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Icariin ameliorates cisplatin-induced cytotoxicity in human embryonic kidney 293 cells by suppressing ROS-mediated PI3K/Akt pathway

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ABSTRACT

Cisplatin, as an effective chemotherapeutic agent, is widely used to treat verious types of cancers. Nephrotoxicity induced by cisplatin seriously limits its clinical application. Icariin, a major and remarkable flavonoid isolated from *Epimedium koreanum*, has been reported to exert anti-oxidative stress and anti-inflammation actions. The purpose of this study is to explore the protective effect and possible mechanism of icariin on cisplatin-induced nephrotoxicity on HEK-293 cells. In this study, icariin pretreatment for 24 h significantly ameliorated cisplatin-induced oxidative stress by reducing levels of malondialdehyde (MDA) and reactive oxygen species (ROS), while increasing level of glutathione (GSH) in HEK-293 cells. Furthermore, icariin pretreatment reduced NF-κB phosphorylation and nuclear translocation in HEK-293 cells followed by decreased secretion of IL-1β, TNF-α, and iNOS, suggesting a suppression of inflammatory response. Moreover, icariin pretreatment significantly reduced cellular apoptosis via reduced levels of Bax, cleaved caspase-3/9, and increased anti-apoptotic protein Bcl-2 in the cells. Importantly, LY294002, a specific PI3K inhibitor, abrogated the anti-apoptosis effect of icariin, implicating the involvement of PI3K/Akt pathway. In summary, icariin prevents cisplatin-induced HEK-293 cell injury by inhibiting oxidative stress, inflammatory response, and cellular apoptosis partly via regulating NF-κB and PI3K/Akt signaling pathways. Icariin may serve as a potential therapeutic target against cisplatin-induced nephrotoxicity.

1. Introduction

Cisplatin is one of the widely used antineoplastic drugs with a wide spectrum in clinical use [1] and represents one of the common anti-tumors drugs to treat diverse malignancies, such as ovarian cancer, bladder tumor, and esophageal carcinoma [2]. Therapeutic effects of cisplatin are dose dependent. Nevertheless, the significant adverse reactions of cisplatin are associated with high dose cisplatin therapy including renal damage, gastrointestinal dysfunction, auditory toxicity, peripheral nerve toxicity and kidney dysfunction [3]. Previous studies have shown that accumulation of cisplatin in tubular cells was 5 times more than that in other tissues, suggesting a major and severe nephrotoxicity [4]. Existing data revealed that oxidative stress, inflammation and apoptosis have been considered as potential mechanisms underlying nephrotoxicity caused by cisplatin-regimen [5]. Cisplatin-induced oxidative stress is an unbalanced redox reaction in kidney cells, resulting in the production of reactive oxygen species (ROS) [6]. However, overproduction of ROS may lead to lipid peroxidation and delayed renal damage [7]. In addition, numerous studies have shown that activated NF-κB may activate many pro-inflammatory cytokines such as TNF-α and IL-1β, and induce inflammatory mediators such as iNOS and COX-2 [8,9]. Furthermore, cisplatin can elevate the level of ROS, which leads to cell injury and mitochondrial dysfunction [10,11]. When cisplatin induces generation of ROS, pro-apoptotic proteins are activated to induce Bax translocation to mitochondrial outer membrane, releasing the cytochrome c into the cytosol [12].

Abbreviations: HEK-293, Human embryonic kidney 293; ROS, Reactive oxygen species; IL-1β, Interleukin-1β; iNOS, Inducible nitric oxide synthase; MDA, Malondialdehyde; GSH, Glutathione; TNF-α, Tumor necrosis factor-α; NF-κB, Nuclear factor-kappa B

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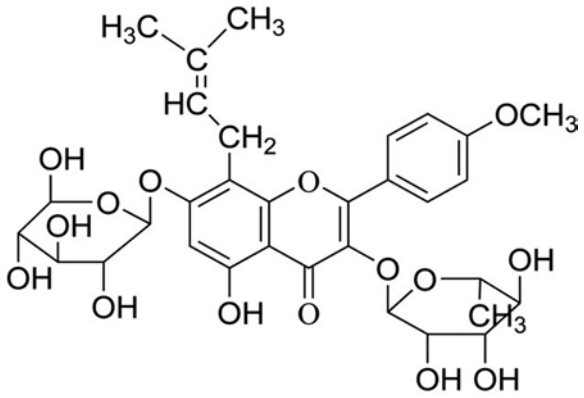

Fig. 1. Chemical structures of icariin.

Upon receiving apoptosis signal from Bax, caspases 9 is initiated and activates downstream caspase-3 to cause apoptosis [13]. More importantly, excessive ROS would damage cellular macromolecules, for example proteins, lipids and DNA, through regulation of signaling pathways such as PI3K/Akt pathway and NF-κB pathway [5,14]. It has been reported that LY294002, an inhibitor of the PI3K/Akt pathway, obviously increased the cytotoxicity of cisplatin in HK-2 cells by enhancing its induction of apoptosis [14].

Epimedium koreanum, is a well-known traditional Chinese herbal medicine. Icariin (Fig. 1), a major flavonoid responsible for the pharmacological actions of Epimedii Herba, was found to be increased significantly after the heating process [15]. Icariin has been shown to have a wide range of pharmacological and biological activities, including anti-neuronal injury [16], promotion of synaptic growth and osteogenesis [17], anti-inflammation, anti-tumor [18], and anti-depression effects [19]. In addition, a report by Deng et al. indicated that icariin had a protective effect on IL-1β-induced human nucleus pulposus cells, and the PI3K/Akt pathway was involved in this effect [20].

