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Original Article

Procyanidin protects HK-2 cells against cisplatin-induced injury through antioxidant action involving Nrf2/HO-1 signaling pathway

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ABSTRACT — Cisplatin (CP) is used as a chemotherapeutic drug for the treatment of various kinds of cancer. However, it is becoming increasingly difficult to ignore its side effects, especially nephrotoxicity which has to do with oxidative stress and inflammation. Procyanidin (PRO) has been proved to be a powerful antioxidant. Therefore, we investigated whether PRO could prevent Cisplatin-induced nephrotoxicity and explored the underlying mechanism. In cellular experiment, reactive oxygen species (ROS), the malondialdehyde (MDA) levels, the activities of total superoxide dismutase (T-SOD) and glutathione peroxidase (GSH-PX) were measured for the assessment of cisplatin-induced oxidative cell damage. CCK-8 reagent and flow cytometry were used to detect the cell viability and apoptosis. Furthermore, the level of oxidative-related protein Nrf2 and HO-1 were carried out by western blot analysis. We found that cisplatin gave rise to the elevated levels of ROS and MDA and the decrease of the activities of T-SOD and GSH-PX with a related lower viability and higher apoptosis in HK-2 cells. Inversely, the pretreatment of PRO mitigated the oxidative damage, promoted the cell viability and lowered the apoptosis, activated nuclear related factor 2 (Nrf2) and elevated the expression of heme oxygenase-1 (HO-1), the above cytoprotection of PRO was blocked by siNrf2 or siHO-1. These results demonstrated that PRO has the potential to prevent cisplatin-induced nephrotoxicity through activation of Nrf2/HO-1 signaling pathway.

Key words: Procyanidin, Cisplatin, Antioxidant action, Nrf2/HO-1 signaling pathway

INTRODUCTION

Cisplatin is one of the most widely used effective drugs for the treatment of solid tumors, including lung, bladder, head and neck, ovarian, and testicular cancers (Fennell *et al*., 2016; Ecke, 2015; Rivelli *et al*., 2015; Bogliolo *et al*., 2015; Koster *et al*., 2013). However, a series of side effects limit its use in clinical applications such as nephrotoxicity, neurotoxicity, ototoxicity and hepatotoxicity *et al*. and the most well-known is the nephrotoxicity (Sooriyaarachchi *et al*., 2014; Hassan *et al*., 2013). Particularly, the nephrotoxicity induced by cisplatin was dose-dependent (Kidera *et al*., 2014). According to statistics, about one-third of patients have kidney damage during the use of cisplatin chemotherapy (Pabla and Dong, 2008). Indeed, even though some precautionary measures including hydration and diuretics can be taken to improve nephroprotection, there is a significant decline in glomerular filtration rate (GFR) after two cycles of chemotherapy based on cisplatin (Hartmann *et al*., 2000; Dugbartey *et al*., 2016). Therefore, treatment of cisplatin induced renal damage is a matter of great important.

The oxidative damage in kidney is well established although the exact mechanics of cisplatin induced neph-

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rotoxicity are not fully understood (Mohamadi Yarijani *et al*., 2018; Zhang *et al*., 2014). Numerous reports indicate that the pathophysiology is closely associated with proximal tubular injury, oxidative stress, inflammation, and kidney vascular injury, in which oxidative stress is considered to be a determinant factor (Ozkok and Edelstein, 2014; Kim *et al*., 2014; Wilmes *et al*., 2011). Reactive oxygen species (ROS) should assume the main responsibility for oxidative injury on account of attacking and modifying proteins, DNA and lipids and activating apoptotic pathway, which comes out of mitochondria, cytochrome P450 enzymes and xanthine oxidase (Oh *et al*., 2016). Moreover, some studies have shown that oxidative injury played an important role in cisplatin-induced hepatic injury (Gao *et al*., 2017) and testicular damage (Almeer and Abdel Moneim, 2018). Hence, decreasing oxidative stress seems to be essential for attenuating cisplatin induced renal damage.

