Formononetin protects against cisplatin-induced acute kidney injury through activation of the PPARα/Nrf2/HO-1/NQO1 pathway

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Abstract. Acute kidney injury (AKI) is characterized by an abrupt deterioration of renal function. Formononetin (FOR) protects against cisplatin (CIS)-induced AKI, and it has various potential pharmacological and biological effects, including anti-inflammatory, antioxidative and anti-apoptotic effects. The current study investigated the role of FOR in CIS-induced AKI. Rats were treated with CIS to establish an AKI model, followed by treatment with FOR. HK-2 cells were treated with CIS, FOR, GW6471 [a peroxisome proliferator-activated receptor α (PPARα) antagonist], eupatilin (a PPARα agonist) and nuclear factor erythroid 2-related factor 2 (Nrf2) small interfering RNA (siNrf2), and cell proliferation and apoptosis were determined by MTT and flow cytometry assays. The mRNA and proteins levels of PPARα, Nrf2, heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) were measured by reverse transcription-quantitative PCR and western blotting. The results demonstrated that FOR attenuated the histopathological changes, the levels of blood urea nitrogen, creatinine, TNF-α and IL-1β, and the MDA content and MPO activity, whereas it enhanced CAT activity in the AKI rat model. Furthermore, FOR and eupatilin promoted cell viability and CAT activity, and increased the levels of PPARα, Nrf2 and HO-1 and NQO1, but suppressed apoptosis and MPO activity, and reduced the levels of MDA, TNF-α and IL-1β in CIS-treated HK-2 cells. Notably, the aforementioned effects were reversed by GW6471 treatment or siNrf2 transfection. In conclusion, FOR protects against CIS-induced AKI via activation of the PPARα/Nrf2/HO-1/NQO1 pathway.

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Abbreviations: FOR, formononetin; CIS, cisplatin; AKI, acute kidney injury; BUN, blood urea nitrogen; PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; MDA, malondialdehyde; MPO, myeloperoxidase

Key words: formononetin, cisplatin, acute kidney injury, peroxisome proliferator-activated receptor α/nuclear factor erythroid 2-related factor 2/heme oxygenase-1/NAD(P)H quinone dehydrogenase 1

Introduction

Acute kidney injury (AKI) is characterized by an abrupt deterioration of renal function and dysregulation of the release of fluids, electrolytes and waste into urine (1), and it will cause life-threatening complications (2). Due to a lack of available therapeutic options, AKI is prevented and the majority of patients with AKI receive only dialysis to replace kidney function (3).

Multiple pathological conditions, such as ischemia-reperfusion, sepsis, trauma and release of nephrotoxic agents, as well as side-effects caused by therapeutic drugs are all responsible for the occurrence of AKI (4). Nephrotoxic AKI is caused by nephrotoxic agents, including drugs with therapeutic uses (5). Nephrotoxic AKI accounts for approximately 33% of all AKI cases (6). As an effective chemotherapeutic agent, cisplatin (CIS) is commonly used for treating hematological and solid tumor malignancies (7). Nephrotoxicity is a principal side effect of CIS treatment (8). Proximal tubule cells can absorb CIS accumulated in all segments of the nephron, which causes severe injury (9). Thus, the efficacy of CIS is limited by its nephrotoxicity, and effective strategies or original drugs to protect patients against the nephrotoxic effect of CIS are urgently required.

Multiple natural products have been identified for the prevention and therapy of renal diseases (10). Formononetin (FOR) is a natural and bioactive isoflavone isolated from herbal medicines, such as *Trifolium pretense*, *Astragalus membranaceus* and *Pueraria lobate* (11). It has recently been reported that FOR prevents CIS-induced AKI (12); however, to the best of our knowledge, the underlying molecular mechanism of FOR in preventing CIS-induced AKI is not clearly understood. Evidence has increasingly demonstrated that CIS-mediated nephrotoxicity is caused by a number of molecular pathways, such as oxidative stress, apoptosis and

inflammatory reactions (8,13). Malondialdehyde (MDA) is an indicator of lipid peroxidation, catalase (CAT) catalyzes the decomposition of hydrogen peroxide into oxygen and water, and myeloperoxidase (MPO) serves as an oxidative marker and an indicator of neutrophil infiltration into kidneys (14). A previous study reported that FOR has various potential pharmacological and biological effects, such as anti-inflammatory (15), antioxidative (16) and anti-apoptotic effects (17). Thus, the present study investigated the effects and protective mechanism of FOR by examining oxidative stress, apoptosis and inflammatory pathways in CIS-induced AKI rats and cells.

It has been demonstrated that FOR protects against rhabdomyolysis-induced AKI (18). FOR prevents against CIS-induced AKI by targeting organic cation transporter 2 and P53; however, to the best of our knowledge, the exact effect and potential mechanism underlying the effects of FOR in CIS-induced AKI remain unclear. Thus, the present study determined the target gene of FOR in CIS-induced AKI. Peroxisome proliferator-activated receptor α (PPARα) is a member of the steroid hormone receptor superfamily (18). Gonzalez-Manan *et al* (19) reported that in C57BL/6J mice fed a high-fat diet, the activation of PPARα is involved in the inhibition of oxidative stress and inflammatory reaction. Wang *et al* (20) demonstrated that activation of PPARα inhibits apoptosis of vascular adventitial fibroblasts. Furthermore, FOR is involved in the regulation of PPARα in ameliorating cholestasis (21).

Previously, it has been revealed that activation of nuclear factor erythroid 2-related factor 2 (Nrf2) improves renal function (22). Upregulation of the Nrf2-mediated signaling pathway protects against CIS-induced renal injury (23). In addition, when cells undergo chemical/oxidative stress, Nrf2 accumulates within the nuclei, in which Nrf2 activates the expressions of antioxidant response element (ARE)-driven genes, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone dehydrogenase 1 (NQO1) (24). The Nrf2/HO-1 signaling pathway protects cells from CIS-induced nephrotoxicity (25).

The current study investigated the protective effects of FOR on CIS-induced AKI and the potential mechanisms, such as via the Nrf2 pathway. It was identified that FOR could serve as a preventative drug in treating CIS-induced AKI.

Materials and methods

Ethics statement. Animal experiments were performed in accordance with the guidelines of the China Council on Animal Care and Use. The use of the animals was approved by the Ethical Committee of Experimental Animals of Zigong First People's Hospital (approval no. ZFPH201904223), and the animal experiments were performed at Zigong First People's Hospital (Zigong, China). All possible efforts were made to minimize pain and discomfort caused to the animals.

Animals and drug treatments. A total of 24 male Wistar rats (body weight, 220-250 g; age, 9-10 weeks) were purchased from the Experimental Animal Center of Zigong First People's Hospital (Zigong, China). All rats were housed in standard cages with a room temperature of 22˚C and humidity of 55%, under a normal 12/12 h light/dark cycle, and provided food and water *ad libitum*. The rats were divided into four different treatment groups, as follows: i) Control group; ii) CIS group; iii) FOR group; and iv) CIS + FOR group, with 6 rats in each group. FOR was purchased from Dalian Meilun Biotech Co., Ltd.

In the control group, vehicle (75 mg/kg 10% hydroxypropyl β-cyclodextrin (Sigma-Aldrich; Merck KGaA) in 500 mM phosphate buffer, pH 7.0) was administrated into the rats by oral gavage once a day for 5 days. On days 3 and 4, the rats were also intraperitoneally injected with normal saline 4 h after vehicle treatment.