Here we have tested the protective effect of icariin on cisplatin-induced nephrotoxicity in HEK-293 cells by inhibition of oxidative stress *via* reducing production of ROS and anti-oxidative enzymes, suppression of inflammatory response *via* deactivation of NF-κB phosphorylation, and alleviation of cell apoptosis *via* restoring PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Sample and reagents

Epimedii Herba (2.0 kg) was purchased from a regular herbal company and identified as *Epimedium koreanum* by Professor Wei Li from the College of Chinese Medicinal Materials of Jilin Agricultural University. Acetonitrile (chromatographic purity) was purchased from TEDIA Corporation. The reference standard including epimedin A, epimedin B, epimedin C, ans icariin (the purities of them > 98.0%) was provided by Shanghai Yuanye Biotechnology Co, Ltd. (Shanghai, China). Cisplatin and Dimethyl sulfoxide (DMSO) were purchased from Sigma Chemicals (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotic (10000U/mL penicillin and 10,000 μg/mL streptomycin) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Biotopped Technology Co., Ltd. (Beijing, China). MDA and GSH assay kits were purchased from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). TNF-α and IL-1β ELISA kits were purchased from the R&D company (Minneapolis, MN, USA). BCA protein assay kit and Hoechst 33258 dye kit were bought from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The rabbit monoclonal anti-mouse PI3K, p-PI3K, Akt, p-Akt, NF-κB p65, p-NF-κB p65, Bax, Bcl-2, caspase-3/9, cleaved-caspase-3/9 and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). iNOS, TNF-α and IL-1β antibodies were obtained from Wanlei Biotechnology Co., Ltd. (Shenyang China). LY294002 was purchased from MedChemExpress Biotech (New jersey, USA). The remaining reagents were analytical pure, and provided by Beijing Chemical Works (Beijing, China).

2.2. Sample preparation and HPLC analysis

According to the proportion prescribed by the Chinese Pharmacopoeia (material ratio 5:1), *Epimedium koreanum* were mixed with suet oil under temperature 90℃ for 10 min and fried the *Epimedium koreanum* to green. Processed and unprocessed sample of *Epimedium koreanum* weighted 50 g were extracted with 500 mL of 70% ethanol for three times by ultrasonic-assisted technique. The filtrates were combined and evaporated under reduced pressure, followed by freeze-drying to obtain powders. Total flavonoids in *Epimedium* were analyzed using HPLC analysis on a Waters e2695 system with UV detector. HPLC analyses were performed through a Hypersil ODS2 column (250 × 4.6 mm, 5 μm) possessed with the chromatographic condition as follows: column temperature of 30 ℃, flow rate of 1.0 mL/min, detection at 270 nm, and the mobile phase of acetonitrile (A) and water (B). A programmed gradient eluting was used as follows: 0 ~ 10 min, 20% A; 10 ~ 35 min, 35% A; 35 ~ 55 min, 55% A; 55 ~ 65 min, 20% A. The chromatographic peaks of four flavonoids were confirmed by their retention times compared to the reference standards containing icariin, epimedin A, epimedin B, and epimedin C. Quantification was performed by the integration of the peaks using the external standard method. The content of individual flavonoid was expressed as mg/g. The determination of each sample was repeated 6 times. The icariin was prepared using the processed *Epimedium* under water and acetonitrile (v/v, 73:27). Fig. 2 shows chromatograms of *Epimedium*.

2.3. HEK-293 cells culture

HEK-293 cells were obtained from the ATCC Cell Bank. The HEK-293 cells were maintained in DMEM medium supplemented with 10% FBS, 100 μg of streptomycin/mL and 100 U of penicillin/mL in humidified atmosphere of 5% CO_2 at 37℃. For passage, the medium was changed every 48 h until the cells became 80 ~ 90% confluent.

2.4. Cells viability assay (MTT assay)

Cell viability was assessed by a quantitative colorimetric assay with MTT. Cells were seeded into 96-well plates and cultured at 37℃ for 24 h, cells were pretreated with various concentrations of icariin for 24 h, and then exposure to cisplatin (20 μM) or not for 24 h. After treatment, 20 μL MTT solution (5 mg/mL) was added to the wells and incubated at 37 ℃ for 3.5 h. Then, 150 μL DMSO was added to each well, and the plates were agitated for 5 min. Finally, the absorbance was measured at 490 nm using a microplate reader (Nano, Germany). The intensity of the color produced was proportional to the number of living HEK-293 cells.

2.5. Oxidative stress biochemical assays

The HEK-293 cells were suspended in 12 mL of fresh medium and seeded at 100 000 cells per well in six-well culture plates, then incubated for 24 h. The study design was to pretreat HEK-293 cells with icariin for 24 h, then treat with or without cisplatin for 24 h. After treatments, cells were scraped, pelleted and re-suspended in 1.0 mL of 100 mM phosphate buffer(pH 7.4). Cell membranes were disrupted and

