European Review for Medical and Pharmacological Sciences 2017; 21: 890-899

Licorice and its active compound glycyrrhizic acid ameliorates cisplatin-induced nephrotoxicity through inactivation of p53 by scavenging ROS and overexpression of p21 in human renal proximal tubular epithelial cells

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Abstract. – OBJECTIVE: Nephrotoxicity is one of the major side effects that limit the use of cisplatin in cancer therapy. Cisplatin-induced apoptosis in renal cells is associated with reactive oxygen species (ROS)-mediated p53 activation. Licorice (*Glycyrrhiza uralensis* Fischer) is one of the most widely used medicinal herbs in Korea, China and Japan. The aim of the study was to evaluate the protective effects of licorice extract (LE) and its active compound glycyrrhizic acid (GA) against cisplatin-induced nephrotoxicity in human renal proximal tubular epithelial (HK-2) cells.

MATERIALS AND METHODS: HK-2 cells were pretreated with LE or GA for 1 h and then treated with 40 μM of cisplatin for indicated times under the serum-free condition. Cell viability was evaluated by MTT assay. Apoptosis was evaluated by flow cytometric analysis and caspase-3 activity. The intracellular ROS levels were determined by DCFH-DA assay. The expression and phosphorylation levels of protein were evaluated by Western blot and densitometry analysis.

RESULTS: When treating HK-2 cells with LE or GA, both of them alleviated cisplatin-induced cytotoxicity and apoptosis. LE and GA inhibited caspase-3 activity and polymerase (PARP) cleavage in cisplatin-treated cells. LE and GA also inhibited p53 expression and its phosphorylation as well as ROS production in cells exposed to cisplatin. Meanwhile, LE and GA enhanced cisplatin-induced p21 expression, which then led to S-phase arrest in cell cycle and limited cell growth. Presumably, increased p21 expression may contribute to cellular prevention from cisplatin-induced apoptosis, because p21 is the key molecule to cytoprotection during cisplatin-induced nephrotoxicity.

CONCLUSIONS: These results suggest that LE and GA ameliorate cisplatin-induced apoptosis through reduction of ROS-mediating p53 activation and promotion of p21 expression in HK-2 cells.

Key Words:
Cisplatin, Glycyrrhizic acid, HK-2 cells, Licorice, Nephrotoxicity, p21, p53, ROS.

Introduction

Cisplatin (*cis*-dichlorodiammine platinum (II)) is a chemotherapeutic agent widely used for the treatment of human cancers[1,2]. However, the chemotherapeutic use of cisplatin is limited by serious side effects, of which nephrotoxicity is the most important[3-6]. Cisplatin nephrotoxicity has been shown to be related to accumulation of cisplatin in kidney, because kidney is a major route of excretion of cisplatin[7]. Cisplatin nephrotoxicity is a complex multifactorial process, of which reactive oxygen species (ROS) and p53 tumor suppressor protein are important factors causing cisplatin nephrotoxi-

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city[8]. Previous studies[9,10] have shown that cisplatin induces oxidative stress by generating ROS in renal cells, which leads to apoptotic cell death through p53 activation. NAC (N-acetyl-cysteine), a scavenger of ROS, and pifithrin-α, a pharmacological inhibitor of p53, attenuated cisplatin-induced p53 activation and subsequent induction of apoptosis in renal cells. Thus, the inhibition of p53 activation may be an important target to alleviate cisplatin nephrotoxicity.

Licorice is derived from the roots of *Glycyrrhiza uralensis* Fisch and has been used in herbal medicine for treatment of sore throat, cough, bronchitis, peptic ulcers, arthritis, and allergic diseases[11]. The bioactive constitutes of licorice are saponins, flavonoids, coumarins, alkaloids, polysaccharides, sitosteron, and aminoacids[12]. Among them, glycyrrhizic acid, a triterpenoid saponin glycoside, is known as the most efficacious composition of licorice[13]. Glycyrrhizic acid, also called glycyrrhizin, consists of one molecule of 18 β-glycyrrhetic acid as aglycon and two molecules of glucuronic acid[13]. Glycyrrhizic acid has antiallergic, antiviral, and anti-inflammatory activities and it is also widely used as a sweetening and flavoring agent in food[14,15]. Several researches[16-18] have reported that licorice and glycyrrhizic acid protect kidney against cisplatin-induced nephrotoxicity. Interestingly, licorice and glycyrrhizic acid exert renoprotective effect against cisplatin-induced oxidative stress through its antioxidative properties[16-18]. Although their antioxidative action may contribute to renal protection against cisplatin nephrotoxicity, the underlying molecular mechanisms remain unclear.