The CIS group was treated as the control group, except that on days 3 and 4, the rats were intraperitoneally injected with CIS (12 mg/kg; Sigma-Aldrich; Merck KGaA) 4 h after vehicle treatment instead of with phosphate buffer. The concentration of CIS to use was determined as previously described (12).

In the FOR group, FOR (75 mg/kg), which has been reported to induce the strongest inhibition of CIS-induced nephrotoxicity previously (12), was administrated into the rats by oral gavage once a day for 5 days. On days 3 and 4, the rats were intraperitoneally injected with physiological saline 4 h after FOR treatment.

In the CIS + FOR group, FOR (75 mg/kg) was administrated into the rats by oral gavage once a day for 5 days. On days 3 and 4, the rats were intraperitoneally injected with CIS (12 mg/kg) 4 h after FOR treatment.

On day 5, 4 h after treatment, all the rats were sacrificed with pentobarbital sodium (165 mg/kg, i.p.) and the heartbeat was checked. The both kidneys and blood (1 ml from the tail vein) were collected, and plasma was separated from the blood. The renal tissue samples were immediately fixed with 10% formalin at room temperature for 24 h, paraffin-embedded, sectioned into 5 μm-thick slides, and then stained with hematoxylin and eosin (H&E). In addition, the renal tissue homogenate was centrifuged at 3,500 x g for 10 min at 4˚C, and MDA content, and CAT and MPO activities were detected using the supernatant. Levels of blood urea nitrogen (BUN), creatinine, TNF-α and IL-1β were also detected in the rat plasma.

Renal histological studies. Paraffin-embedded kidney sections were stained with H&E. Histological changes, such as degree of tubular injury, were visualized and photographs were obtained using a light microscope (magnification, x200). In detail, following dewaxing, the section was stained with hematoxylin solution (cat. no. HHS16; Sigma-Aldrich; Merck KGaA) at room temperature for 10 min. Subsequently, the section were washed and stained with eosin solution (cat. no. 318906; Sigma-Aldrich; Merck KGaA) at room temperature for 5 min. A semi-quantitative pathological grading method was used to assess the degree of tubular injury, including tubular dilation, necrosis and cell membrane bleb formation (26). Two pathologists, who were blinded to the treatment, independently evaluated the histopathological changes of each image using a light microscope (magnification, x200). A total of 24 images of one section were used to calculate the pathological changes (%) according to the injury score (0 for 0%, 1 for <5%, 2 for 5-24%, 3 for 25-74%, 4 for 75-100%), which was based on a system previously reported (26).

Table I. Primary antibodies used for western blotting.

Target protein	Antibody	Cat. no.	Supplier	Working dilution
PPARα	Rabbit anti-PPARα antibody	ab24509	Abcam	1:100
Nrf2	Mouse anti-Nrf2 antibody	ab89443	Abcam	1:1,000
HO-1	Rabbit anti-HO-1 antibody	ab13243	Abcam	1:2,000
NQO1	Mouse anti-NQO1 antibody	ab28947	Abcam	1:1,000
β-actin	Mouse anti-β-actin antibody	ab8226	Abcam	1:1,000

PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1.

Biochemical measurements. The levels of BUN and creatinine in rat plasma were determined using a Urea assay kit (cat. no. C013-2-1) and Creatinine assay kit (cat. no. C011-1-1), respectively. Kidney tissues were rinsed in 100 mg/ml normal saline, then weighed and homogenized. MDA content and activities of CAT and MPO in the homogenate supernatant were determined. MDA in the supernatant of homogenized renal tissues from rats was measured using a MDA assay kit (cat. no. A003-1-2). The CAT enzymatic activity was measured using the CAT assay kit (cat. no. A007-1-1). The activity of MPO was measured by MPO assay kit (cat. no. A044-1-1). All kits were purchased from Nanjing Bioengineering Institute Co., Ltd and used according to the manufacturer's protocols. The levels of TNF-α and IL-1β in the rat plasma were measured using a Rat TNF-α ELISA kit (cat. no. SEKR-0009; Beijing Solarbio Science & Technology Co., Ltd.) and a Rat IL-1β ELISA kit (cat. no. SEKR-0002; Beijing Solarbio Science & Technology Co., Ltd.).

Western blotting. Lysates from renal tissues of the Wistar rats and HK-2 cells were lysed with RIPA lysis buffer (Cell Signaling Technology, Inc.) to isolate proteins, and PPARα, Nrf2, HO-1 and NQO1 proteins were analyzed. Briefly, total proteins of renal tissues were extracted using RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) with a complete protease inhibitor cocktail (cat. no. 539133; Merck KGaA) on ice for 30 min. Subsequently, the supernatant was collected, and the protein concentration was determined using a BCA protein assay kit (cat. no. PC0020; Beijing Solarbio Science & Technology Co., Ltd.). Proteins (30 μg/lane) were then separated by 12% SDS-PAGE (cat. no. P0012A; Beyotime Institute of Biotechnology) and transferred onto PVDF membranes (cat. no. IPVH00010; EMD Millipore). The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline containing 0.5% Tween-20 (w/v) at 37˚C for 1 h. Next, the membranes were probed with the specific primary antibodies listed in Table I at 4˚C overnight, and then washed with TBS containing 0.1% Tween-20. β-actin was used as the reference gene. The membranes were then incubated with HRP-conjugated goat anti-mouse secondary antibody (1:2,000; cat. no. ab205719; Abcam) and HRP-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. ab205718; Abcam) at 37˚C for 1 h. After washing the membranes three times at an interval of 10 min, the signals were visualized using ECL detection kit (Promega Corporation) and Protein expression levels were quantified using ImageJ software (version 1.8.0; National Institutes of Health) and normalized to that of β-actin.

Table II. Primer sequences used for reverse transcription-quantitative PCR analysis of rat samples.

Gene		Primer sequence (5'-3')
PPARα	Forward	AGAGCCCCATCTGTCCTCTC
	Reverse	ACTGGTAGTCTGCAAAACCAAA
Nrf2	Forward	TCTTGGAGTAAGTCGAGAAGTGT
	Reverse	GTTGAAACTGAGCGAAAAAGGC
HO-1	Forward	AGGTACACATCCAAGCCGAGA
	Reverse	CATCACCAGCTTAAAGCCTTCT
NQO1	Forward	AGGATGGGAGGTACTCGAATC
	Reverse	AGGCGTCCTTCCTTATATGCTA
β-actin	Forward	GGCTGTATTCCCCTCCATCG
	Reverse	CCAGTTGGTAACAATGCCATGT

PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from tissue and cells using TRIzol reagent (cat. no. 15596018, Invitrogen; Thermo Fisher Scientific, Inc.), and the mRNA expression levels of PPARα, Nrf2, HO-1 and NQO1 were determined. PrimeScript™ RT Master mix was used for RT (cat. no. RR036B; Takara Bio, Inc.) and incubated at 37˚C for 15 min, followed by incubation at 85˚C for 5 sec. qPCR was performed with a 7300 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using TB Green® Premix Ex Taq™ II (cat. no. RR820Q; Takara Bio, Inc.). The thermocycling conditions were as follows: Incubation at 95˚C for 30 min, followed by amplification at 95˚C for 5 sec and 60˚C for 34 sec for a total of 40 cycles, 72˚C for 30 sec, with a final extension at 72˚C for 90 sec. The expression levels of genes were normalized to that of β-actin using the $2^{-\Delta\Delta Cq}$ method (27). The sequences of primers used are presented in Table II and III.