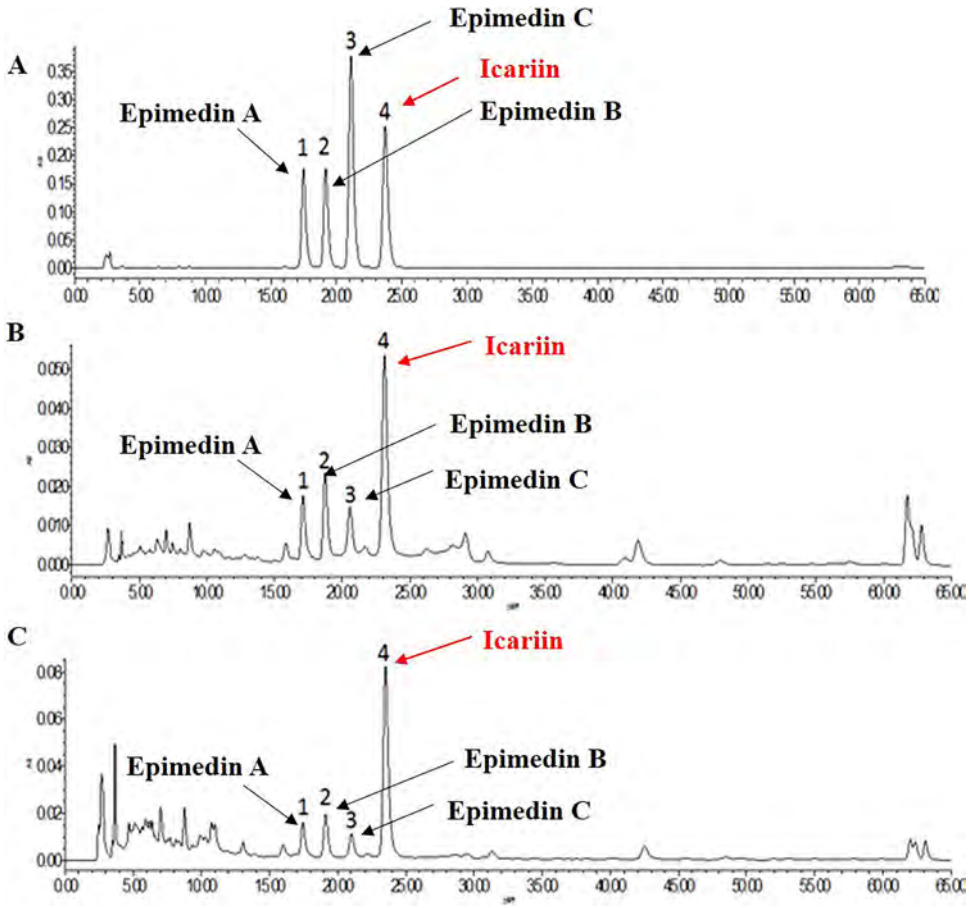

Fig. 2. HPLC chromatogram of standard solution **(A)**. HPLC profiles of raw *Epimedium koreanum* **(B)** and heat-processed *Epimedium koreanum* at 90°C for 10 min **(C)**. (1) Epimedin A, (2) Epimedin B, (3) Epimedin C, (4) Icariin.

the cell lysates centrifuged at 4°C, 10,000 *g*, for 20 min. The supernatant was separated from cell debris and used for subsequent biochemical assay measurements. All measurements were normalized to the protein content of the cell lysates.

2.6. Inflammatory cytokine assays

After experimental treatments, cell membranes were disrupted and the cell lysates centrifuged at 4°C, 10,000 *g*, for 20 min. The supernatant was separated from cell debris by a tissue tearor and used for subsequent measurements. The contents of TNF-α and IL-1β in the supernatant of cells were determined using ELISA assay kits according to the manufacture's protocols (R&D, Minneapolis, MN, USA). The absorbance was measured at 450 nm by ELISA reader (Bio-Rad, California, USA).

2.7. ROS staining

The relative levels of intracellular ROS were determined by a fluorometric assay (DCF-DA assay, Wanlei Biotechnology, Shenyang China) according to the manufactures' protocols. HEK-293 cells were seeded in 6-well microplates, treated HEK-293 cells were incubated with 1 μM DCFH-DA at 37°C for 12 h. Then the medium was removed and cells were washed by PBS twice prior to imaging. Relative DCF fluorescence intensity of treated cells was expressed as a percentage of cisplatin-induced group (Leica TCS SP8, solms, Germany).

2.8. Hoechst 33258 staining

The HEK-293 cell were stained using the Hoechst 33258 solution with 10 μg/mL as described previous. The slides were visualized using a Leica microscope (Leica TCS SP8, solms, Germany). Image-Pro plus 6.0 software (Media Cybernetics, Maryland, and USA) was applied to evaluate the degree of HEK-293 cell apoptosis through quantifying the fragmented and condensed staining.

2.9. Western blotting analysis

Western blot analysis was performed as described previously [21]. Protein samples from HEK-293 cell were separated by 12% SDS-PAGE gel and electrophoretically transferred to PVDF membranes, and then the membranes were blocked with 5% non-fat milk for 3.5 h and incubated with primary antibodies including Bax (1:2000), Bcl-2 (1:2000), caspase-3/9 (1:1000), cleaved-caspase-3/9 (1:1000), PI3K (1:1000), p-PI3K (1:1000), Akt (1:1000), p-Akt (1:1000), INOS (1:500), TNF-α (1:500), IL-1β (1:500), NF-κB (p65) (1:1000), p-NF-κB (p-p65) (1:1000) and GAPDH (1:1000) overnight at 4°C. After that, the membranes were incubated with the secondary antibodies at room temperature for 1 h. Signals were detected using Emitter Coupled Logic (ECL) substrate (Pierce Chemical Co., Rockford, IL, USA). Protein band intensities were analyzed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

2.10. Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The data were analyzed using a two-tailed test or a one-way analysis of variance (ANOVA). GraphPad Prism 6.04 software (GraphPad Software, La Jolla California, USA) was used to create the resulting data charts. The p values less than 0.05 or 0.01 were considered as statistically significant differences.