Nuclear factor-erythroid 2-related factor 2 (Nrf2), an important transcription factor for cells to resist oxidative stress, up-regulates the expression of phase II detoxification enzymes and antioxidant proteins by binding to antioxidant response element (ARE) (Loboda *et al*., 2016). HO-1 is an important Nrf2-regulated antioxidant protein that regulates intracellular ROS levels in response to various stimuli. Previous studies have demonstrated that moringa oleifera leaves extract (Abdou *et al*., 2019) and xanthohumol (Li *et al*., 2018a) could mitigate the oxidative stress via Nrf2/HO-1 signaling pathway. Therefore, the activation of the Nrf2-mediated antioxidant defense system may be an efficient strategy for the prevention and therapy of cisplatin-induced renal damage.

Procyanidins are a family of the proanthocyanidin class of flavonoids. They are oligomeric compounds composed of epicatechin molecules and catechin. PRO is a widely-accepted natural antioxidant that is found in high concentrations in numerous plants such as apples, grapes, cocoa and green tea (Gonzalez-Abuin *et al*., 2015). PRO, as a powerful antioxidant, had been discovered to exert various therapeutic properties against cardiovascular system diseases (Terauchi *et al*., 2014; Ding *et al*., 2012), metabolic diseases (Luan *et al*., 2014; Yin *et al*., 2017) and neoplastic diseases (Mao *et al*., 2016; Dinicola *et al*., 2012; Cedo *et al*., 2014) and there are very few reports on the effects of PRO on cisplatin nephrotoxicity *in vitro*.

On account of its oxidative effects, therefore, we hypothesized that PRO could prevent cisplatin-induced renal damage. In this study, the oxidative effects of PRO against cisplatin and expression of Nrf2 and HO-1 were investigated in human proximal tubule (HK-2) cells. Our results revealed that PRO could prevent cisplatin-induced HK-2 cells injury by mitigating oxidative stress by activating nuclear translocation of Nrf2 and upregulating HO-1 expression.

MATERIALS AND METHODS

Cell Culture and Treatment

HK-2 cells, a human renal proximal tubule cell line, were purchased from the KeyGEN BioTECH (Nanjng, China), and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% heat-inactivated fetal bovine serum (both KeyGEN BioTECH), 0.365g/mL l-glutamine, 80 U/mL penicillin and 80 μg/mL streptomycin, in a 5% CO2 atmosphere at 37°C and subcultured every 2 days in the use of 0.25% trypsin supplemented with 0.53 mM EDTA (KeyGEN BioTECH). Our preliminary study showed that the CP-induced ROS production peaked at 2 hr, and subsequently decreased cell viability at 12 hr. Therefore, we evaluated cellular oxidative stress levels at 2 hr, cell viability and apoptosis at 12 hr after treatment with CP. Specific targeted siRNA (siNrf2 (Santa Cruz, CA, USA) and siHO-1 (Invitrogen, Carlsbad, CA, USA)) were used in this part. Our preliminary study showed the expression of Nrf2 and HO-1 were decreased to approximately 20% of that in the control siRNA-transfected HK-2 cells.

The groups were as follows: (1) control group: the cells were treated with Phosphate-buffered saline (PBS); (2) CP group: cells were treated with 50 μM CP (meilunbio, Dalian, China) for 2 hr or 12 hr; (3) PRO group: cells were treated with 40 μM PRO (meilunbio) for 1 hr; (4) PRO + CP group: cells were treated with 40 μM PRO for 1 hr and then treated with 50 μM CP for 2 hr or 12 hr; (5) siNrf2CTRL + PRO + CP group: Control siRNA were dissolved separately in Optimem I. After 10 min of equilibration at room temperature, each RNA solution was combined with the respective volume of the Lipofectamine 2000 solution, mixed gently and allowed to form siRNA liposomes for 20 min. The primary cultured cells were transfected with the transfection mixture in antibiotic-free cell culture medium for 6 hr, then the cells were treated with 40 μM PRO for 1 hr, finally treated with 50 μM CP for 2 hr or 12 hr; (6) siNrf2 + PRO + CP group: Nrf2 siRNA, PRO, and CP treated cells as above protocol. Vehicle also performed according to the experimental requirements; (7) siHO-1CTRL + PRO + CP group: In brief, the control siRNA was incubated with siRNA transfection reagent for 5 min at room temperature, and then the siRNA–reagent complex was added to the HK-2 cells. After 24 hr, the cells were treated with

40 μM PRO for 1 hr, finally treated with 50 μM CP for 2 hr or 12 hr; (8) siHO-1 + PRO + CP group: HO-1 siRNA, PRO, and CP treated cells as above protocol. Vehicle also performed according to the experimental requirements.

