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Invitrogen Molecular Probes, USA, Eugene, OR) and 5,5 V,6,6 V-tetrachloro-1,1 V 3,3 V-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Invitrogen Molecular Probes, USA, Eugene, OR) [4]. Briefly, HEI-OC1 cells were cultured for 24 h and then treated with 5 Gy RT, 50 μM CDDP and 5 Gy RT-50 μM CDDP for 72 h with or without pretreatment with 50 μm M ALC. After the 72 h incubation, cells were harvested using trypsin-EDTA into tubes and washed with PBS. The cells were treated with 10 μM DCFH-DA and 2 μM JC-1 Working Solution and further incubated for 15 min at 33°C. Flow cytometric analyses (10000 events per sample) of DCFH-DA-treated and JC-1 treated cells were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences, USA) at excitation and emission wavelengths (488 and 530 nm for DCF-DA and 533 and 585 nm for JC-1, respectively) and evaluated using the BD Accuri C6 Software (BD Biosciences,

Table 1. The list of primers used in the real-time PCR experiments

Genes	Primers	
	Forward	Reverse
Nrf2	TCCATTCCCGAATTACAGTGTC	AGTTGCCCACTTCTTTTTCCAG
HO-1	AGATGACACCTGAGGTCAAGCA	GACTCTGGTCTTTGTGTTCCTCT
GST	CGCCACCAAATATGACCTCT	CTGTTGCCCACAAGGTAGT
GPx	GTCCACCGTGTATGCCTTCT	TCTGCAGATCGTTCATCTCG
NQO1	TTCTCTGGCCGATTCAGAGT	AGGCTGCTTGGAGCAAAATA
GR	GACAACCTGACTTCCTTGGG	GAGCACACCAGGCAGAGTTTG
SODII	AGCGGTCGTGTAAACCTCAAT	CCAGAGCCTCGTGGTACTTC
SODIII	AGAGCCTGACAGGTGCAGAG	GCCTATCTTCTCAACCAGGTCA
Catalase	GAGACCTGGGCAATGTGACT	GTTTACTGCGCAATCCCAAT
GCL	AGTTGGAGCAGCTGTATCAGTG	ATGTCAGGGATGCTTTCTTGA
GAPDH	CTCATGACCACAGTCCATGC	GTCATCATACTTGGCAGGTTTCT

Superoxide dismutase (SOD) II, Superoxide dismutase (SOD) III, Catalase and Glutamate Cysteine Ligase (GCL) gene expressions analyses were performed with LightCycler 480 II (Roche, Mannheim, Germany) (**Table 1**). GAPDH expression was used for normalization of the mRNA levels. The evaluation of relative mRNA levels among the treatment groups was carried out using the ΔΔCT method. Data are presented as relative fold changes compared with the control group. The significance gene expressions cut off level is considered as > 2 fold changes.

USA). The red/green fluorescence ratio was introduced to identify living and apoptotic cells. JC-1 exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (≈529 nm) to red (≈590 nm). Hence, mitochondrial membrane depolarization (ΔΨm) is indicated by a decrease in the red/green fluorescence intensity ratio of JC-1. Data are presented as percentages of the fluorescence intensity ratio of control.

Cells were harvested and washed twice with ice-cold phosphate buffered saline after treatments. Subsequently homogenization step, protein concentration was determined using a protein assay kit (Pierce, Thermo Scientific, USA, Rockford). Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid reactive substances (TBARS) by high performance liquid chromatography (HPLC) (CTO-10AS VP, Shimadzu) [6]. Thiobarbituric acid levels were normalized to protein content and expressed as umol/mg protein.

Total RNA was extracted from cells with the use of Tripure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. First-strand cDNAs obtained from total RNAs and they were used for quantitative real-time PCR. cDNA was amplified with Sybr Green I (Roche FastStart DNA Master, Roche, Mannheim, Germany). Nrf2, Heme Oxygenase I (HO-1), Glutathione S Transferase (GST), Glutathione Peroxides (GPx), NAD(P)H: Quinone oxidoreductase 1 (NQO1), Glutathione Reducates (GR),

Statistical analyses were performed using SPSS 15.0 software program (Chicago, IL, USA). All results are expressed as means ± SEM. Continuous variables were compared using 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

In this study 5 Gy RT, 50 μM CDDP and 5 Gy RT-50 μM CDDP treatments significantly inhibited HEI-OC1 cell viability in a time dependent manner as shown in **Figure 1** ($P<0.05$). CDDP-RT combination was decreased the cell viability significantly about 50% at 72 h ($P<0.05$). HEI-OC1 cells were pretreated with 50 μM ALC 30 min and protected from RT, CDDP and CDDP-RT combination induced auditory cell cytotoxicity significantly at 72 h ($P<0.05$) (**Figure 1**).

