

## Original Article

# Oleuropein Reduces Cisplatin-Induced Nephrotoxicity in Human Embryonic Renal Epithelial “GP-293” Cells

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## Abstract

**Background and Aim:** The protective effect of oleuropein, a chief polyphenol compound in olive tree, on cisplatin-induced nephrotoxicity in human cultured renal tubular cells, GP-293, was investigated.

**Materials and Methods:** Cell viability was determined by the MTT assay. Cleaved caspase-3 and Bax: Bcl2 ratios as biochemical parameters of cellular apoptosis, was assessed using western blot analysis.

**Results:** Our data showed that cisplatin in the dose of 55 µg/ml significantly can reduced cell viability, increased caspase-3 activation and Bax: Bcl2 ratio in GP-293 cells after 24hr. Incubation of the cisplatin treated cells, with 20 µg/ml oleuropein decreased the cisplatin-induced cell toxicity, as well as prevented caspase-3 activation and reduced Bax: Bcl2 elevation ratio.

**Conclusions:** Oleuropein protects against cisplatin-induced cellular toxicity. This could be associated with the prevention of cellular apoptosis.

**Keywords:** Cisplatin, Nephrotoxicity, Apoptosis, GP-293 cells, Oleuropein

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## Introduction

Currently, cancer is one of the most common diseases in the world. Cancer can be treated and controlled with the help of various chemotherapy drugs. However, many of these chemotherapy agents, beside of their helpful properties, have severe side effects.

Cisplatin [cis-dichloro diammine-platinum (II)] is a chemotherapy drug used to treat patients with various types of cancer, including testis, ovary, cervix, bladder, lung and esophageal tumors (1-3). Previous studies have shown that cisplatin through the

induction of internal oxidative stress as well as reduction in cellular antioxidant defense system can lead to cellular damage (4, 5). It is notably that, nephropathy induced by cisplatin is the most important reason that reduce the usage of this drugs in cancer chemotherapy (4, 6). It has been shown that 30 percent of patients who treated with cisplatin as an anti-tumor agent, even though they were hydration (for reducing the cisplatin induced nephrotoxicity), needed hospital care because of severe irreversible renal kidney tissue complications due to its oxidative properties (7, 8). Oleuropein is one of the polyphenols which are found abundantly in olive leaf compounds. This polyphenol

can prohibit the membrane lipid oxidation and cardiovascular diseases; also it is effective in improving coronary artery disease and has, anti-arrhythmic effect, as well as improves lipid metabolism (9-11). In addition oleuropein inhibits cellular enzyme disruption due to oxidative stress in chemotherapy situation (12). Further more oleuropein can prevent the cellular toxicity that induced through the high levels of oxidative stress in with cancer patients (12). Altogether, the purpose of this study was to evaluate the possible beneficial properties of oleuropein on nephrotoxic effects of cisplatin in the human cultured epithelial kidney cells, GP-293, as an *in vitro* model of cisplatin induced renal nephropathy.

## Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin– trypsin EDTA and streptomycin solution were obtained from Biosera (BioseraUK). Culture dishes were purchased from SPL Life sciences Inc (SPL, South Korea). Cis-dichloro diammine platinum [II] (cisplatin) powder and 3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Sigma (Sigma, Germany). Primary monoclonal anti- $\beta$ -actin and primary polyclonal anti-caspase 3 antibodies were purchased from Cell Signaling Technology, Inc. (Cell Signaling, USA). Primary polyclonal anti-Bax and primary monoclonal anti-Bcl-2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (SantaCruz, USA).

### Cell culture

Embryonic tubular epithelial GP-293 cells were purchased from National Cell Bank of Iran (NCBI) Pasteur Institute of Iran (Tehran, Iran). Cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 mg/mL). They were maintained at 37°C in 5% CO<sub>2</sub> atmosphere. For experiments, GP-293 cells were grown in plastic culture flasks and used when in exponential growth or were grown to confluent monolayer and replaced with fresh medium for 3 days to reach stationary phase culture. 5000 cells per well seeded in a 96 micro plate well for the MTT assay.