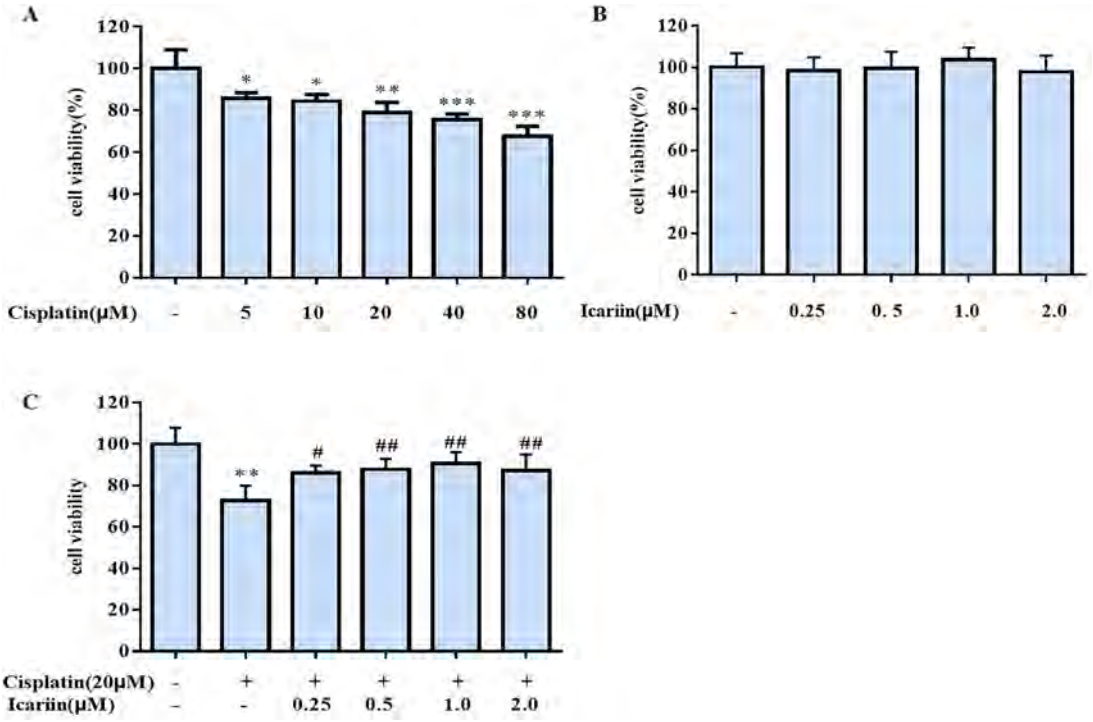

Fig. 3. Effects of different concentration of Icariin or cisplatin on HEK-293 cells viability. The cell viability was assessed by MTT assay. **(A)** HEK-293 cells were treated with cisplatin (0–80 μM). **(B)** HEK-293 cells treated with Icariin (0–2.0 μM). **(C)** HEK-293 cells treated with Icariin and cisplatin (0, 0.25, 0.5, 1.0, 2.0 μM). Data are means ± SD (n = 6). *$p < 0.05$, **$p < 0.01$ *vs.* the control group ***$p < 0.001$ *vs.* the control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* the cisplatin-treated group.

3. Results

3.1. Icariin protected cisplatin-induced cell damage on HEK-293 cell viability

To evaluate the effect of cisplatin or icariin on HEK-293 cells, cell viability was determined using MTT assay. As shown in Fig. 3A, cisplatin exposure for 24 h reduced HEK-293 cell viability in a dose-dependent manner. Compared with normal control group, the experimental groups (5, 10, 20, 40, and 80 μM of cisplatin) exerts significantly statistical differences on cell viability ($p < 0.05$ or $p < 0.01$ or $p < 0.001$). According to the results, 20 μM ($p < 0.01$) was chosen for the subsequent experiments. In Fig. 3B, the result shows that icariin did not affect cell viability in the dose range from 0.25 to 2.0 μM, indicating no toxicity on HEK-293 cells at this dose range. As shown in Fig. 3C, compared with normal control group, icariin with concentration range from 0.25 to 2.0 μM exerted obvious protective effect on cell viability in a dose-dependent manner ($p < 0.05$ or $p < 0.01$).

3.2. Icariin attenuated oxidative stress of HEK-293 cells induced by cisplatin treatment

The effects of icariin on cisplatin-induced oxidative stress were shown in Fig. 4A and B. In the present study, we examined the levels of GSH and MDA in HEK-293 cells. GSH is one of the most important endogenous antioxidants protecting cells against oxidative stress damage. Cisplatin induced a significant reduction in GSH level compared to the normal group ($p < 0.01$). Meanwhile, as a lipid peroxidation marker, the MDA levels were markedly increased after cisplatin exposure ($p < 0.01$). More interestingly, compared to the cisplatin group, icariin at indicated dose range inhibited the overproduction of MDA and increased the GSH level, respectively ($p < 0.05$ or $p < 0.01$).

The degree of oxidative stress after cisplatin exposure was also determined by intracellular ROS. The accumulation of intracellular ROS was detected by fluorescein-labeled dye, DCFH-DA. HEK-293 cells were treated with different concentrations of icariin in the presence of cisplatin and measured for fluorescent intensity. Cells treated with cisplatin resulted in a significant increase in intracellular ROS generation. However, pretreatment with icariin for 24 h reduced the intracellular ROS levels caused by cisplatin exposure. Icariin pretreatments at 0.25, 0.5, and 1.0 μM significantly reduced the production of ROS by 34.11%, 72.14%, and 84.63%, respectively (Fig. 4C and D) ($p < 0.05$ or $p < 0.01$).

3.3. Icariin inhibited inflammation response after cisplatin treatment

To verify whether cisplatin-induced nephrotoxicity was associated with the onset of inflammation, we measured the contents of inflammatory cytokines and mediators by Western blot and ELISA assay kits, such as iNOS, NF-κB, TNF-α and IL-1β. As shown in Fig. 5A-C, cisplatin treatment group resulted in increased protein expressions of iNOS, p-NF-κB, TNF-α and IL-1β in HEK-293 cells, while the administration of icariin at the dose range dramatically reduced these protein expression levels by cisplatin ($p < 0.05$ or $p < 0.01$).

In addition, we measured the levels of TNF- α and IL-1β in culture medium using ELISA kits. As shown in Fig. 5D and E, there were significant increases in TNF- α and IL-1β levels in cisplatin administered groups ($p < 0.01$). Pretreatment with icariin obviously decreased the levels of TNF-α and IL-1β in the cells ($p < 0.05$ or $p < 0.01$).

3.4. Icariin inhibited HEK-293 cell apoptosis after cisplatin treatment

To investigate whether icariin decreased cell apoptosis induced by cisplatin, Hoechst 33258 staining was used to evaluate apoptosis degree of HEK-293 cells in this report. As described in Fig. 6A and B, obvious nuclear fragmentation and condensation of cells were observed in the cisplatin group. However, the cells in icariin pretreatment groups exhibited the regular homogeneous fluorescent intensity and normal