RT, CDDP and CDDP-RT treatments significantly induced apoptosis of HEI-OC1 cells through DNA fragmentation in the cells when compared with the control (**Figure 2**) ($P<0.0\%$). 50 μM ALC prevented against RT, CDDP and CDDP-RT induced apoptotic cell death when compared to their controls at 72 h (**Figure 2**) ($P<0.05$). These results indicated that, this protection came about by inhibiting DNA fragmentation in the cells. Moreover, according to cell viability and apoptosis measurements ALC protected RT, CDDP and CDDP-RT induced hair cell toxicity at the same ratios in the cells.

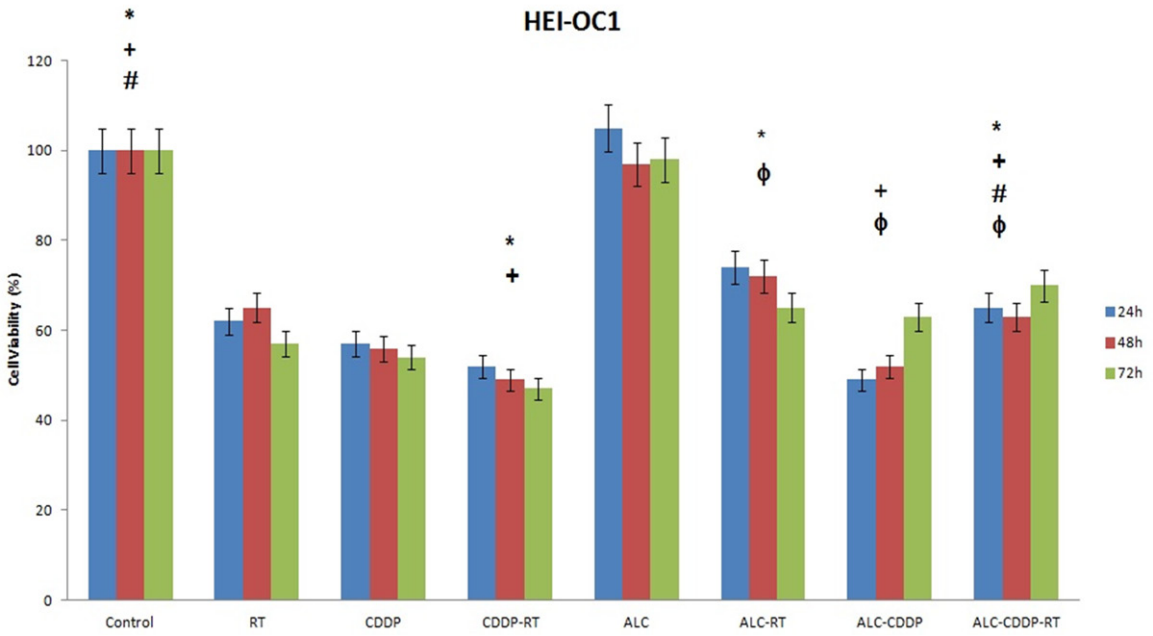

Figure 1. ALC protected HEI-OC1 cells against CDDP, RT and CDDP-RT induced toxicity. Cells were pretreated with 50 μm ALC for 30 min followed by the addition of CDDP (50 μm), RT (5 Gray) and CDDP-RT for 24, 48 and 72 h. Values represent the mean ± SEM of 6 observations (Mann-Whithney-U, P<0.05). (*P<0.05 versus RT, +P<0.05 versus CDDP, #P<0.05 versus CDDP-RT, ϕP<0.05 versus ALC).

Firstly, the cells significantly induced ROS generation and lipid peroxidation with RT, CDDP or RT-CDDP treatments in auditory cells (**Figures 3** and **4**) (P<0.05). Furthermore, HEI-OC1 cells decreased their mitochondrial potential and induced apoptosis as a result of these treatments, when compared with the control group (**Figure 5**). These cells showed a decrease in red fluorescence and an increase in green fluorescence. It suggests that RT, CDDP and RT-CDDP reduced ΔΨm. ALC markedly suppressed the RT-CDDP mediated DCF-DA oxidation and changes in mitochondrial membrane potential (**Figures 3** and **4**) (P<0.05). ALC also inhibited RT-CDDP combination induced lipid peroxidation levels but not to a statistically significant level (**Figure 4**) (P>0.05).