In this study, we investigated the protective effects of licorice and its active compound glycyrrhizic acid against cisplatin-induced nephrotoxicity in human renal proximal tubular epithelial (HK-2) cells and identified the possible molecular mechanism of actions underlying this effect.

Materials and Methods

Regents and Antibodies

cis-Dichlorodiammine platinum (II) (Cisplatin), 2',7'-dichlorofluorescin diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT), glycyrrhizic acid, propidium iodide (PI) and ribonuclease A (RNase A) were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/F12, fetal bovine serum (FBS), trypsin-EDTA and antibiotic-antimycotic solutions were purchased from GIBCO (Grand Island, NY, USA). RIPA lysis buffer, halt protease and phosphatase inhibitor cocktail were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Colorimetric caspase-3 assay kit was purchased from Abcam (Cambridge, MA, USA). Anti-cleaved caspase-3 (p20), anti-PARP, anti-CDK2, anti-p21, anti-p53 and anti-phospho-p53 (Ser15) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-mouse IgG secondary antibodies were purchased from Invitrogen (Carlsbad, CA, USA).

Preparation of Licorice Extract

The roots of licorice (*Glycyrrhiza uralensis* Fischer) were purchased from Gwangmyung Natural Pharmaceutical Co. (Busan, Korea). A voucher specimen (DP-2016-GU) has been deposited in the Department of Pathology, College of Korean Medicine, Wonkwang University. An aqueous extract was prepared by boiling 100 g of licorice with 1 L of distilled water for 2 h and then centrifuged at 2,000 rpm for 15 min to remove the insoluble ingredients. The supernatant was filtered through Whatman No. 4 filter paper in a Buchner funnel under vacuum and stored at -20°C for overnight. Frozen extract was freeze-dried in a vacuum chamber. The yield of extract was 28.12% (w/w).

Cell Culture and Treatment

HK-2 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and routinely cultured in DMEM/F12 (1:1) supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in a humidified 5% CO_2 incubator. HK-2 cells were seeded in a complete growth medium in culture dishes, at a density of 1×10^5 cells/ml for 24 h before treatment with chemicals. The cells were pretreated with different concentrations of licorice extract or glycyrrhizic acid for 1 h, followed by treating with or without 40 μM cisplatin for indicated times under serum-free condition.

Cell Viability Assay

Cell viability was determined by the colorimetric MTT assay. HK-2 cells were seeded into 24-well plates at 5×10^4 cells/well. After treatment,

the cells were incubated with 0.5 mg/ml of MTT in serum-free medium for 4 h at 37ºC. The purple MTT-formazan crystals were dissolved in DMSO and then measured the amount of formazan at 570 nm using SpectraMAX 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Caspase-3 Activity Assay

The activity of caspase-3 was determined by colorimetric caspase-3 assay kit according to the manufacturer's protocol. Briefly, the cells were incubated with cell lysis buffer on ice for 10 min and centrifuged at 10,000 rpm for 1 min. The supernatants were incubated with reaction buffer and 4 mM DEVD-p-NA substrate at 37ºC for 2 h in the dark condition. The colorimetric release of p-nitroaniline from DEVD-p-NA substrate was measured at 405 nm using SpectraMAX 250 microplate reader.