Measurement of cellular ROS levels

CM-H2DCFCA, a ROS sensitive fluorescent dye was used to measure ROS levels. To evaluate the effect of PRO on cisplatin-induced ROS production, HK-2 cells were cultured in 96-well plates. Cultures were incubated for 30 min at 35°C in the appropriate experimental medium containing CM-H2 DCFDA (10 μM). Fluorescence intensities were measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a microplate fluorescence reader.

Measurement of T-SOD and GSH-PX activities, as well as MDA levels

Cells were cultured in a 75 cm^2 flask and culture supernatant was used for the detection. The content of MDA was used to assess the oxidant levels of the HK-2 cells with an MDA assay kit (Jiancheng Bioengineering Institute, Nanjing, China) and activities of T-SOD and GSH-PX were measured to evaluate the antioxidants by using T-SOD and GSH-PX assay kits (Jiancheng Bioengineering). The measurement method is in strict accordance with the manufacturer's instructions.

Measurement of HK-2 cell viability

Cell viability was evaluated using the CCK-8 assay kit (KeyGEN BioTECH). In brief, HK-2 Cells were cultured in 96-well plates (approximately 1 × 104 cells/well) and incubated for 24 hr. We added 10 μL of CCK-8 reagent to each well and cells were incubated for 2 hr at 37°C. Measuring the optical density at 450 nm with a spectrophotometer.

Flow cytometry

Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime Institute of Biotechnology, Shanghai, China) was used for the detection of apoptotic HK-2 cells. Briefly, 195 μL Annexin V-FITC binding buffer was added to the cell pellet followed by 5 μL FITC-conjugated Annexin V and 10 μL propidium iodide (PI), then cells were incubated for 15 min at room temperature in the dark. Finally, the samples were analyzed with a flow cytometer.

Extraction of nuclear and cytoplasmic proteins

Cultured cells were harvested and used for the extraction of nuclear and cytoplasmic proteins using a nuclear/cytoplasmic protein extraction kit (Beyotime) according to the manufacturer's instructions. The protein concentrations were measured with a BCA Protein Assay Kit (Beyotime).

Western blot analysis

Equal amounts of protein lysates were separated by 12% SDS-PAGE gel and transferred onto polyvinylidene fluoride membranes. The membranes were blocked in blocking reagent (Beyotime) for 1 hr and then incubated overnight at 4°C with primary antibodies against Nrf2 (rabbit monoclonal 1: 1000; Abcam Inc., Cambridge, MA, USA), HO-1 (mouse monoclonal 1: 500; Abcam), Histone H3 (rabbit monoclonal 1: 500; Abcam) or β-actin (mouse monoclonal polyclonal 1: 1,000; Abcam). The membranes were then incubated with a mouse anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology Inc., Dallas, TX, USA) for 2 hr at room temperature followed by the detection with the enhanced chemiluminescent (Beyotime). The results were normalized to the expression of Histone H3 or β-actin.

Statistical Analysis

Data are expressed as the means ± SD. Difference of data was analyzed by single factor analysis of variance (ANOVA) with Tukey's multiple comparison (parametric tests) and considered significant when the $p < 0.05$.