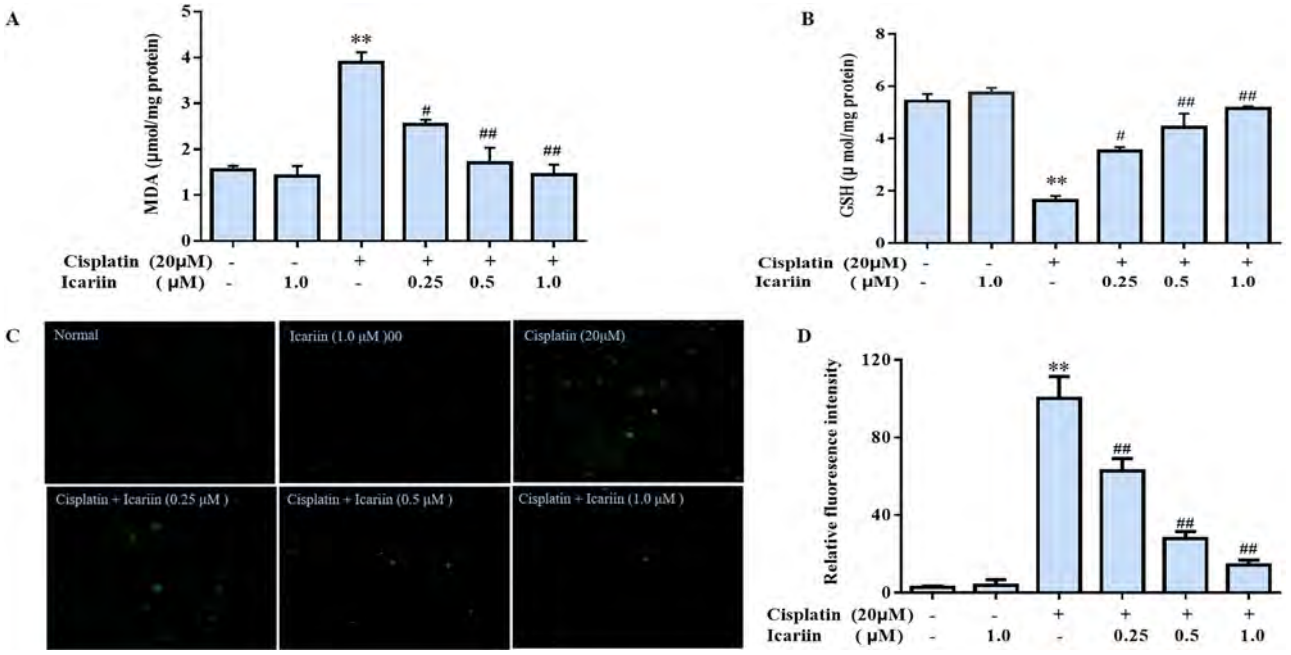

Fig. 4. Effects of different concentrations of Icariin against cisplatin-induced oxidative stress. Effects of icariin on the levels of **(A)** MDA and **(B)** GSH in HEK-293 cells. Effects of icariin with different concentrations on ROS generation in cisplatin-induced HEK-293 cells **(C)**. Data are means ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control group; $^{\#}p < 0.05$ or $^{\#\#}p < 0.01$ *vs.* cisplatin-treated group.

contours ($p < 0.05$ or $p < 0.01$).

To further measure the extent of apoptosis, we evaluated the expression levels of cleaved caspase-9/3, pro-apoptotic factor Bax and anti-apoptotic factor Bcl-2 in all experimental groups by Western blotting analysis. As shown in Fig. 6C-E, cisplatin significantly increased the protein expression levels of Bax and cleaved caspase-9/3,

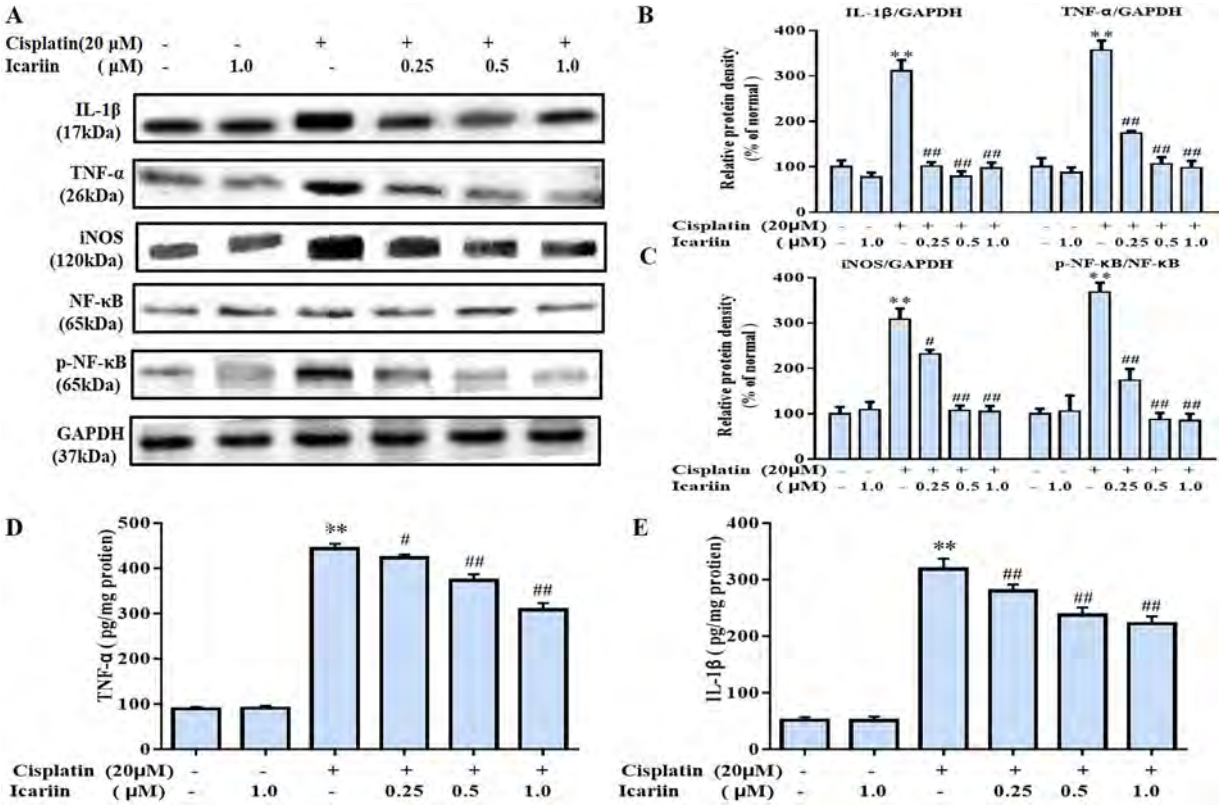

Fig. 5. Effects of Icariin against cisplatin-induced cytotoxicity by regulating inflammation response. The protein expressions of IL-1β, TNF-α, iNOS, NF-κB(p65) and p-NF-κB(p65) were measured by Western blotting with specific primary antibodies, and GAPDH protein level was used as a loading control (**A**). Quantification of relative protein expression was performed by densitometric analysis (**B** and **C**). Icariin decreased TNF-α (**D**) and IL-1β (**E**) levels in Cisplatin-induced cytotoxicity of HEK-293 cells. All data are expressed as mean ± SD (n = 3 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs.* cisplatin-treated group.