Flow Cytometric Analysis

Apoptosis and cell cycle progression were monitored by quantitating cellular DNA content after staining with PI. The cells were fixed with ice-cold 70% ethanol for 1 h, washed with phosphate buffered saline (PBS) (pH 7.4) and then incubated with 10 μg/ml PI solution containing 100 μg/ml RNase A at 37ºC for 1 h in the dark condition. DNA contents were analyzed using FACSCalibur flow cytometer and CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

Reactive Oxygen Species Production Assay

The relative levels of reactive oxygen species were (ROS) were determined by DCFH-DA fluorescence assay. The cells were seeded into 96-well microplates (1×10^4 cells/well) for 24 h and then incubated in HBSS (pH 7.4) solution containing 25 μM DCFH-DA at 37ºC for 1 h. After incubation, the cells were washed with HBSS (pH 7.4) and treated with 40 μM cisplatin under HBSS (pH 7.4) for the indicated time periods. Relative DCF fluorescence intensity was determined with the excitation wavelength of 485 nm and emission wavelength of 538 nm using SpectraMAX M2 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western Blot Analysis

The cells were lysed in RIPA lysis buffer containing 1% halt protease and phosphatase inhibitor cocktail. Cell lysates were centrifuged at 14,000 rpm in a refrigerated microcentrifuge for 15 min, and then protein concentrations of supernatants were determined using a Bradford assay (Bio-Rad, Hercules, CA, USA). An equal amount of total protein samples were separated by SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane. After transfer, nonspecific binding sites were blocked 5% non-fat dry milk in TBS containing 0.05% Tween-20 (TBS-T). The membranes were once washed with TBS-T, probed with primary antibodies (all 1:1000 in 3% BSA in TBS-T) overnight at 4°C, and finally incubated with HRP-conjugated secondary antibodies (all 1:2000 in 3% BSA in TBS-T). The protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Inc. Waltham, MA, USA) with the FluorChem E-System (ProteinSimple, San Jose, CA, USA). Densitometric analysis was performed by using Quantity One software (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

All data are presented as means ± standard deviation (SD) of at least three independent experiments. Comparisons between two groups were performed by Student's t-test in Microsoft Office Excel (Microsoft, Redmond, WA, USA). A p-value of less than 0.05 was considered statistically significant.

Results

Effects of Licorice Extract and Glycyrrhizic Acid on Cisplatin-Induced Cytotoxicity

To determine whether licorice extract (LE) and glycyrrhizic acid (GA) have cytotoxicity to HK-2 cells, their effects on cell viability were evaluated using MTT assay. The inhibitory effects of LE (50-400 μg/ml) or GA (1-10 μM) alone on cell viability after 24 h treatment were not significant (Figure 1A). We next examined whether the LE and GA could have the cytoprotective effects of LE and GA against cisplatin-induced cytotoxicity of HK-2 cells. The cells were incubated with 40 μM of cisplatin for 24 h after treatment with different concentrations of LE or GA and then examined for cell viability and percentage of apoptotic cells. Cell viability was significantly increased a dose-dependent manner after 24 h of co-treatment with LE or GA (Figure 1A). Treatment with both LE or GA also showed a significant reduction of percentage of apoptotic cells (Sub-G1 peak) in