Table III. Primer sequences used for reverse transcription-quantitative PCR analysis of HK-2 cells.

Gene		Primer sequence (5'-3')
PPARα	Forward	ATGGTGGACACGGAAAGCC
	Reverse	CGATGGATTGCGAAATCTCTTGG
Nrf2	Forward	TCAGCGACGGAAAGAGTATGA
	Reverse	CCACTGGTTTCTGACTGGATGT
HO-1	Forward	AAGACTGCGTTCCTGCTCAAC
	Reverse	AAAGCCCTACAGCAACTGTCG
NQO1	Forward	GAAGAGCACTGATCGTACTGGC
	Reverse	GGATACTGAAAGTTCGCAGGG
β-actin	Forward	CATGTACGTTGCTATCCAGGC
	Reverse	CTCCTTAATGTCACGCACGAT

PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1.

Cell culture. HK-2 (human kidney proximal tubule) cells were purchased from the American Type Culture Collection and incubated in DMEM/F12 (cat. no. BNCC342221; BeNa Culture Collection) containing 10% fetal bovine serum (cat. no. 11011-8611; Beijing Solarbio Science & Technology Co., Ltd.) and 1% Penicillin-Streptomycin Liquid (cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.) with 5% CO_2 at 37˚C.

To investigate the role of PPARα in FOR preventing CIS-induced injury of HK-2 cells, GW6471 (cat. no. G5045; Sigma-Aldrich; Merck KGaA), which is a selective PPARα antagonist, was used to inhibit PPARα in HK-2 cells. The cells were pre-treated with normal saline or 25 μM GW6471 for 30 min, and then cultured with 10 or 25 μM FOR for 2 h at room temperature. Next, the cells were treated with 25 μM CIS for 24 h. The treatments for different cell groups were as follows: i) Control group, cells treated with PBS; ii) CIS group, cells treated with 25 μM CIS; iii) CIS + FOR-L group, cells treated with 25 μM CIS and 10 μM FOR; iv) CIS + FOR-H group, cells treated with 25 μM CIS and 25 μM FOR; and v) CIS + FOR-H + GW6471 group, cells treated with 25 μM CIS, 25 μM FOR and 5 μM GW6471.

Transfection. The expression of Nrf2 was inhibited using Nrf2 small interfering RNA (siRNA; siNrf2) to further investigate the potential mechanism of PPARα in FOR prevention of HK-2 cells treated with CIS. The siNrf2 (5'-GACATGGAT TTGATTGACATACT-3') and siRNA negative control (siNC; 5'-CACTTGAATCCGACGGATTTGCA-3') were purchased from Guangzhou RiboBio Co., Ltd. HK-2 cells transfected with siNC or siNrf2 served as the negative control group or siNrf2 group, respectively. The cells, cultured in 6-well culture plates in serum-free OPTI-MEM medium (Gibco; Thermo Fisher Scientific, Inc.), were transfected with 100 nM siNC or 100 nM siNrf2 using Lipofectamine (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h. The next day, the cells were incubated in fresh medium for a further 48 h, and then subjected to the following treatments.

Following cell transfection with siNrf2, the cells were pre-treated with 50 μg/ml eupatilin (PPARα agonist; cat. no. HY-N0783; MedChemExpress) for 24 h, and then cultured with 25 μM FOR for 2 h at room temperature. Eupatilin was used to treat the cells for the purpose of comparing its effect with that of FOX on CIS injury. Subsequently, the cells were cultured with 25 μM CIS for 24 h. The treatments for different cell groups were as follows: i) siNC, transfection with siNC only; ii) siNC + CIS, siNC transfection and CIS (25 μM) treatment; iii) CIS + eupatillin + siNC, siNC transfection with CIS (25 μM) and eupatillin (50 μg/ml) treatment; iv) CIS + eupatillin + siNrf2, siNrf2 transfection with CIS (25 μM) and eupatillin (50 μg/ml) treatment; v) CIS + FOR-H + siNC, siNC transfection with CIS (25 μM) and FOR (25 μM) treatment; and vi) CIS + FOR-H + siNrf2, siNrf2 transfection with CIS (25 μM) and FOR (25 μM) treatment. The concentrations of FOR and CIS to be used were selected as previously described (8).

MTT assay. The cells ($2x10^4$ cells/well) were plated in 96-well plates, and cell proliferation was determined using a MTT kit (cat. no. IM0280; Beijing Solarbio Science & Technology Co., Ltd.). Briefly, following the corresponding treatments, MTT solution (20 μl; 5 mM) was added to each well of the 96-well plates and incubated for a further 4 h. The absorbance was measured at 570 nm using a microplate reader.

Flow cytometry assay. Apoptosis of the treated cells was measured by flow cytometry using an Annexin V-FITC Apoptosis Detection kit (cat. no. CA1020; Beijing Solarbio Science & Technology Co., Ltd.). The collected cells were washed with cold sterile PBS, counted and resuspended in 1 X binding buffer, then incubated with Annexin V-FITC and propidium iodide in the dark at room temperature for 15 min. The cells were then further diluted using PBS to 500 μl, and cell apoptosis was evaluated using a flow cytometer and FlowJo software (version 7.6.1; FlowJo LLC).

Biochemical measurements of the cells. MDA, CAT enzymatic activity and the activity of MPO in HK-2 cells were measured using the same methods as described for tissues. The levels of TNF-α and IL-1β in HK-2 cells were measured by Human TNF-α ELISA kit (cat. no. SEKH-0047; Beijing Solarbio Science & Technology Co., Ltd.) and Human IL-1β ELISA kit (cat. no. SEKH-0002; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions.

Statistical analysis. The data are presented as the mean ± standard deviation. All experiments were performed in triplicate. Statistical significance in the study was analyzed by one-way ANOVA followed by Tukey's post hoc test and Bonferroni's correction. $P<0.05$ was considered to indicate a statistically significant difference. The analyses were performed using SPSS 17.0 software (SPSS, Inc.).

Results

FOR protects against CIS-induced nephrotoxicity in the rat model. As presented in Fig. 1A, epithelial cell swelling, vacuolar degeneration and massive necrosis in the proximal tubules