### Cell viability assay

The cell viability was explored with MTT assay. This method is based on reduction of 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) formazan. MTT (0.5mg/ml) was added to the 96-well plates and the cells were incubated for 2 hr at 37°C. After medium removing, the resulting formazan was solubilized in dimethyl sulfoxide (DMSO) (100 $\mu$ l per well). The optical density was determined at 570nm by an automatic microplate reader (Eliza MAT 2000, DRG Instruments, GmbH). Results were expressed as percentages of control. Control cells were grown in DMEM and the other cells (cisplatin treated) grown in DMEM with 54 $\mu$ M cisplatin. For protein extraction, cells were cultured in a 6 plate well and permitted to attach and grow for 24h. Then the cells were incubated with cisplatin contained medium and effective dose of oleuropein for 24h.

### Immunoblot analysis

GD-293 cells were homogenized in ice-cold buffer [10mM Tris–HCl (pH 7.4), 1mM EDTA, 0.1% SDS, 0.1% Na-deoxycholate, 1% NP-40] supplemented with protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). The homogenate was centrifuged at 14,000 RPM for 15 min at 4°C. The protein content of the supernatant was determined by the Bradford assay (Bio-Rad Laboratories, Muenchen, Germany). Of protein were resolved electrophoretically on a 12% SDS-PAGE gel at equivalent amounts and stained with Ponceau S as a loading control, the protein resolved were transferred to polyvinylidene fluoride (PVDF) membranes (Roch, Germany). After overnight blocking with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (blocking buffer, TBS-T, 150mM NaCl, 20mM Tris–HCl, 0.1% Tween 20, pH7.5), the PVDF membranes were probed with rabbit monoclonal antibody to caspase-3 [Cell Signaling Technology, 1:1000 overnight at 4°C]; Bax and Bcl-2 [Santa Cruz Biotechnology, 1:1000 for 3h at room temperature]. After washing in TBS-T (3 times, 5 min), the blots were incubated for 1 hour at room temperature with a horseradish peroxidase-conjugated secondary antibody (1:15,000, GE Healthcare Bio-Sciences Corp., 1:15,000). All antibodies were diluted in blocking buffer. Via the ECL system the antibody–antigen complexes were detected and exposed to Lumi-Film

chemiluminescent detection film (Roch, Germany). To analyze the values of Bax, Bcl-2, Caspase 3 and b-actin band intensity and their expression, Lab Work analyzing software was used (UVP, UK).  $\beta$ -actin immunoblotting (antibody from Cell Signaling Technology, Inc., Beverly, MA; 1:1000) was used to control for loading.

#### Statistical analysis

All results were presented as mean  $\pm$  SD. The difference in mean cell viability between groups was analyzed by one-way ANOVA, followed by the Newman-Keuls test. The values of protein (Bax, Bcl-2 and cleaved caspase 3) band densities were expressed as tested protein/b-actin ratio for each sample. The average values of different experimental groups were compared by one-way ANOVA, followed by the Newman-Keuls test.  $P < 0.05$  was considered significant.