RESULTS

PRO reduced the levels of oxidative stress through activation of Nrf2/HO-1

The effect of PRO on cellular ROS levels induced by cisplatin were examined using HK-2 cells. The cellular ROS production induced by cisplatin in the PRO + CP group were significantly lower than those in the CP group ($p < 0.01$) (Fig. 1(a)). MDA, as is well-known, is a major product of lipid peroxidation, which is widely used as a major marker for oxidative stress (Tongqiang *et al*., 2016). The MDA levels of HK-2 cells in the CP group were markedly increased compared to those in the control group ($p < 0.01$) (Fig. 1(b)). However, the MDA levels in the PRO + CP group were significantly decreased in comparison with those in CP group ($p < 0.01$) (Fig.1(b)). T-SOD and GSH-PX act as antioxidants and play an important role in protecting cells from oxidative damage. We found that the activities of T-SOD and GSH-PX were apparently decreased in the CP group when compared with the control group ($p < 0.05$) (Fig. 1(c) and 1(d)). However, the activities of T-SOD and GSH-PX in the

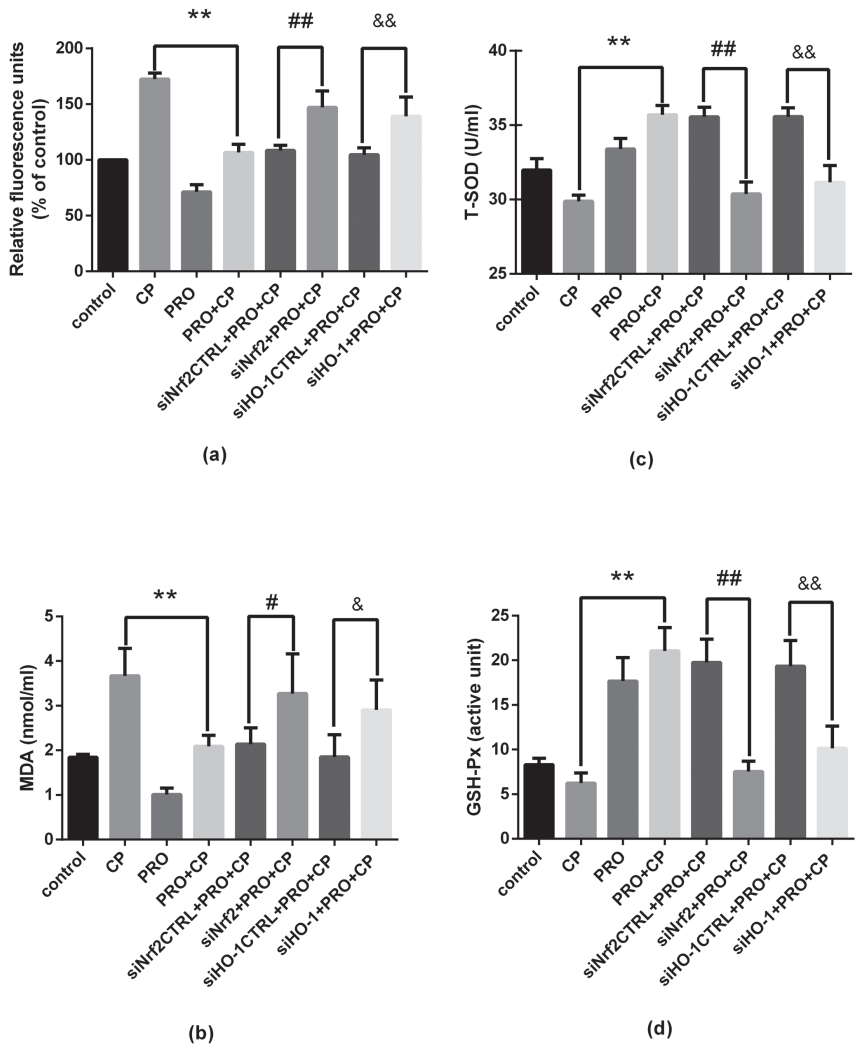

Fig. 1. Effects of PRO on oxidative stress levels of renal tubular epithelial cells injured with CP. Cells were transfected with siNrf2, siNrf2CTRL, siHO-1 or siHO-1CTRL siRNA. The levels of ROS and MDA and the activities of T-SOD and GSH-Px were determined by commercial assay