ROS generation and mitochondrial membrane potential was affected by RT and CDDP treatment alone in the cells (P<0.05). Moreover, lipid peroxidation levels were significantly increased due to RT and CDDP (P<0.05). ALC protected cochlear cells by inhibiting ROS generation; lipid peroxidation and variation in mitochondrial membrane potential from both RT and CDDP induced oxidative stress in the groups at the same ratios (**Figures 3-5**) (P<0.05).

The transcription factor of Nrf2 is a major regulator genes encoding phase II detoxifying and antioxidant genes such as HO-1, GST, GPx, NQO1, GR, SOD II, SOD III, Catalase and GCL. In this study, these gene expressions of mRNA levels were determined by the RT-PCR method. Nrf2 and all of antioxidant these genes mainly induced with RT-CDDP combinations treatments at 72 h incubation as compared with control (**Figure 6**) (P<0.05). RT alone induced GPx and SODIII expressions were increased slightly when compared with the control group (P<0.05). ALC-RT combinations induced SODIII and NQO1 gene expressions when compared to control group (P<0.05).

CDDP treatment did not show any Nrf2 and target gene expressions after 72 h incubations time point except that SODII expressions decreased in comparison with control (P<0.05). ALC-CDDP combinations prominently increased the level of expressions of GST and Nrf2 genes while the level of NQO1, GR and HO-1 gene expressions decreased (P<0.05). CDDP-RT combination was induced HO-1, GPx, SODIII, Catalase and SODII gene expressions slightly (P<0.05). ALC protected the HEI-OC1 cells especially from CDDP-RT combination induced