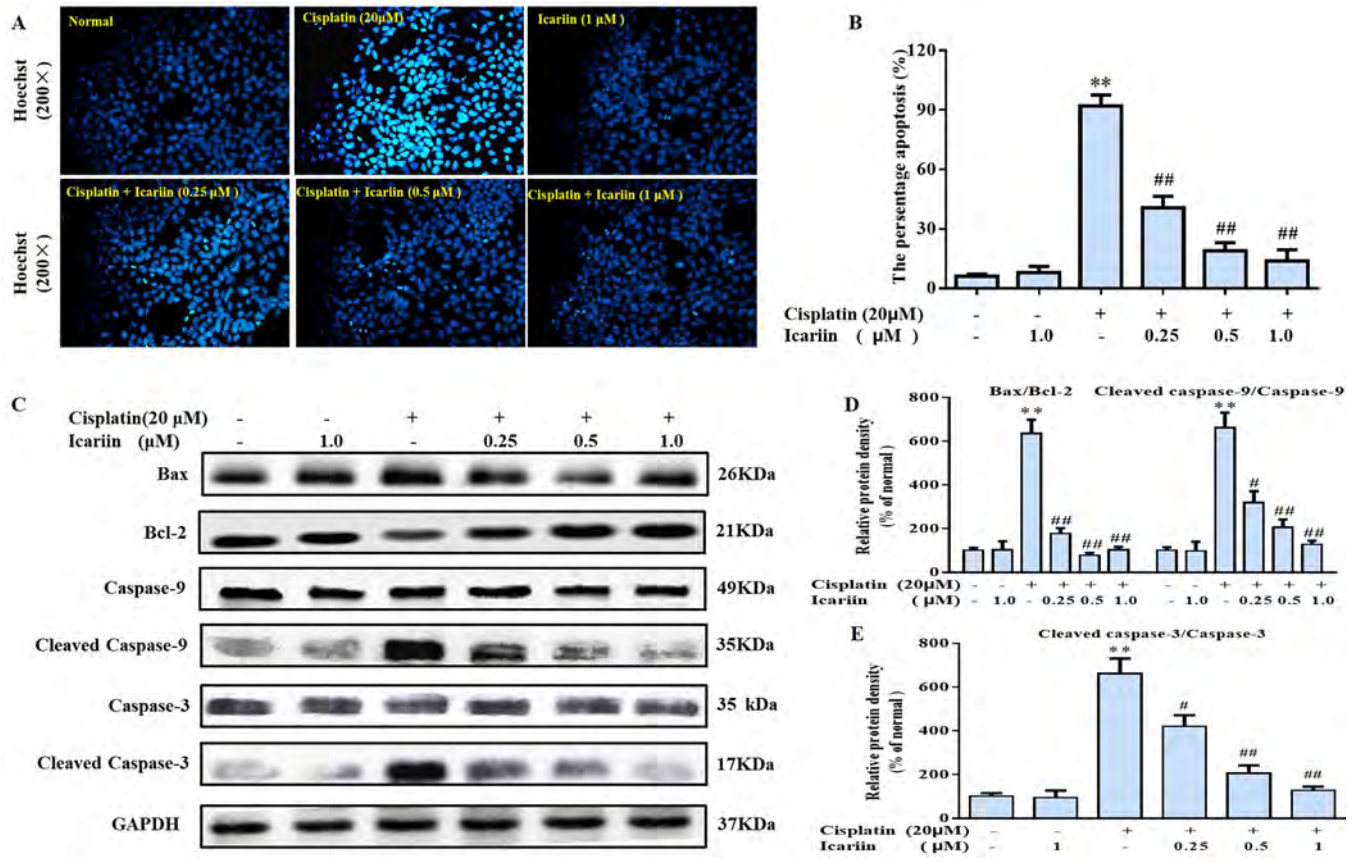

Fig. 6. Effects of Icariin against cisplatin-induced cytotoxicity by regulating apoptotic. HEK-293 cells were stained with Hoechst 33258 (200 ×) (**A**). In the Hoechst 33258 staining, white arrows indicate apoptotic cells. Percentage of Hoechst 33258 -positive (**B**). The protein expressions of Bax, Bcl-2, Cleaved-caspase-9/3 and Caspase-9/3, were measured by Western blotting with specific primary antibodies, and GAPDH protein level was used as a loading control (**C**). Quantification of relative protein expression was performed by densitometric analysis (**D** and **E**). All data were expressed as mean ± S.D., $n = 3$. * $p < 0.05$, ** $p < 0.01$ *vs*. control group; # $p < 0.05$, ## $p < 0.01$ *vs*. cisplatin-treated group.

while decreased the protein expression level of Bcl-2 ($p < 0.05$ or $p < 0.01$). However, different concentrations of icariin significantly increased expression level of Bcl-2 and decreased the expression levels of cleaved-caspase 9/3 and Bax in HEK-293 cells ($p < 0.05$ or $p < 0.01$).

3.5. Icariin activated the PI3K/Akt pathway that is inhibited by cisplatin

To verify the specific mechanism of cisplatin-induced apoptosis, LY294002, an inhibitor of the PI3K/Akt, was employed to treat the cells together with icariin. Treatment of cells with LY294002 (5 μM) prior to the addition of cisplatin and icariin, the cell viability was measured by MTT assay.

As shown in Fig. 7A, LY294002 significantly exacerbates the viability loss of cells exposed to cisplatin ($p <$ 0.05). As previously, icariin alleviated cisplatin-induced cytotoxicity; whereas addition of LY294002 significantly inhitited the protective effect of icariin (Fig. 7B and C) ($p <$ 0.05 or $p <$ 0.01). In addition, icariin pretreatment at a dose of 1.0 μM significantly increased Bcl-2 protein expresion, and significantly reduced Bax and cleaved caspase-3 protein expression as compared to that in the cisplatin group. And, the regulating effect of icariin was significantly inhibited by LY294002 (Fig. 7B and D) ($p <$ 0.05 or $p <$ 0.01). Overall, PI3K inhibitor LY294002 reversed the protective effects of icariin on cisplatin-stimulated HEK-293 cells. The results suggested that the antiapoptotic action of icariin might be mediated through PI3K/Akt signaling pathway.