kits. PRO decreased ROS(a) and MDA (b) production, and increased T-SOD (c) and GSH-Px (d) activity. (a, b, c, d) Knockdown of Nrf2 or HO-1 by siRNA reduces the positive effects of PRO against cisplatin-induced oxidative stress. Results were expressed as mean ± SD (n = 5). $^{**}p < 0.01$ versus the CP group; $^{\#\#}p < 0.01$ versus the siNrf2CTRL + PRO + CP group; $^{\#}p < 0.5$ versus the siNrf2CTRL + PRO + CP group; $^{\&\&}p < 0.01$ versus siHO-1CTRL+ PRO + CP group; $^{\&}p < 0.05$ versus the siHO-1CTRL + PRO + CP group.

PRO + CP group were significantly increased compared with the CP group ($p < 0.01$) (Fig. 1(c) and 1(d)). The findings showed that PRO exhibited a protective effect against CP-induced oxidative injury. HK-2 cells were transfected with siNrf2, siNrf2CTRL, siHO-1 or siHO-1CTRL siRNA, and then treated with PRO and CP. The results showed that the knockdown of Nrf2 or HO-1 partially reversed the inhibitory effects of PRO on the CP-induced increased oxidative stress and decreased antioxidant activity (Fig. 1(a), 1(b), 1(c) and 1(d)).

PRO-mediated HK-2 cell protection against CP-induced injury through the activation of Nrf2/HO-1

The cell viability in CP group was markedly decreased compared with that in control group ($p < 0.01$) (Fig. 2(c)), while the rate of apoptosis induced by CP was markedly increased ($p < 0.01$) (Fig. 2(a) and 2(b)). On the con-