## Results

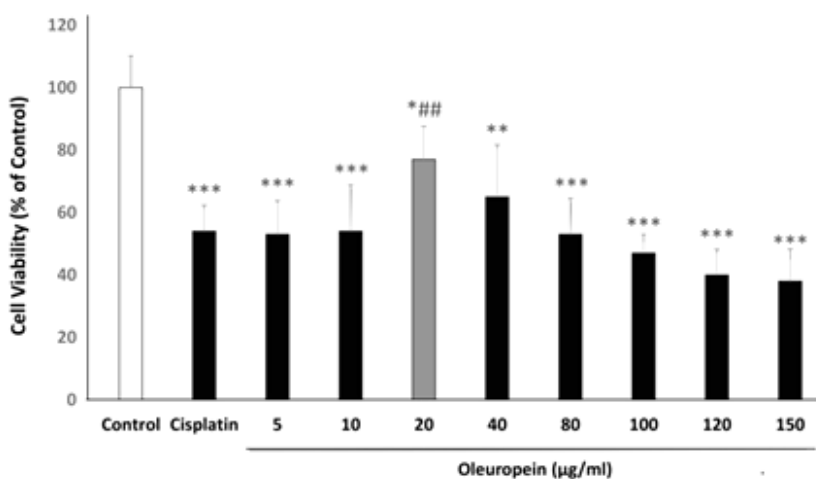
#### Analysis of cell viability

We first analyzed the effects of various cisplatin (40, 45, 50, 55 and 60  $\mu$ M) concentrations on the viability of GP-293 cells via MTT assay. Our data shows that cisplatin could decrease the viability of GP-293 cells to 50 percent as dose of 55  $\mu$ M. The toxic effect observed in this dose of cisplatin, was used as optimum dose for damaging the cells and assessing the protective effects of the oleuropein pretreatment

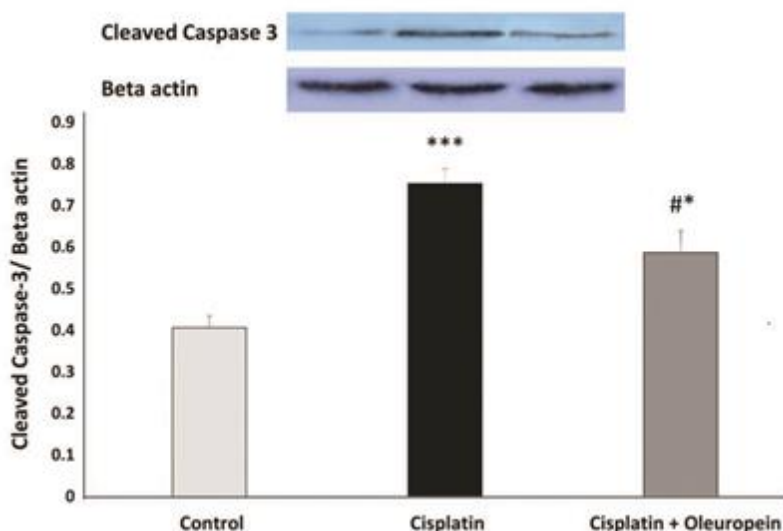
(data not show). As shown in Figure 1, oleuropein in the dose of 20  $\mu$ g/ml significantly inhibited cisplatin toxicity in GP-293 cells after 24hr; whereas, oleuropein could not prevent cell toxicity in other concentrations (Figure 1).

#### The effect of oleuropein on cleaved caspase 3, Bax and Bcl-2 in GP-293 cells in the presence of cisplatin

We examined caspase-3 activation, Bax and Bcl-2 using western blot analysis to evaluate the potential activity of oleuropein on preventing apoptosis after cisplatin induced cellular toxicity in GP-293 cells. The GP-293 cells were categorized to several groups including control, cisplatin and cisplatin plus effective concentrations of oleuropein. There was not any significant difference in the expression of the Bcl-2 between cisplatin and cisplatin + Oleuropein treated groups (Figure 3-C). In addition, a significant increase in Bax protein was observed in cisplatin group compare to control group (figure 3-B). It is notable that elevated amount in Bax excretion was decreased in cisplatin + Oleuropein group (figure 3-C). As shown in figure 2 and 3-A, 24hr. incubation with cisplatin enhanced expression of cleaved caspase-3 (Figure 2) and Bax/Bcl2 ratio (Figure 3-A) compared to the control groups. Furthermore, treatment of GP-293 cells with 20  $\mu$ M of oleuropein was significantly reduced cisplatin-induced up-regulation of cleaved caspase-3 (Figure 2) and



**Fig. 1.** Effects of different doses of oleuropein on cisplatin-treated GP-293 cell viability. Cisplatin reduced cell viability and oleuropein (20  $\mu$ g/ml) protected the GP-293 cells from cisplatin cell damage. Data are expressed as mean  $\pm$  SEM;  $n = 6-8$  wells for each group; \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus control cells. # $P < 0.05$  compared to cisplatin-treated cells.