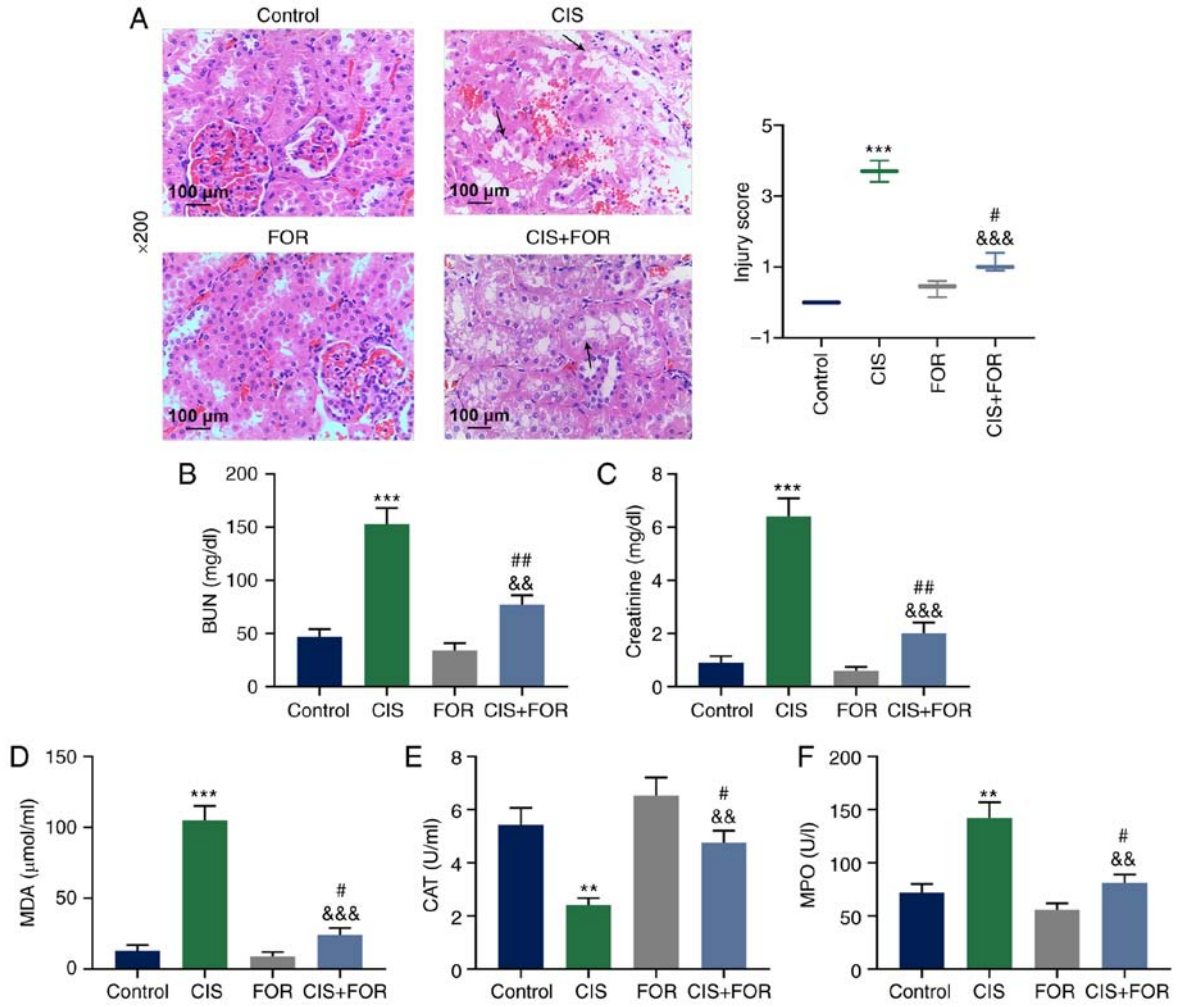

Figure 1. FOR ameliorates CIS-mediated acute kidney injury in a rat model. (A) Kidney histopathological examination was performed by hematoxylin and eosin staining in the rat model (magnification, x200). Arrows indicate areas of severe renal necrosis. The changes in (B) BUN and (C) creatinine levels in plasma, (D) MDA level, and (E) CAT and (F) MPO activities in kidney were ameliorated by FOR following treatment with CIS. Data are presented as mean ± standard deviation. **P<0.01, ***P<0.001 vs. Control. $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. CIS. $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. FOR. FOR, formononetin; CIS, cisplatin; BUN, blood urea nitrogen; MDA, malondialdehyde; MPO, myeloperoxidase; CAT, catalase.

were observed in rats treated by CIS, and these effects were not observed in the CIS + FOR treatment group. Furthermore, FOR treatment significantly reduced the CIS-increased injury score in renal tissues compared with the CIS group (P<0.00; Fig. 1A).

The levels of BUN and creatinine in the plasma were significantly increased in the CIS group compared with the control group (P<0.001), but significantly reduced in the CIS + FOR group compared with the CIS group (P<0.001; Fig. 1B and C).

The level of MDA in the rats was greatly increased by CIS treatment, but was significantly reduced in the CIS + FOR group (P<0.001; Fig. 1D). Furthermore, the CAT activity was significantly reduced by CIS treatment, but significantly increased in the CIS + FOR group (P<0.01; Fig. 1E). The MPO activity of the rats was significantly increased by CIS treatment, and was significantly reduced by FOR treatment (P<0.01; Fig. 1F). The levels of TNF-α (Fig. 2A; P <0.001) and IL-1β (Fig. 2B, P<0.001) were significantly increased by treatment of rats with CIS (P<0.001), and were significantly decreased in the CIS + FOR group (P<0.001; Fig. 2A and B).

FOR increases the expression levels of PPARα, Nrf2, HO-1 and NQO1, which are reduced by CIS in the rat models. As presented in Fig. 2C and D, compared with the control group, CIS treatment significantly suppressed the mRNA and proteins levels of PPARα, Nrf2, HO-1 and NQO1 in the rats (P<0.05), and these levels were significantly reversed by FOR treatment (P<0.001).

FOR protects HK-2 cells against CIS-induced injury in a PPARα-dependent manner. As presented in Fig. 3A, CIS treatment significantly suppressed proliferation of HK-2 cells at 12 (P<0.05), 24 (P<0.01) and 48 h (P<0.001) compared with the control group; however, this suppression was significantly reversed by FOR-L treatment at 48 h (P<0.05) and by FOR-H treatment at 24 (P<0.05) and 48 h (P<0.001). Notably, the promotive effect of FOR-H at 24 and 48 h on cell proliferation was significantly prevented by GW6471 treatment at 48 h (P<0.001).

As presented in Fig. 3B, the cell apoptosis rate of HK-2 cells was significantly increased in the CIS group (P<0.001), but was reduced by FOR-L (P<0.01) and FOR-H (P<0.001) in