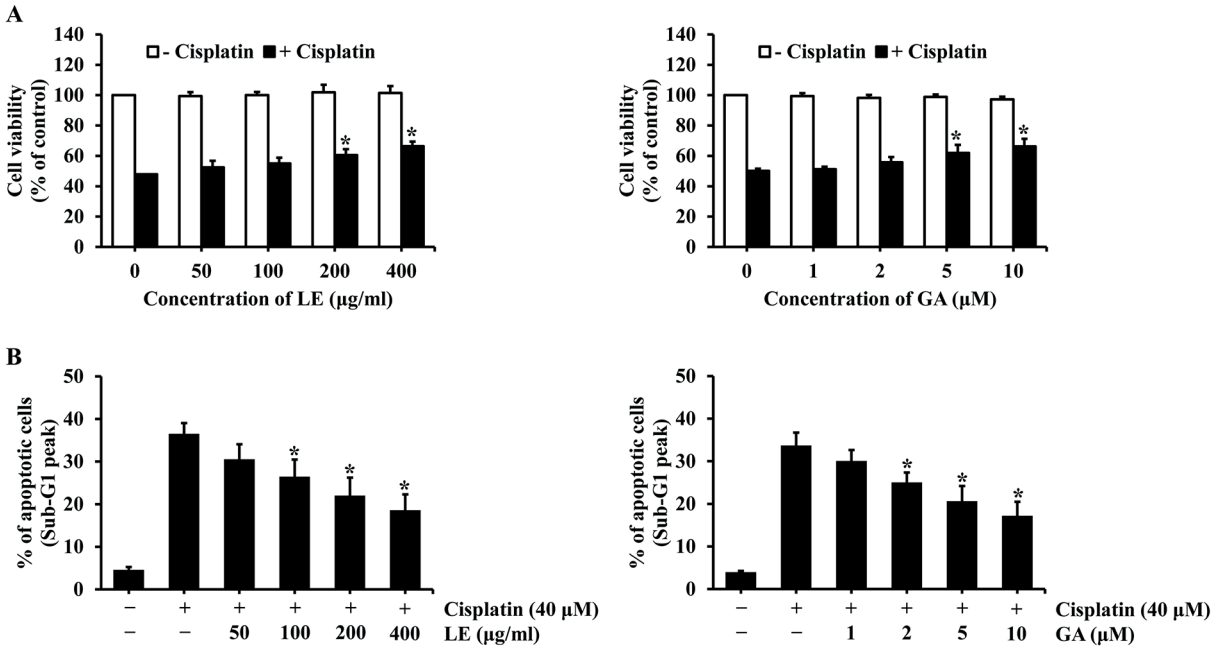

Figure 1. The effects of licorice extract and glycyrrhizic acid on cisplatin-induced cytotoxicity in HK-2 cells. ***(A)*** The cells were incubated with different concentrations of LE or GA in the absence or presence of 40 μM of cisplatin for 24 h. Cell viability was measured by MTT assay and expressed as percentage of cell survival in drug-treated cells relative to untreated cells. ***(B)*** The cells were pretreated with different concentrations of LE or GA for 1 h and then exposed with 40 μM of cisplatin for 24 h. Apoptotic cells were quantified on the PI histogram as a hypodiploid peak (Sub-G1). Data are the means of three different experiments. $^{*}p<0.05$ vs. cisplatin-treatment alone.

cells exposed to cisplatin as compared to cells treated with cisplatin alone (Figure 1B). These results indicate that LE and GA inhibit cisplatin-induced cytotoxicity in HK-2 cells.

Effects of Licorice Extract and Glycyrrhizic Acid on Cisplatin-Induced Apoptotic cell Death

To determine whether LE and GA have anti-apoptotic effects on cisplatin-induced apoptosis of HK-2 cells, the cells were incubated with 40 μM of cisplatin for 24 h after treatment with different concentrations of LE or GA. Then, the cells were examined for the activation of caspase-3, which is a key protein in execution of apoptosis[19], and cleavage of poly (ADP-ribose) polymerase (PARP), which is a well-characterized substrate of caspase-3[20]. As shown in Figure 2, the pretreatment with LE or GA significantly reduced the activity of caspase-3 and the levels of cleaved caspase-3, which is activated form of caspase-3, in cells exposed to cisplatin. The inhibitory effect of LE and GA on cisplatin-induced caspase-3 activity was evidently shown at concentrations up to 100 μg/ml and 2 μM, respectively (Figure 2A). Similarly, LE and GA reduced proteolytic cleavage of PARP to form an 89-kDa fragment (Figure 2B). These results indicate that LE and GA protect HK-2 cells against apoptotic cell death induced by cisplatin.

Effects of Licorice Extract and Glycyrrhizic acid on Cisplatin-Induced ROS Production and p53 Activation

Cisplatin induces oxidative stress by generating ROS in renal cells, which leads to the activation of apoptotic pathways[9,10]. Licorice has been reported to have antioxidant activity[21]. Antioxidant activity of licorice is mainly attributed to its potent antioxidants such as glycyrrhizic acid and flavonoids[21]. Therefore, we examined the scavenging effects of LE and GA treatments on cisplatin-induced ROS production of HK-2 cells. The cells were incubated with 40 μM of cisplatin for indicated times after treatment with LE or GA, and the intracellular ROS levels were then measured. As shown in Figure 3A, the cisplatin-induced generation of ROS was significantly suppressed by pretreatment with LE and GA.