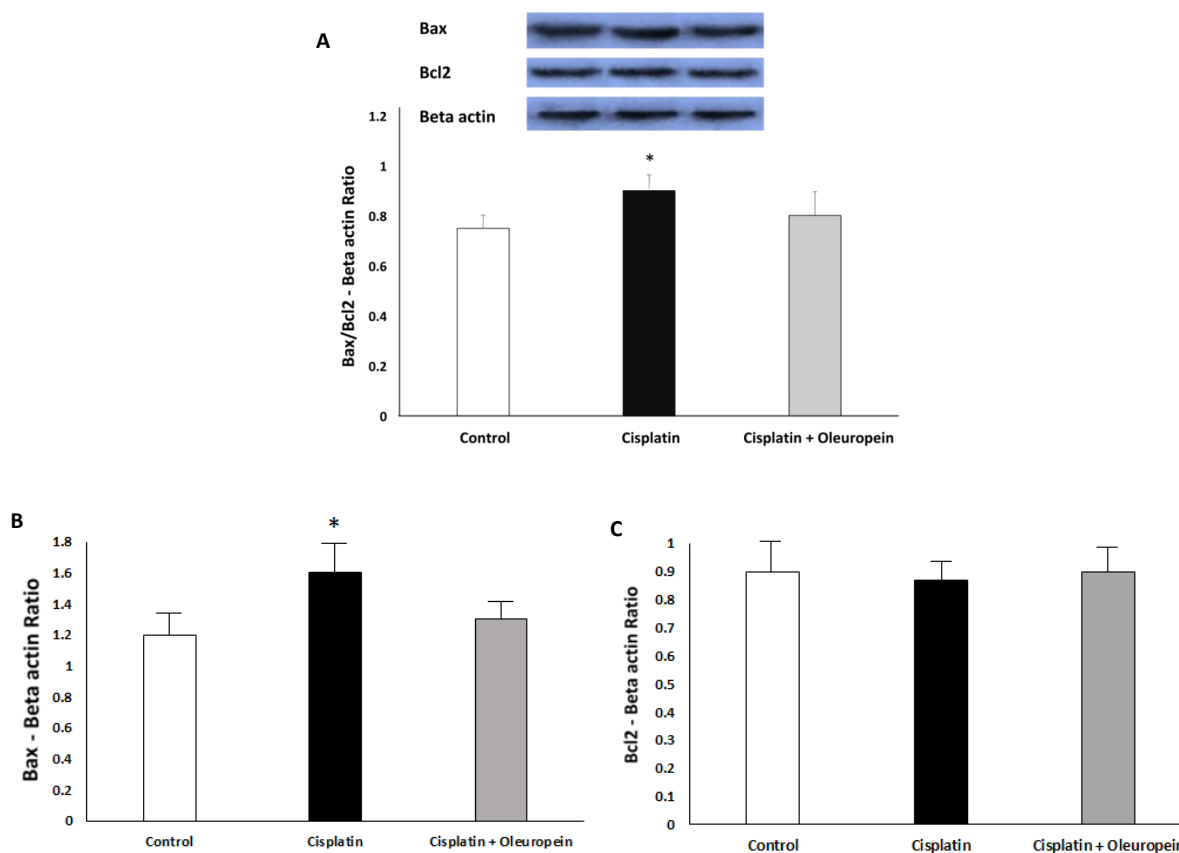
**Fig. 2.** The activation of caspase 3 protein in GP-293 cells exposed to cisplatin and cisplatin plus effective dose of oleuropein for 24h which was studied by Western blot. Each value in the graph is the mean  $\pm$  SEM band density ratio for each group. Beta-actin was used as an internal control. \* $P < 0.05$  and \*\*\* $P < 0.001$  compared to control cells. # $P < 0.05$  compared to cisplatin-treated cells.