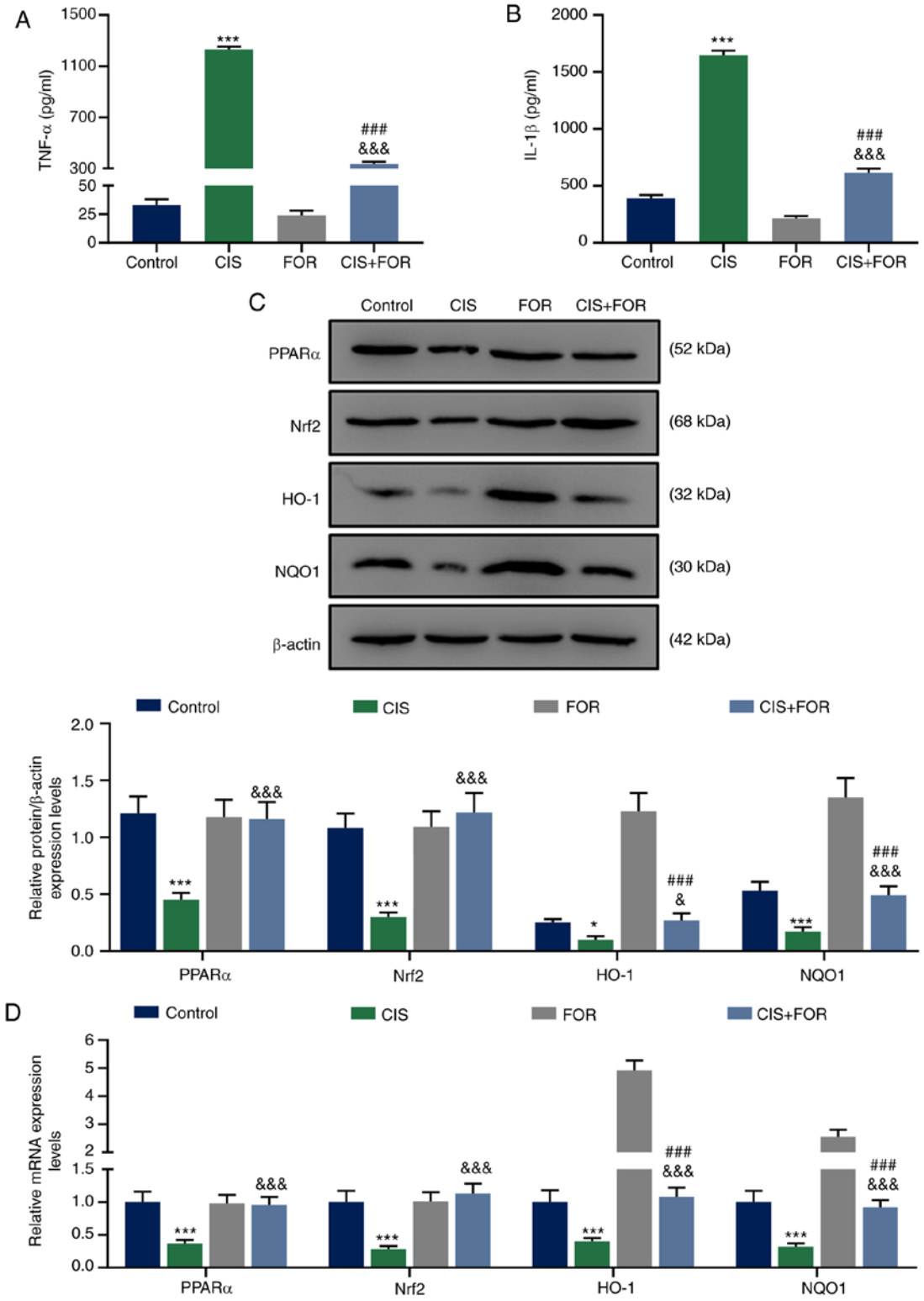

Figure 2. FOR increases CIS-reduced TNF-α and IL-1β levels and expressions of PPARα, Nrf2, HO-1 and NQO1 in the rat model. FOR increased CIS-reduced (A) TNF-α and (B) IL-1β levels. FOR increased the CIS-reduced (C) protein and (D) mRNA expression levels of PPARα, Nrf2, HO-1 and NQO1. Data are presented as mean ± standard deviation. *P<0.05, ***P<0.001 vs. Control. $^{\&}$P<0.05, $^{\&\&\&}$P<0.001 vs. CIS. $^{\#\#\#}$P<0.001 vs. FOR. FOR, formononetin; CIS, cisplatin; PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1.

a dose-dependent manner. However, these effects of FOR-H were reversed by GW6471 treatment (P<0.001).

As presented in Fig. 3C, the MDA level in HK-2 cells was significantly increased in the CIS group (P<0.001), but was reduced by FOR-L (P<0.05) and FOR-H (P<0.01) in a dose-dependent manner. However, the effect of FOR-H on MDA level was significantly reversed by GW6471 treatment (P<0.01). As shown in Fig. 3D, CIS treatment significantly

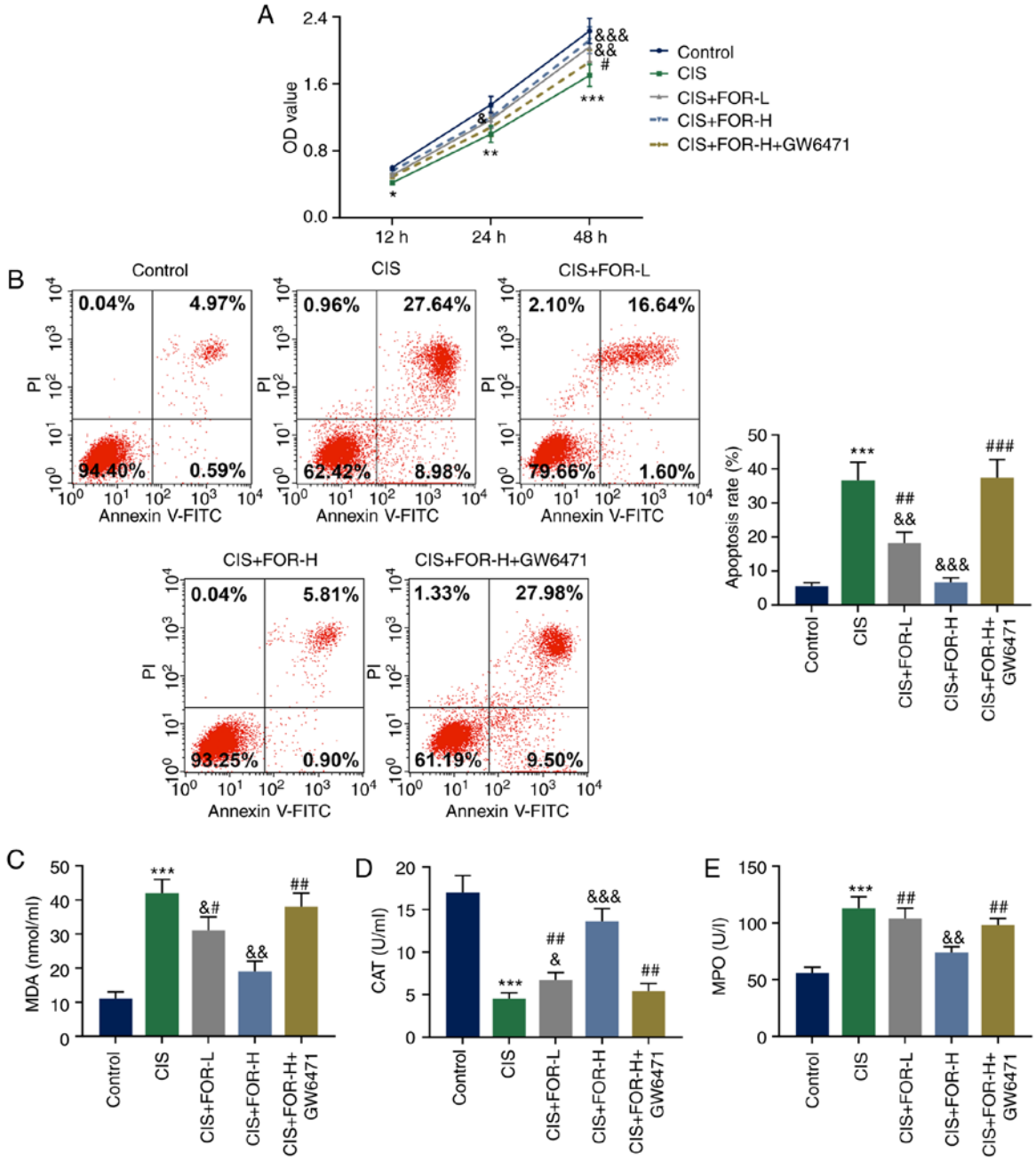

Figure 3. FOR protects against CIS-induced injury of HK-2 cells in a PPARα-dependent manner. (A) FOR increased the CIS-reduced proliferation in a PPARα-dependent manner. (B) FOR reduced the CIS-increased apoptosis in a PPARα-dependent manner. (C) FOR reduced the CIS-increased MDA level in a PPARα-dependent manner. FOR increased the CIS-reduced (D) CAT and (E) MPO activities in a PPARα-dependent manner. Data are presented as mean ± standard deviation. *P<0.05, **P<0.01, ***P<0.001 vs. Control. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. CIS. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. CIS + FOR-H. FOR, formononetin; CIS, cisplatin; PPARα, peroxisome proliferator-activated receptor α; MDA, malondialdehyde; MPO, myeloperoxidase; CAT, catalase; PI, propidium iodide; OD, optical density; L, low dose; H, high dose.