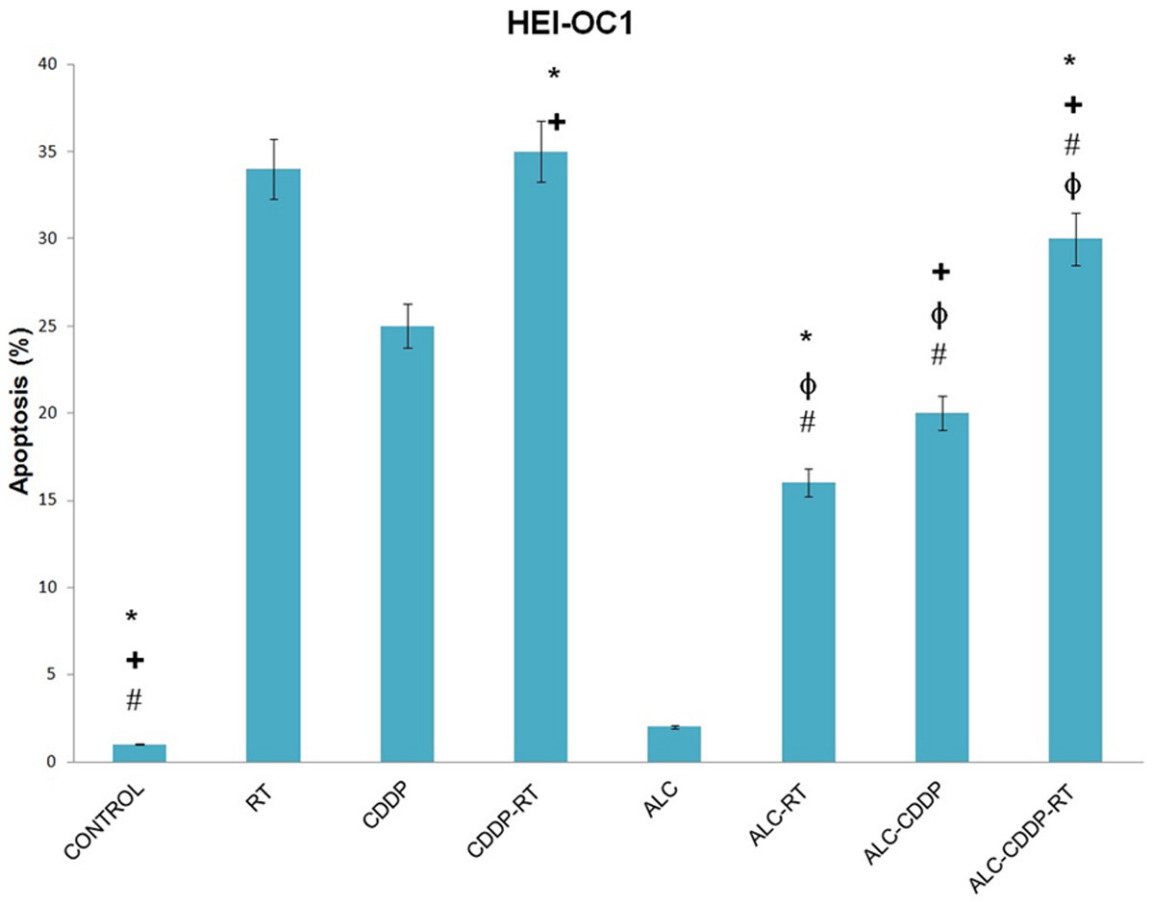

Figure 2. Effect of ALC on CDDP, RT and CDDP-RT induced apoptotic cell death in auditory cells. After pretreatment with 50 μm Acetyl-L-carnitine (ALC), auditory cells were protected from 50 μm cisplatin (CDDP), 5 Gray radiation (RT) and CDDP-RT induced apoptotic cell death at 72 h. Values represent the mean ± SEM of 6 observations (Mann-Whithney-U, p<0,05). (*P<0.05 versus RT, +P<0.05 versus CDDP, #P<0.05 versus CDDP-RT, ϕP<0.05 versus ALC).

toxicity trough inducing Nrf2 and related anti-oxidant gene expressions (**Figure 6**) (P<0.05). ALC-CDDP-RT combinations induced almost all gene expressions of Nrf2, GR, SODII, Catalase, GPx, and mainly NQO1, SODIII, HO-1 and GST genes except GCL when compared to the control group (P<0.05).

Discussion

Ototoxicity is the main dose limiting side effect for children or adult patients when a combination of RT and CDDP is used in the clinic. However, chemoradiation is the major risk for hearing loss especially in childhood. Formation of platinum metabolites with the radiation induced free oxygen radicals together may cause further damage through increasing DNA damage to hair cells [3]. Furthermore, the main mechanism behind the ototoxicity of the CDDP-RT combination is a possible augmentation of apoptosis. This study revealed that CDDP, RT and CDDP-RT combinations inhibited cochlear cell viability and induced apoptotic cell death. ROS generation, lipid peroxidation and mitochondrial membrane potential changes were determined, linked to CDDP, RT and CDDP-RT combinations in cochlear cells. However, researchers have studied many potential chemoprotective agents that reduce the adverse reactions induced by cisplatin or radiotherapy including ebselen [3], resveratrol [12], erdosteine [4] and bucillamine [13].

ALC is a natural anti-oxidant compound that has essential roles in intermediary metabolism linked to the facilitating essential fatty acid entry and exit from mitochondria. Previous studies demonstrated that L-carnitine or ALC gave protection from RT related toxicities *in*