Because cisplatin induces apoptotic cell death through ROS-mediated p53 activation in renal

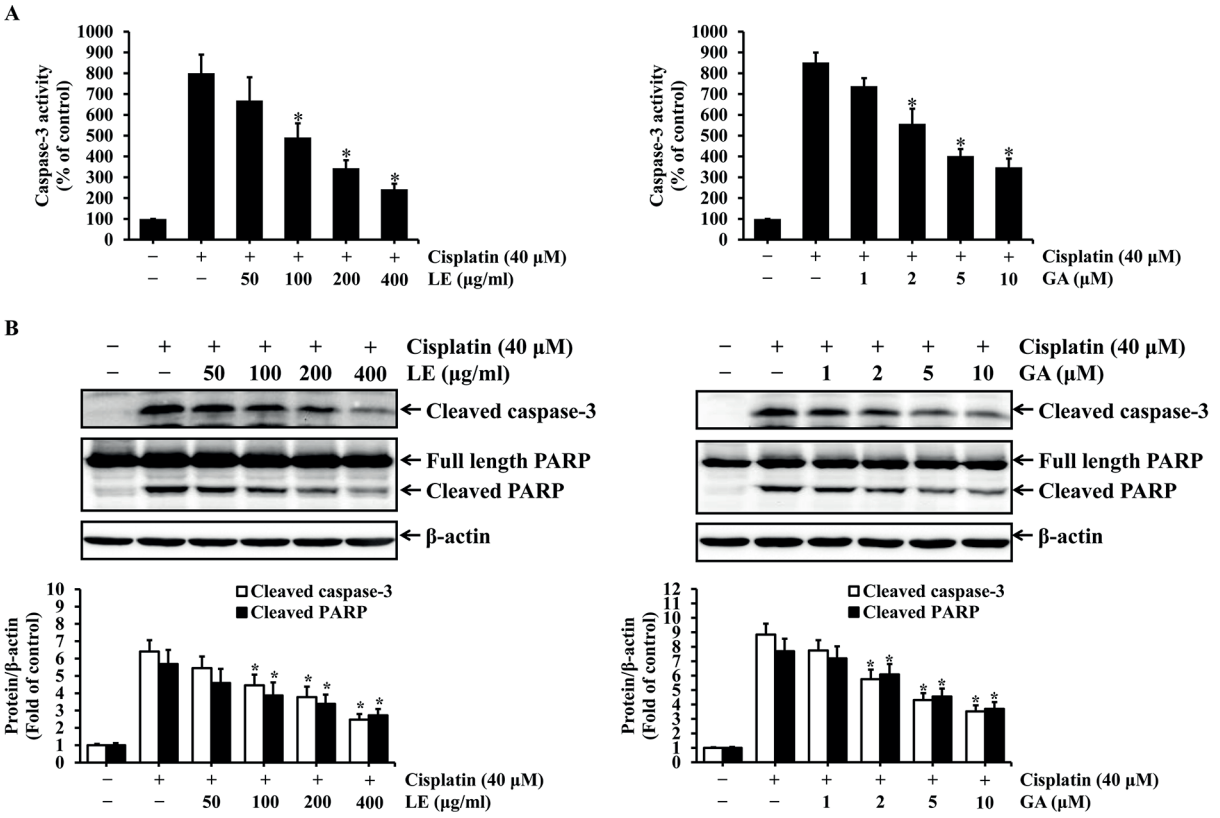

Figure 2. The effect of licorice extract and glycyrrhizic acid on cisplatin-induced caspase-3 activation and PARP cleavage in HK-2 cells. The cells were pretreated with different concentrations of LE or GA for 1 h and then exposed to 40 μM of cisplatin for 24 h. ***(A)*** Caspase-3 activity was measured using caspase-3 colorimetric assay kit. ***(B)*** The levels of cleaved caspase-3 and PARP were examined by Western blot analysis using anti-cleaved caspase-3 (p20) and anti-PARP antibodies. The density of the protein band was expressed as the ratio to β-actin protein. Data are the means of three different experiments. $^{*}p<0.05$ vs. cisplatin-treatment alone.

cells[9,10], we next examined whether ROS-scavenging activity of LE and GA could affect the ROS-dependent activation of p53 in HK-2 cells. As shown in Figure 3B, the expression of p53 and its phosphorylation, which are directly associated with p53 activation, were markedly reduced by pretreatment with LE or GA in cells exposed to cisplatin. These results indicate that LE and GA can attenuate cisplatin-induced apoptotic cell death, at least in part, by inactivation of p53 through scavenging ROS in HK-2 cells.