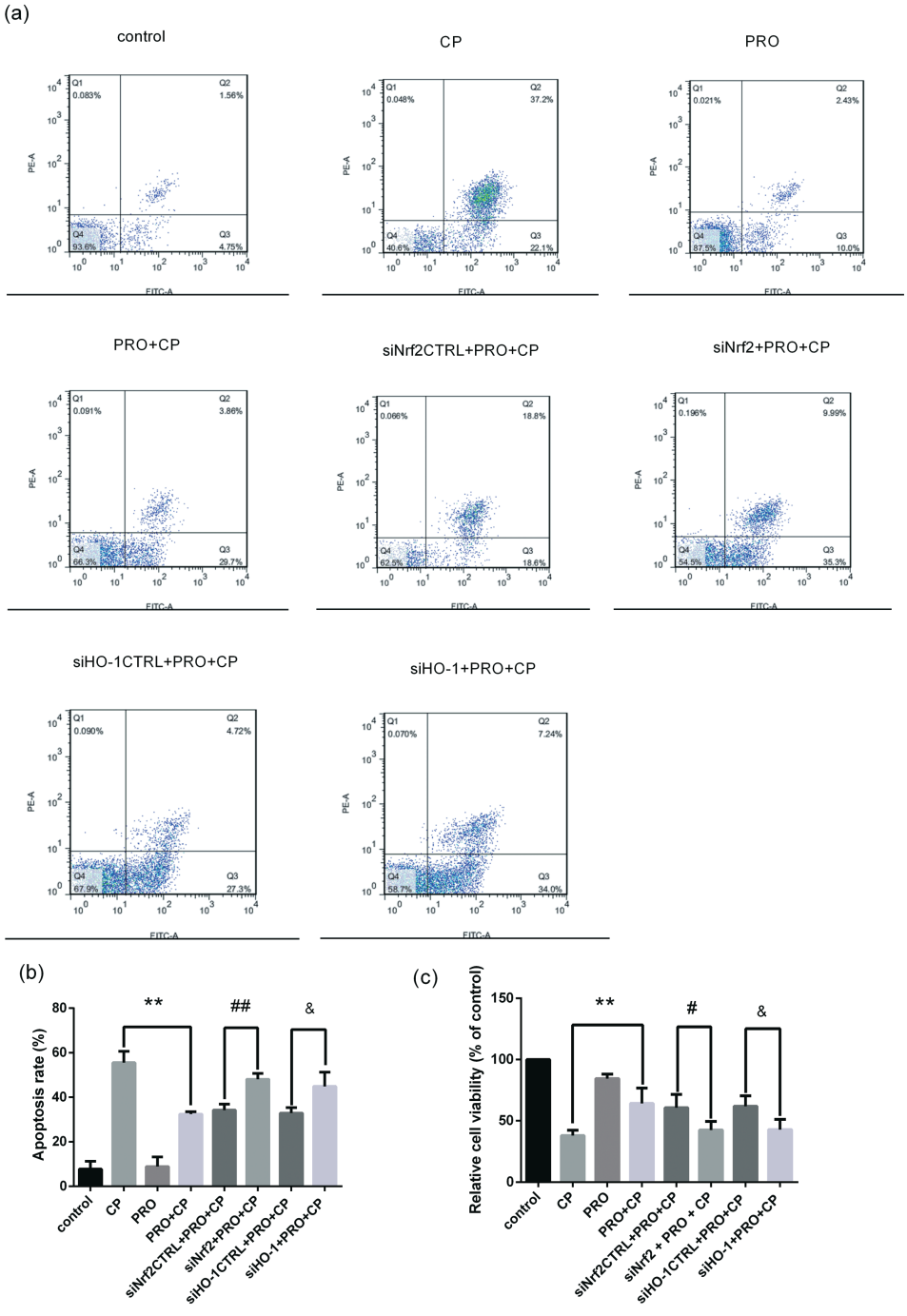

Fig. 2. Effects of PRO on apoptosis and viability of renal tubular epithelial cells injured with CP. (a, b) Flow cytometry to determine cell apoptosis and quantitative analysis of apoptotic cells. PRO significantly protected against cisplatin-induced cell apoptosis and the protective effect was reversed after cisplatin exposure in Nrf2-deficient or HO-1-deficient cells. (c) CCK-8 assay kit to evaluate cell viability. PRO markedly improved cell viability, but the supportive effect of PRO was blunted after cells were transfected with siNrf2 or siHO-1. Results were expressed as mean ± SD. $^{**}p < 0.01$ versus the CP group; $^{\#}p < 0.05$ versus the siNrf2CTRL + PRO + CP group; $^{\#\#}p < 0.01$ versus the siNrf2CTRL + PRO + CP group. $^{\&}p < 0.05$ versus the siHO-1CTRL + PRO + CP group.