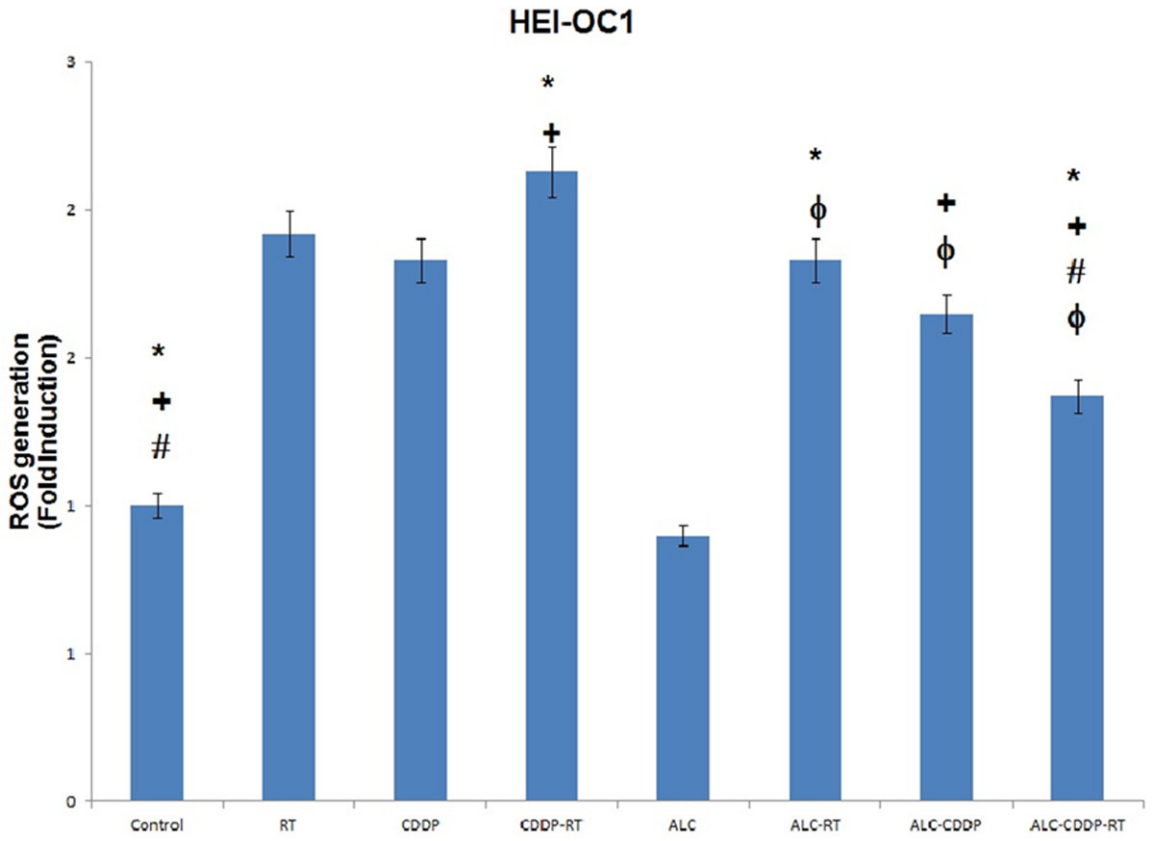

Figure 3. Pharmacological inhibition of CDDP, RT and CDDP-RT induced ROS generation in HEI-OC1 cells with ALC. Cells were treated with 50 uM ALC for 30 min and further incubated with 50 uM CDDP, 5 Gy RT and CDDP-RT for 72 h. After 72 h, the level of intracellular ROS was monitored using a fluorescent probe, DCFH-DA. The data represented the mean ± S.E.M. of six independent experiments (Mann-Whithney-U, $P<0.05$). ($^{*}P<0.05$ versus RT, $^{+}P<0.05$ versus CDDP, $^{\#}P<0.05$ versus CDDP-RT, $^{\phi}P<0.05$ versus ALC).

vivo and *in vitro* in different ways [2, 9, 14-16]. Another *in vivo* study showed that hepatic and lung toxicity due to total body irradiation was prevented by ALC through the activation of an antioxidant defense mechanism [14]. Furthermore, L-carnitine protected the brain, retinal, renal, ovary and ileal tissues from damage caused by irradiation in short and long time periods by up-regulation of antioxidant enzyme activities and inhibiting apoptotic cell death mechanism [2, 15, 16]. Up to date, only Altas and et al. has shown that L-carnitinine has protective effect against RT induced damage in cohlea, *in vivo* [10]. They showed that intraperitoneal L-Carnitine treatment reduced the degeneration of stria vascularis and hair cells in total cranial irradiated guinea pigs. ALC has also immunostimulatory effects on CDDP or RT induced damages shown by *in vivo* and *in vitro* studies [1, 8]. However, how ALC protects auditory cells from CDDP, RT or CDDP-RT-induced damage in cochlear cells remains unclear.

In this study, ALC markedly suppressed RT-CDDP mediated ROS generation and mitochondrial membrane potential changes in auditory cells. RT-CDDP induced lipid peroxidation levels were inhibited by ALC. Moreover, RT-CDDP induced apoptotic cell death decreased with ALC pretreatment of HEI-OC1 cells. The protective mechanism of ALC from RT-CDDP induced toxicity might be related to mitochondrial stability of apoptotic cell death. Additionally, under stress conditions such as CDDP and RT chemoradiation of cells, Nrf2 translocate from the cytoplasm into the nucleus to stimulate transcription of heme oxygenase-1 and other cytoprotective enzymes through binding to antioxidant responsive elements. Therefore, Nrf2 and related antioxidant gene expressions were also determined in this study through RT, CDDP or CDDP-RT combination induced auditory cell damage model. One study showed that after 24 h CDDP treatment of auditory cells erdosteine increased the downstream