4. Discussion

Cisplatin is one of the most effective and common used antineoplastic drug that is widely employed to treat patients with solid-organ cancers [22]. Nephrotoxicity is the major limitation in cisplatin based chemotherapy. Cisplatin treatment causes significant nephrotoxicity among other serious dose-limiting adverse effects [23]. The pathogenesis of cisplatin-induced nephrotoxicity involves the crosstalk of complex signal pathways leading to increased oxidative stress and inflammation in kidney during the treatment, as well as apoptosis of kidney tubular cells [5]. Thus far, there are very few effective approaches available to control cisplatin-induced nephrotoxicity.

In this study, we assessed the protective effects of icariin on cisplatin-induced nephrotoxicity using HEK-293 cell model. Results showed that icariin administration reduced cisplatin-induced oxidative stress, apoptosis and inflammation, which were implicated in the pathogenesis of renal dysfunction by cisplatin. The underlying molecular mechanism involved a reduction of apoptosis regulated by PI3K/Akt-mediated effector caspase-9/3 and Bcl-2 family proteins, a suppression of inflammatory factors including IL-1β, TNF-α, iNOS and an inactivation of NF-κB.

Oxidative stress has been known as an important factor to promote cisplatin-induced nephrotoxicity through increasing the accumulation of intracellular ROS [24–26]. Under normal physiological conditions, cells control ROS level through balancing the generation of ROS and their elimination by scavenging system [27]. Previous reports have displayed that cisplatin damages anti-oxidant defense mechanisms

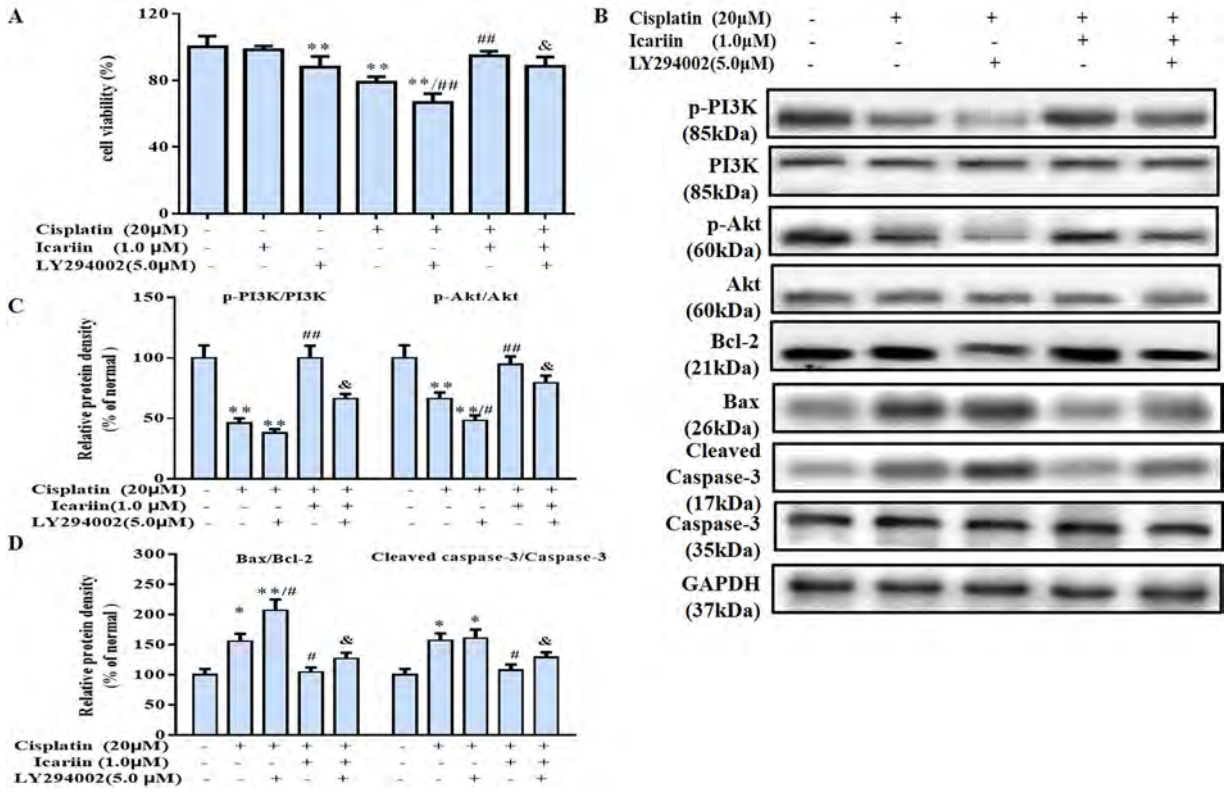

Fig. 7. LY294002 blocks the protective effect of Icariin against cisplatin-induced injury to HEK-293 cells. HEK-293 cells were treated with cisplatin, Icariin and LY294002. The HEK-293 cells were pretreated with LY294002 for 1 h and then incubated with 20 μM cisplatin for 24 h. Cell viability was assessed by MTT assay **(A)**. The protein expressions of PI3K, p-PI3K, Akt, p-Akt, Bax, Bcl-2, Cleaved-caspase-3, Caspase-3, were measured by Western blotting with specific primary antibodies, and GAPDH protein level was used as a loading control (**B**). Quantification of relative protein expression was performed by densitometric analysis (**C and D**). All data were expressed as mean ± S.D., $n = 3$. * $p < 0.05$, ** $p < 0.01$ *vs.* control group; $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ *vs.* cisplatin-treated group, $^{\&}$ $p < 0.05$, $^{\&\&}$ $p < 0.00$ *vs.* cisplatin and icariin treated group.

followed an apparent reduction in the level of GSH and elevation in MDA content [28]. In our report, supplementation with icariin evidently repressed the elevation of MDA and GSH contents in cisplatin-treated HEK-293 cells, respectively. In addition, icariin pretreatment significantly attenuated the increase in ROS levels by 15.37%. Collectively, icariin exerted significant anti-oxidative effect.