Bax/Bcl2 ratio (Figure 3-A).

## Discussion

The present study demonstrated that oleuropein pretreatment of human cultured embryonic tubular epithelial cells (GP-293) induces tolerance against Cisplatin Nephrotoxicity. In the present study, cisplatin enhanced the caspase-3 activity and the Bax: Bcl-2 ratio that is in accordance with our previous studies and other investigations in this field (13, 14). All these effects of cisplatin were abolished by oleuropein pretreatment. Our results indicate that oleuropein pretreatment leads to resistance against cisplatin cell injury which is associated with the decrease of caspase-3 activity and the Bax: Bcl-2 ratio. Therefore, it is important to know possible mechanisms that oleuropein can protect the renal tubular cells against cytotoxic effects of cisplatin. Many studies show that ROS (reactive oxygen species), particularly hydroxyl radicals, accumulate rapidly following cisplatin administration (15-17). On the other hand, oxidative stress induced by ROS, has been implicated in renal damage under various pathological stipulations (7, 18). According to previous studies, cisplatin administration leads to renal injury in the proximal tubule region of the

kidney nephrons (19). One of the important mechanisms in the development of acute renal toxicity of cisplatin is oxidative damage that is caused by ROS and the most important of them is the hydroxyl radical. The reactive species cause lipid peroxidation, protein and DNA damage and, in this way, tubular damage and acute renal toxicity occurred (4). A large amount of studies shows that oxidative stress such as cisplatin chemotherapy is closely linked to apoptosis in renal nephrotoxicity (4, 13, 14). It seems that the use of diet supplements containing antioxidant compounds, such as ascorbic acid or vitamin E attenuate the free radical oxidation complications by preventing the intracellular molecule calcification and free radical absorption (10). Oleuropein, the bitter compound, the major constituent of the secoiridoid family of compounds in the olive tree, has been shown to be a strong antioxidant and anti-inflammatory effects (20). Also, some studies have shown that oleuropein can prevent cardiac disease by protecting membranes from lipid oxidation (8, 21). Oleuropein may prevent the free radical generation by its ability to chelate metal ions, such as Fe, and Cu which catalyze free radical formation reactions, as well as its ability to inhibit some inflammatory enzymes, such as lipoxygenases, without affecting the cyclo-oxygenase pathway (20). In addition, it was found that oleuropein was capable of



**Fig. 3.** Western blot analysis of Bax protein (B), Bcl-2 protein (C) and Bax/Bcl-2 ratio (A) in GP-293 cells exposed to cisplatin and cisplatin plus effective dose of oleuropein for 24h. Data are expressed as mean  $\pm$  SEM; n=6–8 wells for each group; \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001 versus control group.

preventing the generation of reactive oxygen species by intact leukocytes, without evidence of toxicity (22). It has been shown that oleuropein can scavenge the superoxide anions and inhibit the respiratory burst of neutrophils resulted from hypochlorous acid-derived radicals (22). In addition, oleuropein scavenged hydroxyl radicals (23). Oleuropein was also reported to be potent scavengers of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical (20).

## Conclusion

The results obtained from this investigation support the idea that oleuropein have potentially beneficial effects on Cisplatin induced apoptosis and cellular toxicity. This beneficial effects, at least part, is possibly due to the antioxidant property of oleuropein. Further basic and clinical studies is essential to examine the safety profile of various doses of oleuropein, and more basic scientific studies are needed to improve our knowledge of the

molecular mechanisms underlying any therapeutic efficacy of this bioflavonoid compound

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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