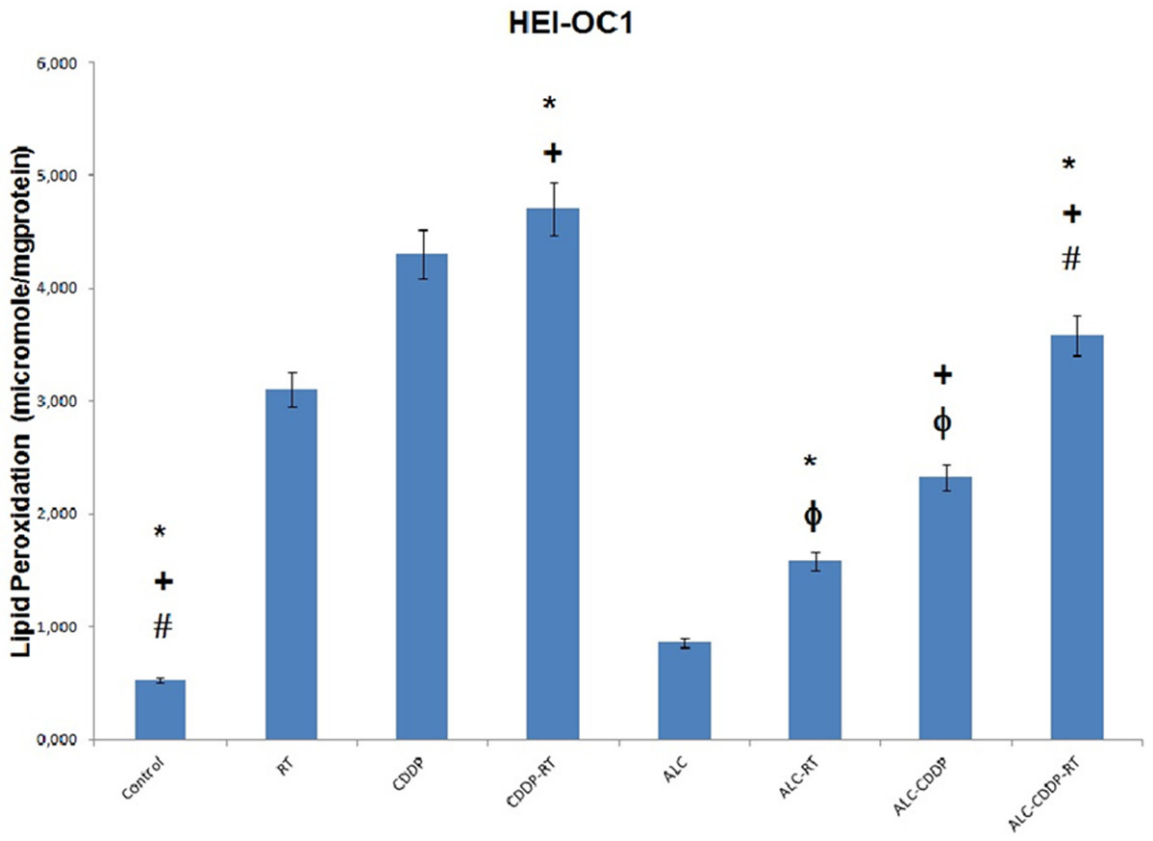

Figure 4. Effect of ALC on CDDP, RT and CDDP-RT induced lipid peroxidation. Lipid peroxidation induction (HPLC analysis) at 72 h in HEI-OC1 cells with CDDP (50 uM), RT (5 Gy) and CDDP-RT were determined. Values represent the mean ± SEM of 3 observations.

of Nrf2 antioxidant genes of HO-1 and SOD II and glutathione biosynthesis related genes of glutathione synthetase [13]. In agreement with this study and related to CDDP ototoxicity we showed that ALC-CDDP treatment significantly induced Nrf2 and GST gene mRNA expressions, while CDDP alone did not affect these genes in our ototoxicity model. There are some differences between these studies regarding the doses and incubation times of CDDP treatments. Another agent flunarazine only increased the expression of an endogenous molecule, HO-1 that protects against oxidative stress, in HEI-OC1 cells linked to Nrf2 [5]. However, mRNA expression profiles of other Nrf2-regulated genes, such as NQO1, catalytic and modifier subunits of GCL, GSTm-1, and GSTA4, were not affected by flunarizine in the presence of cisplatin. Kim et al. [13] showed that bucillamine also prevented cisplatin induced auditory cell death by Nrf2 and related to redox regulation of GSH system of genes such as catalytic subunit of GCL. Similar to our study, Kim et al. [3] showed that ebselen considerably increased the mRNA expression levels of HO-1 and NQO1 in cisplatin-treated HEI-OC1 cells.

Up to date there was no study about the possible protective molecular mechanism of carnitine against radiation induced auditory cell damage in the literature. In the present study, we firstly showed that ALC inhibited oxidative stress and apoptotic cell death induced by RT or RT-CDDP. Moreover, this protection was elucidated by Nrf2 related mechanism in auditory cells. ALC-RT induced SODIII and NQO1, while RT alone induced GPx and SODIII gene mRNA expressions. ALC significantly showed the maximum protective effect on the Nrf2 and mediated antioxidant gene expressions when the cells were exposed to chemoradiation (RT-CDDP) together. The approximately all of the Nrf2 and target gene expressions were induced by ALC pretreatment after RT-CDDP treatment in auditory cells. The most increased genes were GST,