Effects of Licorice Extract and Glycyrrhizic Acid on Cell Cycle Progression and Expression of p21 and Cyclin-Dependent Kinase 2

Cisplatin-induced apoptosis in HK2-cells was attenuated by both LE and GA, but the levels of cell viability in LE or GA plus cisplatin-treated cells were lower than expected. It is assumed that LE and GA, together with their cytoprotective effects, may also induce growth inhibition, which is an essential requirement for cell cycle progression, in cisplatin-treated HK-2 cells. To validate the growth inhibitory effects of LE and GA on HK-2 cells exposed to cisplatin, we examined the cell cycle distribution of HK-2 cells treated with cisplatin in the presence of LE or GA. The cells were incubated with 40 μM of cisplatin for 24 h following pretreatment with LE or GA, and DNA content was then measured. As shown in Figure 4A, the distributions of S-phase in LE or GA plus cisplatin-treated cells were 18.4% and 18.1%, respectively, which was higher than estimated in untreated and cisplatin-treated cells.

Treatment with both LE and GA resulted in S-phase arrest in HK-2 cells exposed to cisplatin. Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), the activity of which is regulated by cyclins and CDK inhibitors[22]. Particularly, the S-phase progression requires CDK2, which can be inhibited by the

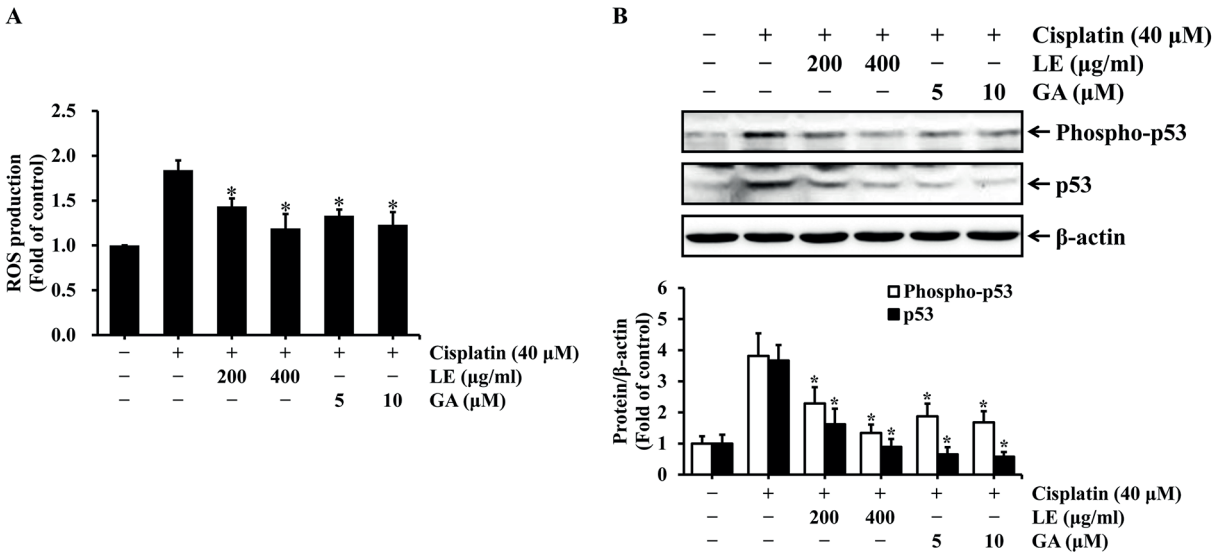

Figure 3. The effect of licorice extract and glycyrrhizic acid on cisplatin-induced ROS production and p53 activation in HK-2 cells. The cells were pretreated with different concentrations of LE and GA for 1 h and then exposed to 40 μM of cisplatin for 4 h (ROS production assay) or 8 h (Western blot analysis). ***(A)*** The intracellular ROS production was measured using DCFH-DA fluorescence dye. ***(B)*** The levels of p53 phosphorylation and expression were examined by Western blot analysis using anti-phospho-p53 (Ser15) and anti-p53 antibodies. The density of the protein band was expressed as the ratio to β-actin protein. Data are the means of three different experiments. $^{*}p<0.05$ vs. cisplatin-treatment alone.