suppressed CAT activity in HK-2 cells (P<0.001), which was significantly reversed by FOR-L (P<0.05) and FOR-H (P<0.01) treatment in a dose-dependent manner, and this effect of FOR-H was reversed by GW6471 treatment (P<0.01). As presented in Fig. 3E, CIS treatment significantly enhanced the MPO activity in HK-2 cells (P<0.001), which was significantly reduced by FOR-H treatment (P<0.01), and GW6471 treatment significantly reversed this effect of FOR-H (P<0.01).

As shown in Fig. 4A and B, CIS treatment significantly increased the levels of TNF-α (P<0.001) and IL-1β (P<0.001) in HK-2 cells, which were reversed by FOR-L (P<0.01) and FOR-H (P<0.001). However, GW6471 treatment significantly reversed this effect of FOR-H (P<0.001).

FOR increases the expression levels of PPARα, Nrf2, HO-1 and NQO1, which are inhibited by CIS, in PPARα-dependent manner. As presented in Fig. 4C and D, CIS treatment significantly reduced the mRNA and proteins levels of PPARα (P<0.001), Nrf2 (P<0.001), HO-1 (P<0.001) and NQO1 (P<0.001) in HK-2 cells, which was reversed by FOR-L (P<0.05) and FOR-H treatment (P<0.01). Furthermore, GW6471 treatment significantly reversed the effect of FOR-H of these expression levels (P<0.001).

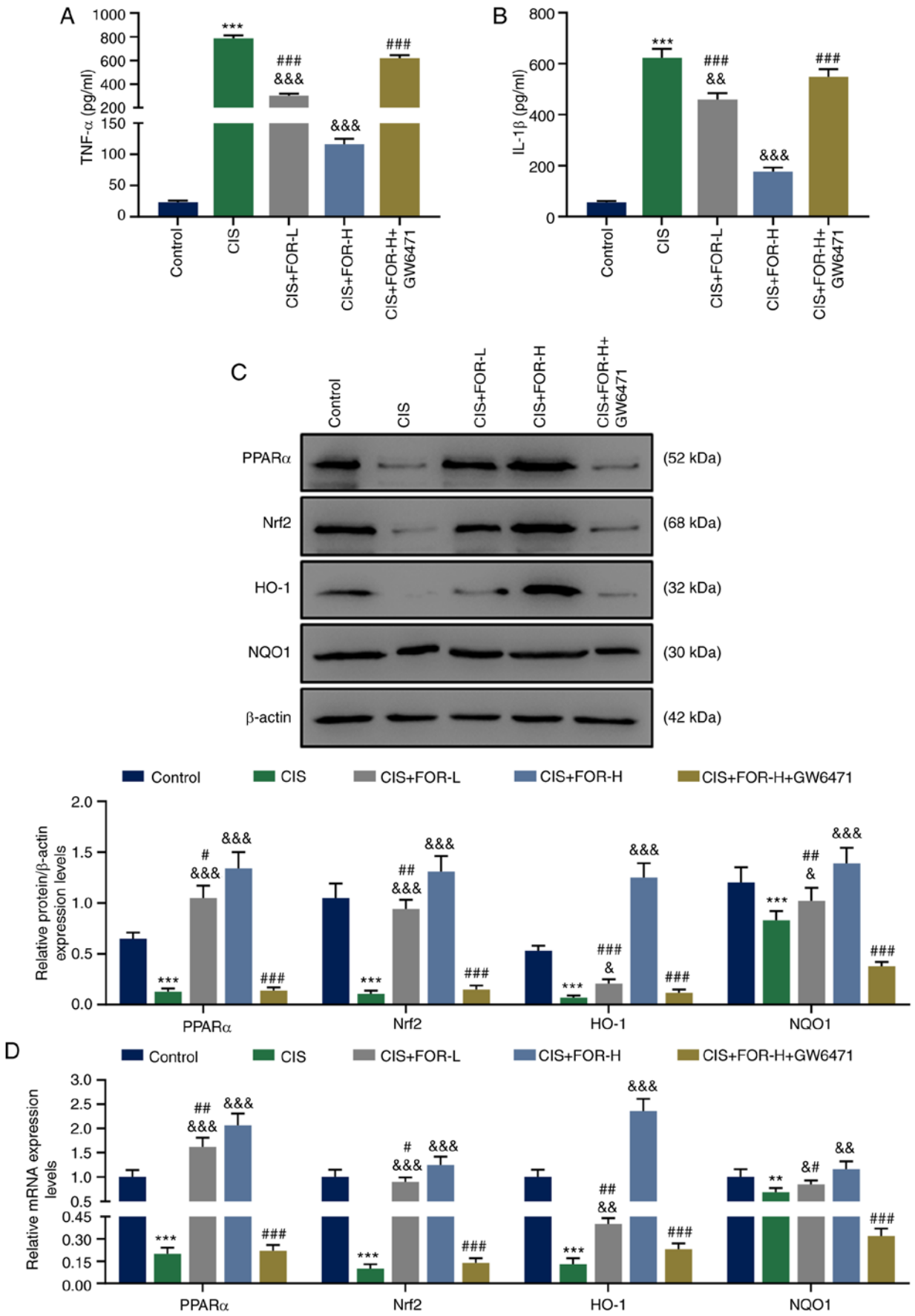

Figure 4. FOR protects against CIS-induced injury and expression levels of PPARα, Nrf2, HO-1 and NQO1 in HK-2 cells in a PPARα-dependent manner. FOR reduced the CIS-increased (A) TNF-α and (B) IL-1β levels in a PPARα-dependent manner. FOR increased the CIS-reduced (C) protein and (D) mRNA levels of PPARα, Nrf2, HO-1 and NQO1 in a PPARα-dependent manner. Data are presented as mean ± standard deviation. **P<0.01, ***P<0.001 vs. Control. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. CIS. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. CIS + FOR-H. FOR, formononetin; CIS, cisplatin; PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1; L, low dose; H, high dose.