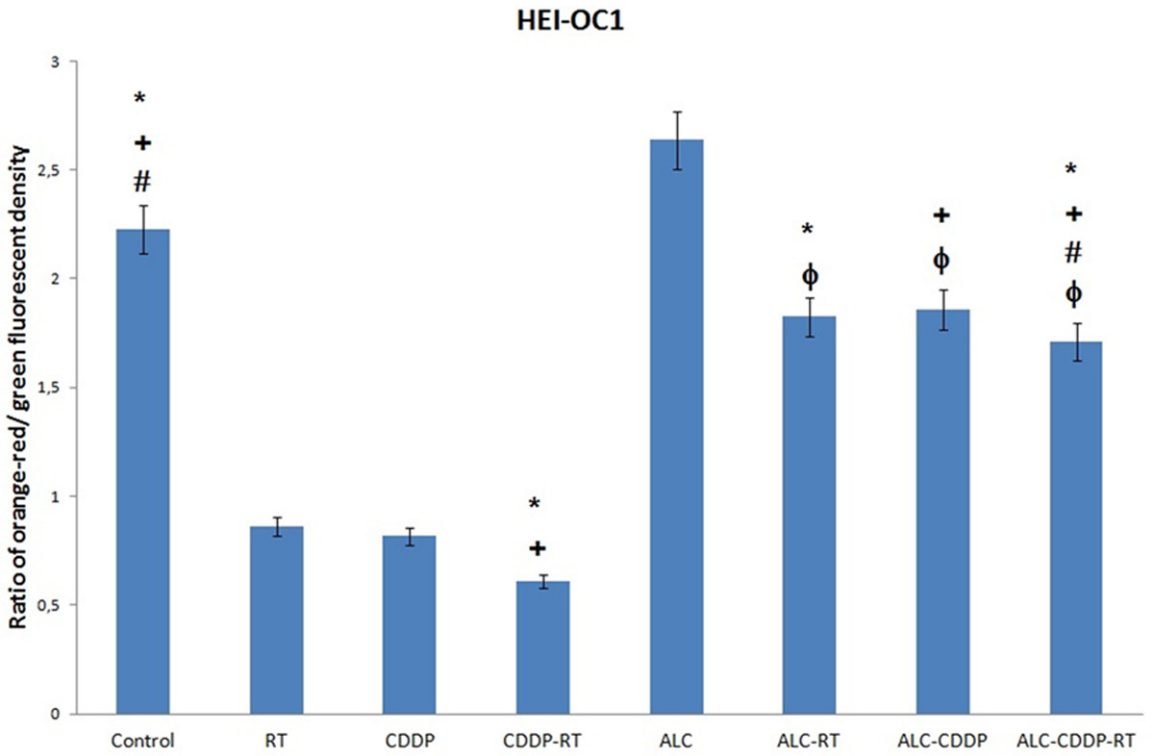

Figure 5. Effect of ALC on CDDP, RT and CDDP-RT induced mitochondrial membrane potential (ΔΨm) changes assed by JC-1 assay. Data are expressed as mean ± S.E.M of 6 observations. RT (5 Gy), CDDP (50 μm) and CDDP-RT reduced mitochondrial membrane potential at 72 h in HEI-OC1 cells. ALC was increased decline of mitochondrial membrane potential in cells with CDDP, RT and RT-CDDP combination (Mann-Whithney-U, $P<0.05$). ($^{*}P<0.05$ versus RT, $^{+}P<0.05$ versus CDDP, $^{\#}P<0.05$ versus CDDP-RT, $^{\phi}P<0.05$ versus ALC).

HO-1, SODIII and NQO1 with ALC against to RT-CDDP. Furthermore, Nrf2, GR, SODII, Catalase and GPx gene expressions were induced with a combination of ALC-RT-CDDP. Our previous study revealed that ALC has anti-inflammatory features and activating anti-apoptotic Akt1 expressions might be related to Nrf2 pathway in this protection [1]. This is the first study to show that ALC protected auditory cells against RT-CDDP mediated ototoxicity. This was achieved through Nrf2 and detoxifying target gene expressions.

In conclusion, CDDP, RT and CDDP-RT resulted in oxidative stress and apoptosis and altered Nrf2 related gene expressions in auditory cells. Inhibition of oxidative stress and upregulation of Nrf2 related gene expressions in the presence of ALC significantly reduced oxidative stress. Our data suggests that ALC has protective effects on CDDP, RT and CDDP-RT induced ototoxicity by regulating Nrf2 related phase II detoxifying and antioxidant genes. Therefore, ALC might be a promising protective agent against RT or CDDP-RT induced ototoxicity like as CDDP. Further *in vivo* translational molecular mechanistic studies must be done concerning Acetyl-L-carnitine and the toxic side effects of radiation and cisplatin.