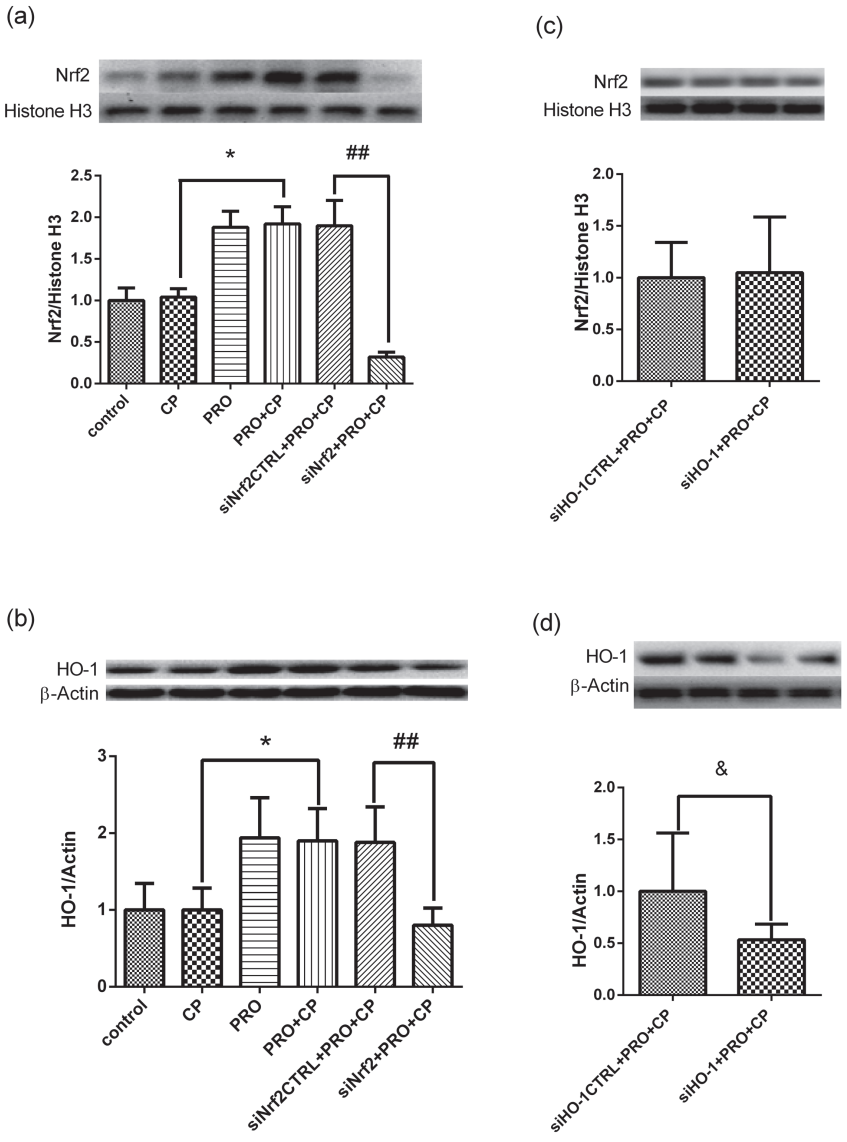

Fig. 3. Effects of PRO on Nrf2 and HO-1 expression of renal tubular epithelial cells injured with CP. The protein level and statistical data of nuclear Nrf2 (68KDa) and HO-1 (32KDa). The expression of Nrf2 was normalized to the Histone H3. The expression of HO-1 was normalized to the β-actin. Western blot analysis at 12 hr after being treated with cisplatin or vehicle. PRO could activate the expression of Nrf2 and elevate the expression of HO-1, which was suppressed by siNrf2. siHO-1 could only suppress the expression of HO-1 but not Nrf2. Results were expressed as mean ± SD. *$p < 0.05$ versus the CP group; ##$p < 0.01$ versus the siNrf2CTRL + PRO + CP group; &$p < 0.05$ versus the siHO-1CTRL + PRO + CP group.

trary, the cell viability in CP+PRO group was significantly increased compared with that in CP group ($p < 0.01$) (Fig. 2(c)), and the apoptosis rate was significantly decreased ($p < 0.01$) (Fig. 2(a) and 2(b)). The above findings indicated that PRO could protect against CP-induced cell apoptosis and enhance the cell viability. HK-2 cells were transfected with siNrf2, siNrf2CTRL, siHO-1 or siHO-1CTRL siRNA, and then treated with PRO and CP. The results showed that the knockdown of Nrf2 or HO-1 partially reversed the inhibitory effects of PRO on the CP-induced increased apoptosis rate and decreased cell viability (Fig. 2(a), 2(b) and 2(c)).

Effects of PRO on CP-induced HK-2 cell expression of nuclear-Nrf2 and HO-1

Only in the nuclear fraction, Nrf2 can exert antioxidative effects. As is shown in Fig. 3(a) and 3(b). The expression of nuclear-Nrf2 and HO-1 in PRO+CP group was markedly promoted compared with the group only treated with CP. Moreover, we found that the level of nuclear-Nrf2 and HO-1 in the group pretreated with PRO alone was also significantly increased. SiNrf2 significantly reversed PRO-induced protein expression of nuclear-Nrf2 and HO-1, but siHO-1 only reversed PRO-induced protein expression of HO-1 but not nuclear-Nrf2 (Fig. 3(c) and 3(d)). The above results suggested that PRO-induced antioxidative and cell-protective activity was mediated via the activation of Nrf2/HO-1 signaling pathway.