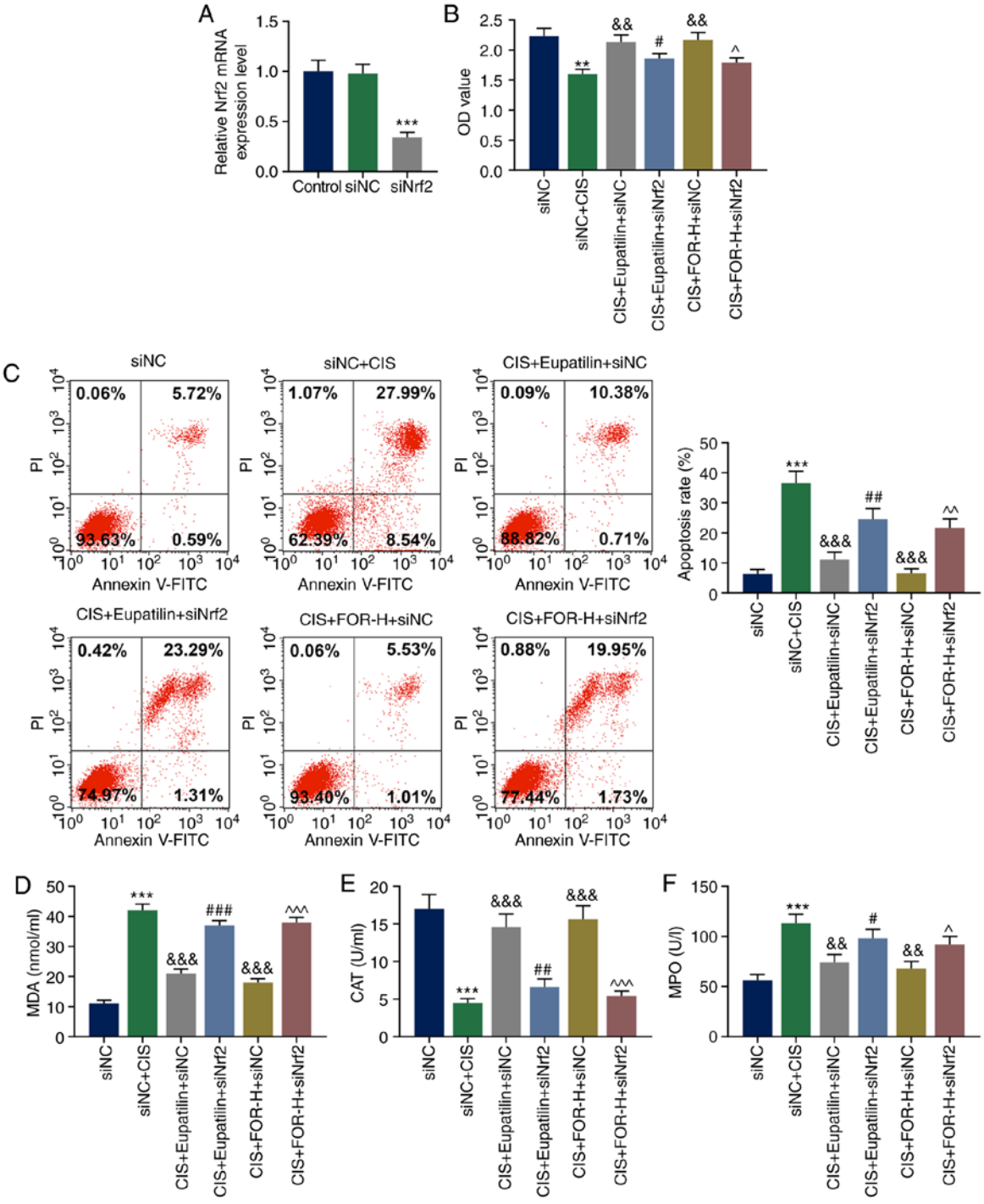

Figure 5. Nrf2 silencing effectively reverses the protective effect of eupatilin or FOR on CIS-induced injury of HK-2 cells. (A) Nrf2 mRNA expression was significantly downregulated in HK-2 cells by transfection with siNrf2. (B) Eupatilin or FOR increased the CIS-reduced proliferation in a Nrf2-dependent manner. (C) Eupatilin or FOR reduced the CIS-increased apoptosis in a Nrf2-dependent manner. (D) Eupatilin or FOR reduced the CIS-increased MDA level in a Nrf2-dependent manner. Eupatilin or FOR increased the CIS-reduced (E) CAT and (F) MPO activities in a Nrf2-dependent manner. Data are presented as mean ± standard deviation. **P<0.01, ***P<0.001 vs. siNC. $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. siNC + CIS. $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. CIS + Eupatilin + siNC. $^{\wedge}$P<0.05, $^{\wedge\wedge}$P<0.01, $^{\wedge\wedge\wedge}$P<0.001 vs. CIS + FOR-H + siNC. Nrf2, nuclear factor erythroid 2-related factor 2; FOR, formononetin; CIS, cisplatin; MDA, malondialdehyde; MPO, myeloperoxidase; CAT, catalase; PI, propidium iodide; OD, optical density; H, high dose; si, small interfering RNA; NC, negative control.

Nrf2 silencing effectively reverses the protective effect of eupatilin or FOR on CIS-induced injury of HK-2 cells. Following transfection with siNrf2, the Nrf2 mRNA expression was significantly downregulated in siNrf2 cells (P<0.001; Fig. 5A). As shown in Fig. 5B and C, the CIS-inhibited decrease in viability and increase in apoptosis of HK-2 cells were greatly attenuated by eupatilin (P<0.01) or FOR treatment (P<0.01), but significantly reversed by Nrf2 silencing (P<0.05). As presented in Fig. 5D-F, eupatilin (P<0.01) or FOR treatment (P<0.01) significantly attenuated the CIS-promoted MDA level and MPO activity, and the CIS-suppressed CAT activity. However, these effects were reversed by Nrf2 silencing (P<0.05). As shown in Fig. 6A and B, eupatilin (P<0.001) or FOR treatment (P<0.001) significantly attenuated the