There is growing evidence showing that ROS-mediated apoptosis is one of the important pathological mechanisms of cisplatin-induced nephrotoxicity [5,7]. Excessive ROS accumulation would damage cellular macromolecules such as proteins through activation of a series of signaling cascades, including mitochondrial, apoptotic and PI3K/Akt pathways [27]. When cisplatin accumulates in the mitochondrial matrix, it causes a large amount of ROS production and mitochondrial dysfunction, leading to increased mitochondrial permeability, pro-apoptotic factor release and initiate apoptosis [29]. The Bcl-2 family of proteins has a crucial role in the apoptotic mechanism of mitochondrial pathway [30], which includes pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 [31]. Bax is capable of activating caspase-9, which is responsible for proteolytic activation of caspase 3 involved in the cleavage of a group of proteins. In contrast, Bcl-2 exerts its anti-apoptotic activity by inhibiting the translocation of Bax to mitochondria [32–34]. In this study, icariin markedly decreased the cellular apoptosis confirmed by Hoechst 33258 staining in comparison with cisplatin control group. We observed increases in Bax and cleaved caspase 9/3 and decrease in Bcl-2 expression levels in cisplatin group. Importantly, icariin effectively reversed above alterations on Bax, caspase 9/3 and Bcl-2, suggesting the protective effect of icariin on cisplatin-induced apoptosis.

In addition, ROS has been shown to play an important role in renal tubulointerstitial inflammation and activation of NF-κB [35]. Many studies have shown that the pathological process of cisplatin-induced renal cell apoptosis is related to the release of many inflammatory mediators such as TNF-α and IL-1β [36,37]. A study by Deng et al. has demonstrated that icariin has a significant anti-inflammatory effect [20]. Therefore, we hypothesized that icariin might exert anti-inflammatory effects on cisplatin-induced nephrotoxicity. As expected, the results showed that compared with the control group, pretreatment with icariin significantly decreased the expression levels of phosphorylated NF-κB and iNOS, TNF-α, and IL-1β proteins. In conclusion, these results suggest that icariin may act as an anti-inflammatory drug for cisplatin-induced nephrotoxicity.

To further analyze the specific protective mechanisms of icariin in cisplatin-induced cytotoxicity, we analyzed the expressions of PI3K/Akt signaling pathway-related proteins in this experiment. The PI3K/Akt pathway plays a vital role in proliferation, aging, survival, and apoptosis [38]. Nevertheless, excessive activation of PI3K/Akt pathway may lead to neoplastic lesion [39]. Icariin stimulates the PI3K/Akt pathway to exert antiapoptotic action [40,41], antioxidative stress [42] and promotion of cell differentiation [43]. The protective effect of icariin against LPS-induced acute inflammatory responses is achived through PI3K/Akt signaling pathway [44]. A study has shown that 25-Hydroxyl-protopanaxatriol protected cardiomyocytes against H_2O_2-induced apoptosis, in which molecular mechanism of action was summarized as inhibition of pro-apoptotic protein Bax and activation of anti-apoptotic protein Bcl-2 *via* PI3K/Akt pathway [45]. Treatment with LY294002, a specific PI3K/Akt pathway inhibitor, abolished the protective effect of 25-Hydroxyl-protopanaxatriol [45]. Similarly, in our study, the PI3K/Akt pathway was inhibited after cisplatin exposure, and the combination of LY294002 and cisplatin enhanced this inhibition. Icariin treatment attenuated cisplatin-induced inhibition of PI3K/Akt pathway.

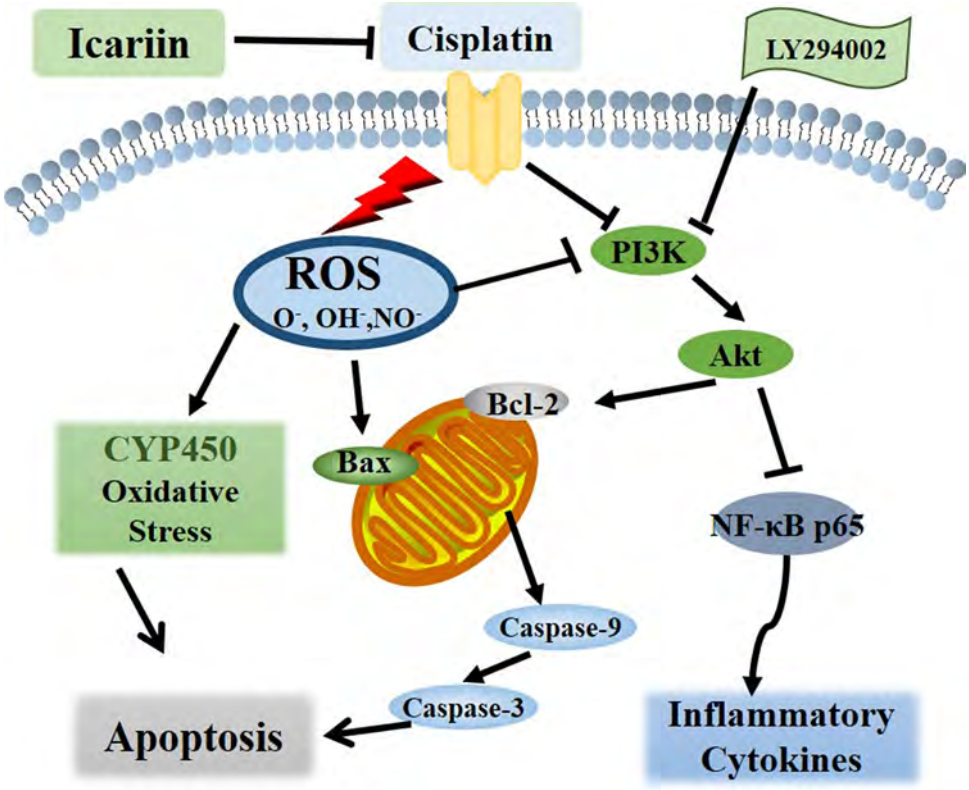

Fig. 8. The schematic diagram of molecular mechanism underlying ameliorative effects of icariin against cisplatin-induced cytotoxicity.

PI3K inhibitor LY294002 may reduce the protective effect of icariin.

In summary, we conclude that icariin has anti-oxidative stress, anti-inflammatory and anti-apoptosis effects. Main mechanism of action is mediated by ROS-mediated PI3K/Akt pathway (Fig. 8).

Conflicts of interest

The authors declare no conflict of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2018.11.108.

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