DISCUSSION

Cisplatin was first discovered by Michele Peyrone in 1845. Until he demonstrated the anti-tumor effect of cisplatin in a mice model of sarcoma (Rosenberg and VanCamp, 1970), it became the cornerstone of treatment of various cancers today. Among numerous solid tumors, the chemotherapy based on cisplatin is particularly effective against testicular cancer, which is the most common cause of death in male patients with cancer at the age of 20 to 40 (Koster *et al*., 2013). Many recent studies have reported that antioxidants including betaine supplementation (Hagar *et al*., 2015), S-allylcysteine (Gomez-Sierra *et al*., 2014), C-phycocyanin (Fernandez-Rojas *et al*., 2014) and vetiver oil (Sinha *et al*., 2015) have positive effects on renoprotection against cisplatin to some extent. However, there are no studies have been conducted to evaluate the effect of PRO on cisplatin-induced renal damage. The experiment revealed that PRO decreased the cell apoptosis and enhance the cell viability of renal tubular cells induced by cisplatin. Our research showed that PRO vastly reduced cisplatin-induced nephrotoxicity in HK-2 cells.

Nephrotoxicity is the major limitation of cisplatin chemotherapy and acute kidney injury is the most serious cisplatin-induced nephrotoxicity which occurs in 20-30% of patients (Oh *et al*., 2016). Our study revealed that, in line with previous studies, treatment with cisplatin lead to significant nephrotoxicity *in vitro* (Jin *et al*., 2015). Oxidative damage is a major factor in kidney injury induced by cisplatin. In vitro and *in vivo* studies have demonstrated that PRO has the potential to protect against oxidative stress (Kim *et al*., 2013; Chen *et al*., 2013). In agreement with previous researches, the experiment indicated that after being treated with PRO, the levels of ROS and MDA were suppressed, the activities of T-SOD and GSH-PX were promoted. The study showed that PRO exerted antioxidative effects to protect renal tubular cells against cisplatin-induced kidney injury.

Nrf2, a transcription factor falls within the cap 'n' collar (CNC) family, is a major regulator of the antioxidant cell response, regulating the expression of about 1055 genes which involves not only antioxidant defenses but also detoxifying responses and cell proliferation (Antunes Dos Santos *et al*., 2018). In ordinary circumstances, Nrf2 locates in the cytoplasm complexed with its repressor Kelch-like ECH-associated protein 1 (Keap1) at a relatively level. Once cells are exposed to oxidative stress, Nrf2 translocates into the nucleus and activates the transcription of genes containing ARE, such as heme oxygenase-1 (HO-1), glutathione S-transferase (GST) and thioredoxin reductase (TrxR). After being activated, the ARE-containing genes play their biologic role in antioxidant (Li *et al*., 2018b; Gan and Johnson, 2014; Tang *et al*., 2018). The significant effects of Nrf2 against oxidative stress-induced diseases have been verified. Wu J *et al*. found that the activation of Nrf2 could attenuate H_2O_2-induced acute lung injury model (Wu *et al*., 2018). Wang J *et al*. also discovered that the activation of Nrf2-mediated signaling pathway could protect the kidney function of diabetic nephropathy rats (Wang *et al*., 2018). In the present study, we found that PRO induced the activation of Nrf2 and led to a significant increase in HO-1 expression and decreased the levels of the oxidative stress. Furthermore, the knockdown of Nrf2 or HO-1 reversed its protective effects.

It is inspiring that our study reveals that PRO is closely related to cisplatin-induced renal damage. Moreover, the renoprotection relys on the activation of Nrf2. Thus we speculate that other Nrf2 activating agents, such as paeonin (Xiao *et al*., 2018), polydatin (Zeng *et al*., 2018), salviae (Li *et al*., 2018b) and umbelliferone (Yin *et al*., 2018), may be available for renal oxidative damage. There is no doubt that natural product PRO provides a new approach for treatment of nephrotocicity and it is a novel and significant discovery in the process of probing new therapeutic agents to improve survival rate of patients with cisplatin chemotherapy. Therefore, animal experiments will be essential to further confirm the effect of preventing kidney damage and other mechanisms underlying the activation of Nrf2/HO-1 signaling pathway.

In conclusion, PRO can mitigate the cisplatin-induced renal oxidative damage through the activation of Nrf2/HO-1 pathway.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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