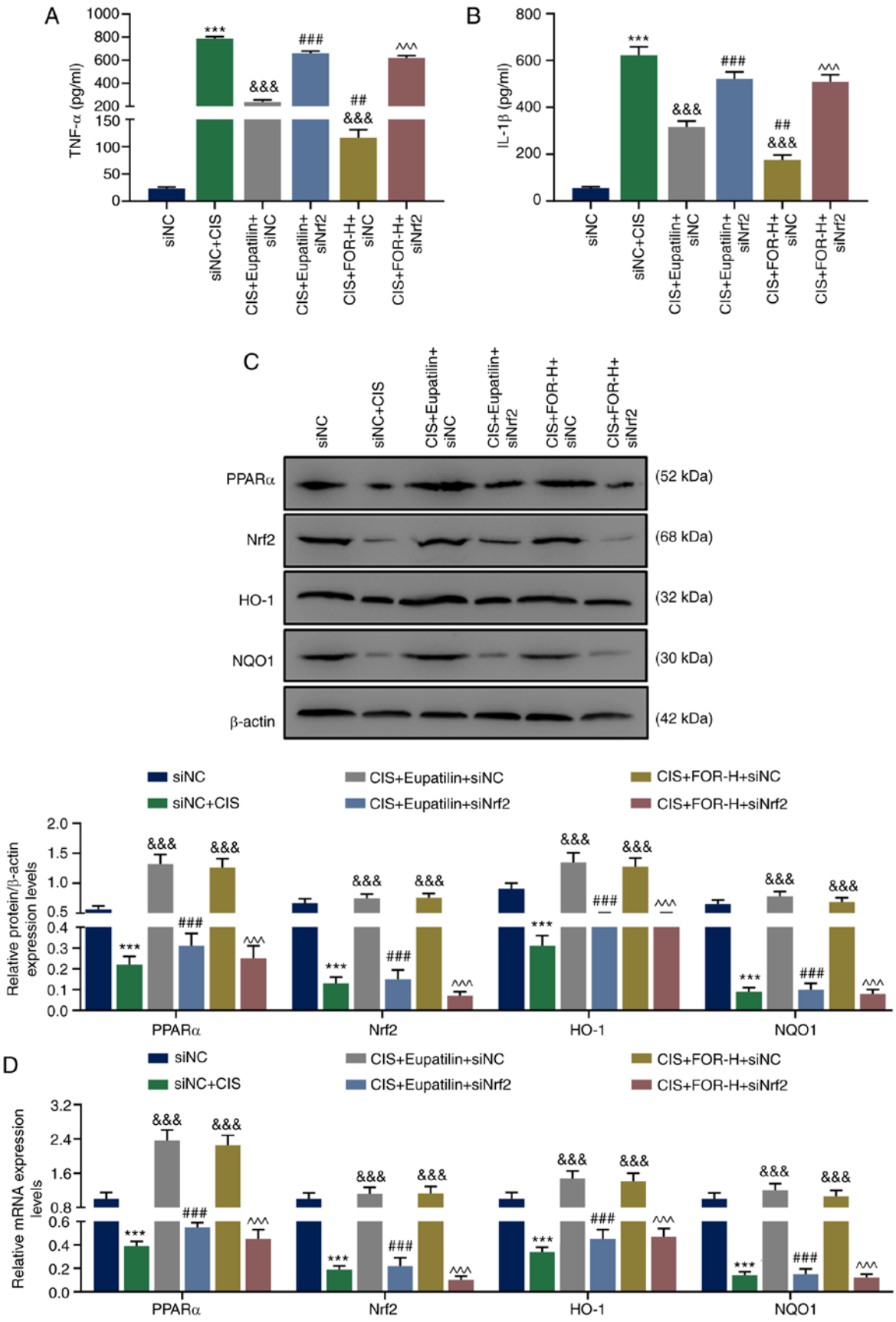

Figure 6. Nrf2 silencing effectively reverses the protective effect of eupatilin or FOR CIS-induced injury of HK-2 cells, and reverses the stimulative effect of eupatilin or FOR on the CIS-inhibited PPARα/Nrf2/HO-1/NQO1 pathway in HK-2 cells. Eupatilin or FOR reduced the CIS-induced increases of (A) TNF-α and (B) IL-1β levels in a Nrf2-dependent manner. Eupatilin or FOR increased the CIS-induced reduced (C) protein and (D) mRNA expression levels of PPARα, Nrf2, HO-1 and NQO1 in a Nrf2-dependent manner. Data are presented as mean ± standard deviation. ***P<0.001 vs. siNC. $^{\&\&\&}$P<0.001 vs. siNC + CIS. $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. CIS + Eupatilin + siNC. $^{\wedge\wedge\wedge}$P<0.001 vs. CIS + FOR-H + siNC. Nrf2, nuclear factor erythroid 2-related factor 2; FOR, formononetin; CIS, cisplatin; H, high dose; si, small interfering RNA; NC, negative control; PPARα, peroxisome proliferator-activated receptor α; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; NQO1, NAD(P)H quinone dehydrogenase 1.

CIS-increased TNF-α and IL-1β levels, which were reversed by Nrf2 silencing (P<0.001).

Nrf2 silencing effectively reverses the stimulatory effect of eupatilin or FOR on the CIS-inhibited PPARα/Nrf2/HO-1/NQO1 pathway in HK-2 cells. As presented in Fig. 6C and D, eupatilin (P<0.001) and FOR treatment (P<0.001) significantly increased the mRNA and proteins levels of PPARα, Nrf2, HO-1 and NQO1 of HK-2 cells, which were previously reduced by CIS; however, this was reversed by Nrf2 silencing (P<0.001).

Discussion

The level of CIS in the kidney is the highest in the proximal tubule (28). Proximal tubular cell injury and death are responsible for CIS-induced AKI (29), and proximal tubular cell death is associated with high morbidity and mortality (30). Therefore, in order to relieve kidney injury of patients with AKI and investigate the protective effect of FOR on CIS-induced AKI, the present study examined kidney and proximal tubular cells under CIS condition. The present study found that FOR protected against CIS-induced nephrotoxicity, as supported by improved renal function, attenuated histopathological changes of the rat model, and improved cell viability, reduced apoptosis, oxidative stress, and inflammatory reaction of renal proximal tubular cells.

PPARα protects against metabolic and inflammatory derangements associated with AKI (31). Therefore, GW6471 (a selective PPARα antagonist) was used to treat renal proximal tubular cells to investigate the effect of PPARα on FOR in preventing HK-2 cells from CIS-induced injury. The results demonstrated that FOR protected against CIS-induced injury of HK-2 cells and increased the expression levels of PPARα, Nrf2, HO-1 and NQO1, which were previously reduced by CIS, in a PPAR-α-dependent manner.

In response to natural Nrf2 activators, oxidative stress, inflammation and kidney disease are induced in animal models, while Nrf2 silencing amplifies these pathogenic pathways (32). Zoja *et al* (33) also reported that blocking Nrf2-activation promotes oxidative stress, inflammation and the development of tissue damage in the kidney. Targeting Nrf2 ameliorates oxidative stress and inflammatory reactions in kidney disease (34). Furthermore, FOR upregulates Nrf2 *in vivo* and *in vitro* to protect against AKI (18). The role of Nrf2 in FOR protecting HK-2 cells from CIS-induced injury was further investigated, and it was identified that FOR treatment or upregulated PPAR-α protected HK-2 cells against CIS-induced injury in an Nrf2-dependent manner, indicating that FOR treatment protects HK-2 cells against renal injury induced by CIS through activation of the PPARα/Nrf2 pathway.

Aladaileh *et al* (35) reported that FOR upregulates Nrf2/HO-1 signaling to prevent oxidative stress, inflammatory reaction and kidney impairment in methotrexate-induced rats. Upregulated Nrf2/ARE/HO-1 signaling suppresses oxidative stress, inflammatory reactions and apoptosis in rat kidneys exposed to cyclophosphamide (36). Gang *et al* (37) indicated that NQO1 protects cells against renal failure induced by CIS. The present study demonstrated that the PPARα, Nrf2, HO-1 and NQO1 expression levels previously reduced by CIS were reversed by FOR or upregulated PPAR-α in an Nrf2-dependent manner. Therefore, FOR treatment protected HK-2 cells against CIS-induced renal injury through activation the of α/Nrf2/HO-1/NQO1 pathway.

The current findings demonstrated that the protective effects of FOR on CIS-induced AKI were associated with activation of the PPARα-Nrf2-HO-1/NQO1 pathway. However, whether FOR could also clinically reduce the tumoricidal efficacy of CIS remains unclear; therefore, more experiments are needed to investigate the combined effects of FOR and CIS on patients with AKI and the associated molecular mechanisms. Additionally, other better alternative products, such as PPAR agonists, may be applied as first line therapy for AKI in future study. The present study focused both on cells and animals, supporting the results of each other. Certainly, studies could be conducted more deeply both in cells and animals in the future.

Taken together, the protective effect of FOR against CIS-induced AKI, and improvement of renal function and alleviation of histopathological changes were associated with the reduced oxidative stress, inflammatory reaction, and activation of the PPARα-Nrf2-HO-1/NQO1 pathway. The current research demonstrated that FOR is a promising drug for preventing CIS-injured AKI.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YH and JM substantially contributed to the conception and design of the study. WL, LP, YC and QZ acquired, analyzed and interpreted the data. YH and JM drafted the article and critically revised it for important intellectual content. YH agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Experimental Animals of Zigong First People's Hospital, Zigong, China (approval no. ZFPH201904223).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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