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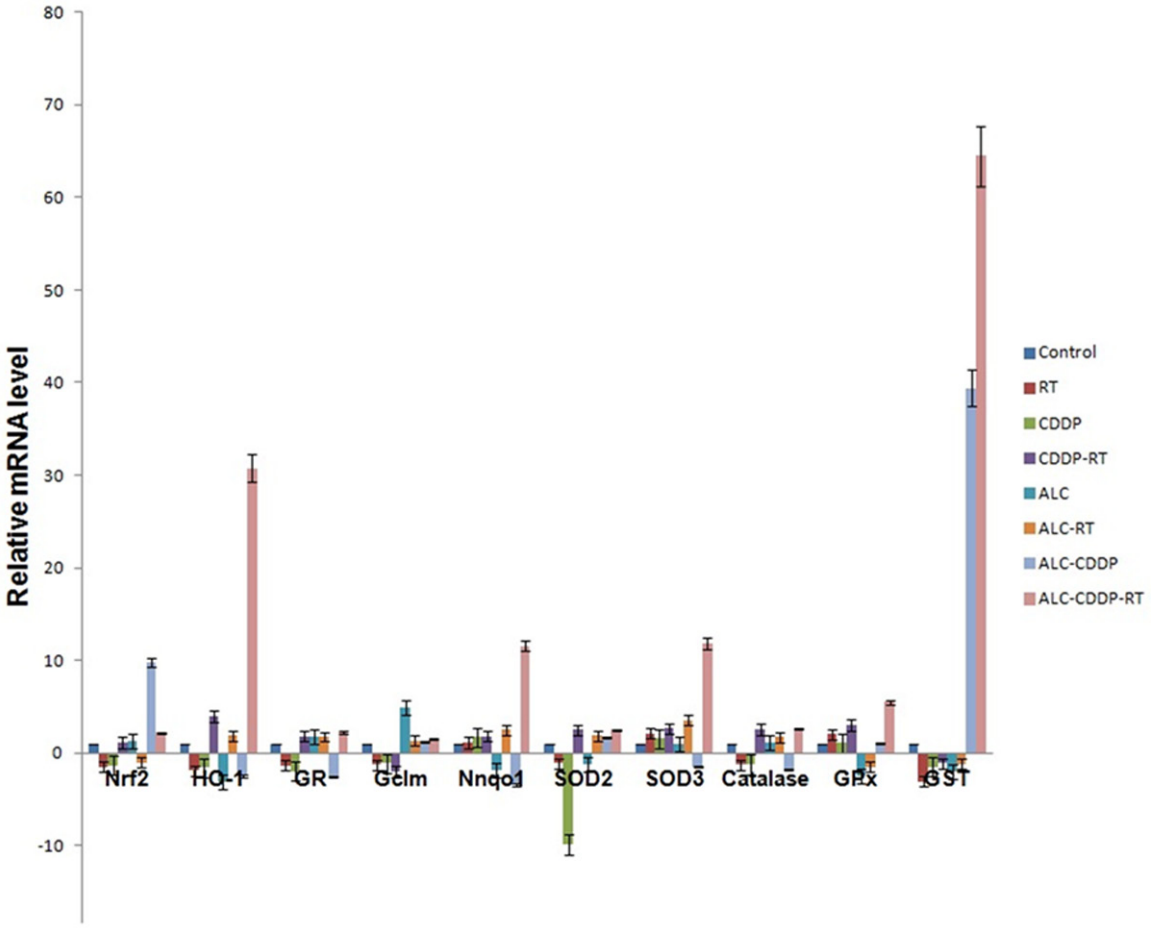

Figure 6. Effect of ALC on CDDP, RT and CDDP-RT induced Nrf2 and target gene expressions. Aceytl-L-Carnitine (ALC) induces the relative expression levels of Nrf2 and phase II antioxidants and detoxifying genes at 72 h in HEI-OC1 cells treated with CDDP and mainly CDDP-RT. Cells were pretreated with 50 μm ALC for 1 h followed by the addition of 50 μm CDDP, 5 Gy RT and CDDP-RT at 72 h. Expression levels of Nrf2, mHO-1, Nnqo1, SOD3 and GST were significantly increased by the treatment with ALC as compared with CDDP and CDDP-RT at 72 h ($P<0.05$).

Otology (June 30- July 3, 2015) as poster presentations.

Disclosure of conflict of interest

None.

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