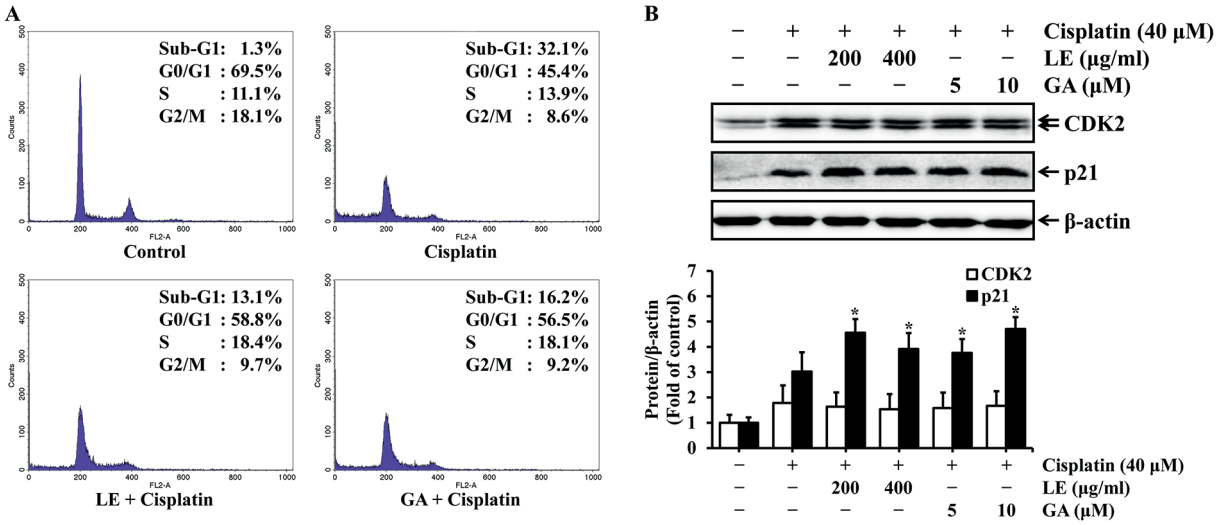

Figure 4. The effect of licorice extract and glycyrrhizic acid on cell cycle progression, and expression of CDK2 and p21 in cisplatin-treated HK-2 cells. ***(A)*** The cells were pretreated with different concentrations of LE or GA for 1 h and then exposed to 40 μM of cisplatin for 24 h. Cell cycle distribution was determined using flow cytometry with PI staining. ***(B)*** The expression levels of CDK2 and p21 were examined by Western blot analysis using anti-CDK2 and anti-p21 antibodies. The density of the protein band was expressed as the ratio to β-actin protein. Data are the means of three different experiments. $^{*}p<0.05$ vs. cisplatin-treatment alone.

CDK inhibitor p21[23]. Accordingly, we examined the expression levels of CDK and p21 in LE or GA plus cisplatin-treated HK-2 cells. As shown in Figure 4B, LE or GA co-treatment induced overexpression of p21 in cisplatin-treated cells, with no significant change in the expression level of CDK2. These results indicate that LE and GA can induce S-phase arrest by overexpression of p21 in HK-2 cells exposed to cisplatin

Discussion

Nephrotoxicity is one of the main side effects that limit the therapeutic efficacy of the anticancer drug cisplatin[1-6]. Cisplatin nephrotoxicity is mainly associated with an increase in oxidative stress, which ultimately leads to renal cell death and irreversible kidney dysfunction[10]. Licorice is one of the most widely used herbal medicines and has anti-inflammatory, anti-viral, anti-microbial, and antioxidative properties[24]. Glycyrrhizic acid is the main biologically active component of licorice and is found to exhibit potent antioxidant activity[21]. Several studies[16-18] have shown that licorice and glycyrrhizic acid have a protective effect against cisplatin-induced nephrotoxicity by their antioxidant activity. However, it is unclear how their antioxidant activity can alleviate cisplatin nephrotoxicity. The p53 can be activated by ROS during cisplatin nephrotoxicity and contributes to renal cell injury and death, so scavenging ROS with NAC could prevent cisplatin-induced p53 activation and subsequent induction of apoptosis in renal cells[9,10]. In the present study, we demonstrated for the first time one of the protective mechanisms behind the protective effects of licorice and glycyrrhizic acid against cisplatin-induced apoptosis in human renal proximal epithelial cells. Licorice and glycyrrhizic acid inhibited the cisplatin-induced ROS production and p53 activation, and consequently inhibited caspase-3 activation and PARP cleavage in HK-2 cells. These results indicate that licorice and glycyrrhizic acid ameliorate cisplatin-induced nephrotoxicity in HK-2 cells, at least in part, through inactivation of p53 by decreasing ROS production. In addition to their anti-apoptotic effects, both licorice and glycyrrhizic acid also have anti-proliferative effects on HK-2 cells exposed to cisplatin.

Licorice and glycyrrhizic acid have limited benefit to minimize loss of cell viability despite their renoprotective effect on cisplatin-induced apoptosis of HK-2 cells. Thus, it is most likely that LE and GA may induce growth inhibition, which is associated with cell cycle arrest, in cisplatin-treated HK-2 cells. Our data show that licorice and glycyrrhizic acid induced S-phase arrest caused by enhancing p21 expression in HK-2 cells exposed to cisplatin. Generally, cell cycle progression is controlled by changes in CDK activity, which is in turn regulated by several cyclins and CDK inhibitors[22]. Particularly, S-phase progression is directed by CDK2/Cyclin A complex, which can be inhibited by p21[23]. Cell cycle-related proteins are major molecular regulators of renal cell death and survival during cisplatin nephrotoxicity[25]. Interestingly, the balance between p21 and CDK2 is important in cisplatin nephrotoxicity; while p21 plays a cytoprotective role, CDK2 promotes renal cell death during cisplatin nephrotoxicity[26-29]. Licorice and glycyrrhizic acid induced overexpression of p21 in HK-2 cells exposed to cisplatin, but did not affect the expression level of CDK2. Also, licorice and glycyrrhizic acid increased p21 expression in cisplatin-treated cells, despite the decreased expression level of p53. Generally, p21 expression is regulated by p53-dependent and p53-independent mechanisms[30]. This means that expression of p21 induced by licorice and glycyrrhizic acid was independent of p53 in HK-2 cells exposed to cisplatin. These results suggest that licorice and glycyrrhizic acid may inhibit cisplatin-induced apoptosis in HK-2 cells by overexpression of p21 other than inactivation of p53 through scavenging ROS.

On the other hand, licorice or glycyrrhizic acid might interfere with the therapeutic efficacy of cisplatin because p21 plays a key role in the protection against cisplatin nephrotoxicity, but p21 is also associated with cisplatin resistance in cancer cells[26,27,31]. Lee et al[16] have reported that the combination of the licorice and cisplatin diminish the therapeutic efficacy of cisplatin in mouse colon carcinoma. In our study, p21 expression was only induced when licorice and glycyrrhizic acid were combined with cisplatin in HK-2 cells. Therefore, it is assumed that the interference of licorice in the therapeutic efficacy of cisplatin may be associated with p21 expression.

Conclusions

Licorice and its active compound glycyrrhizic acid ameliorate cisplatin-induced nephrotoxicity in HK-2 cells. Also, we have identified potential mechanisms by which licorice and glycyrrhizic acid may inhibit cisplatin-induced nephrotoxicity through overexpression of p21 other than inactivation of p53 by scavenging ROS. On the other hand, licorice or glycyrrhizic acid might diminish the therapeutic efficacy of cisplatin because p21 is associated with cisplatin resistance in cancer

cells. Consequently, further studies are required to explore in more detail the interaction between cisplatin and licorice or glycyrrhizic acid, particularly in light of the possible consequences for cancer therapy.

Acknowledgment
This study was supported by Wonkwang University in 2015.

Conflict of interest
The authors declare no conflicts of interest.

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