Cycloartenyl Ferulate Inhibits Paraquat-Induced Apoptosis in HK-2 Cells With the Involvement of ABCC1

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ABSTRACT
Nephrotoxicity induced by chemicals such as paraquat (PQ) is a common clinical phenomenon; therefore, searching for drugs with renal protective effect is of a great practical significance. Our previous investigation found that cycloartenyl ferulate (CF) can antagonize the cytotoxic effect of PQ, and recent studies also revealed a variety of bioactivities of CF. However, specific molecular mechanisms underlying the protective effect of CF have not been explored yet. HPLC detection of PQ content indicated that CF reduced PQ accumulation in HK-2 cells and thereby improved cell survival. Western blot results showed that both PQ and CF did not affect the expression of ABCB1; however, while PQ suppressed the expression of ABCC1, CF upregulated ABCC1 expression and thereby reversed the inhibitory effect of PQ on ABCC1 expression. Meanwhile, HK-2 cells did not express ABCG2. When the expression of ABCC1 was knocked down with siRNA, the inhibitory effect of CF on intracellular PQ accumulation was blocked. Further flow cytometric analysis showed that while PQ significantly induced the appearance of sub-G1 apoptotic peak in cells, CF evidently inhibited apoptosis. TUNEL-DAPI double-staining also detected that PQ significantly induced the occurrence of DNA fragmentation in cells, whereas CF effectively inhibited the effect of PQ. Further results showed that ABCC1 siRNA effectively abolished the protective effect of CF on PQ-induced apoptosis. Taken together, these data demonstrated that in HK-2 cells, CF could antagonize PQ-induced toxicity with the involvement of regulation of ABCC1 protein expression, which provides a new strategy for treatments of nephrotoxicity. J. Cell. Biochem. 9999: 1–9, 2015. © 2015 Wiley Periodicals, Inc.

KEY WORDS: NEPHROTOXICITY; PARAQUAT; CYCLOARTENYL FERULATE; ABCC1; APOPTOSIS

Kidney is the most important excretory organ in the body, as well as one of the main organs metabolizing exogenous toxins. Thus, there is a high incidence of renal injury caused by drugs, etc. According to statistics, 19–33% of clinical acute renal injuries are caused by drugs. Renal tubules are usually the main target where renal toxicity occurs, and some drugs may cause toxic reaction in renal tubular cells through pathways such as damaging mitochondrial function, interfering with renal tubular transport, enhancing oxidative stress, generating free radicals, etc. [Hall, 2013]. Therefore, renal toxicity is currently a research hotspot in the field.

PQ is a presently most widely used highly efficient herbicide in the world. However, it has a relatively strong toxicity to humans and animals, and there are frequent incidences of death event caused by acute PQ poisoning due to skin contact, inhalation, and wrongful ingestion [Dinis-Oliveira et al., 2008]. Data have shown that patients suffered from acute PQ poisoning are prone to acute renal injury, mainly affecting the renal tubules and interstitium, which may occur within hours after the poisoning and last for a long period; Renal fibrosis may occur in some patients and further progress into chronic renal dysfunction [Pavan, 2013; Wunnapuk

Guang-Liang Hong and Jia-Ming Liu contributed equally to this study.
Grant sponsor: key construction academic subject of Zhejiang Province; Grant number: 2012-XK-A28;
Grant sponsor: key discipline construction project of colleges and universities in Zhejiang Province;
Grant sponsor: Natural Science Foundation of Zhejiang Province; Grant number: LQ14H1500002; Grant sponsor: key research project of Traditional Chinese Medicine in Zhejiang province; Grant number: 2015ZZ015.
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Manuscript Received: 31 March 2015; Manuscript Accepted: 9 September 2015
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 00 Month 2015
DOI 10.1002/jcb.25370 © 2015 Wiley Periodicals, Inc.
et al., 2013]. Kim et al. [2009] studied 278 patients and confirmed that kidney injury occurred in all patients after PQ poisoning. For kidney is the main organ for PQ excretion, impaired renal function results in a reduced PQ clearance, which may exacerbate the injuries to other organs. Clinical studies have shown that renal injury is an independent risk factor leading to the death of PQ-poisoning patients [Kim et al., 2009]. Currently, this disease has no clinically effective drugs and mainly depends on blood purification treatment, which brings not only damages to the body but also high costs to the patients. Therefore, to actively explore drugs that can effectively intervene with renal injury caused by PQ poisoning is of a great significance for preventing excretion of toxic substances and improving the prognosis of patients.

With the research and development of drugs, many compounds of natural origin have been found to have unique physiological activities. On this basis, a large number of drugs with special treatment effect have been developed. Natural active substances have features including high activity, low adverse reactions, and unique effect. CF is a typical triterpene alcohol unique in rice bran oil. Recent studies have shown that CF can directly remove reactive oxygen species (ROS), inhibit ROS generation via regulating cell membrane NAD(P) oxidase system, inhibit nuclear factor-kappaB (NF-κB) activity, and reduce the activities of COX-2 (cyclooxygenase-2) and iNOS (inducible nitric oxide synthase) and the expression of proinflammatory cytokines TNF-α (tumor necrosis factor-α), IL-1β (interleukin-1β), and IL-6 [Islam et al., 2011]. The studies of Kong et al. [2009] indicated that CF promoted apoptosis in SW480 human colon adenocarcinoma cells and sensitized the metastatic SW620 cells to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis. Thus, CF has various bioactivities such as anti-oxidant, anti-inflammatory, and anti-tumor among other activities, with a wide range of medicinal value [Islam et al., 2011; Hong et al., 2013; Mizushima et al., 2013]. For instance, Mizushima et al. [2013] showed that CF could inhibit mammalian DNA polymerase and suppresses inflammation. Oka et al. [2010] found that CF effectively attenuated mast cell degranulation. Interestingly, our previous studies also found that CF could effectively antagonize PQ-induced renal toxicity [Hong et al., 2013] and thus may form an effective renal protective drug. Its effectiveness and mechanisms warrant further investigations.

Meanwhile, preliminary experiments in this study also showed that nuclear factor E2-related factor-2 (NRF2)-antioxidant response element (ARE) pathway played a crucial role in CF antagonizing PQ-induced injury in HK-2 cells [Hong et al., 2013]. NRF2-ARE signaling pathway consisting of NRF2 and its downstream ARE is an important defensive signal transduction pathway against internal and external stimuli such as oxidation, chemical, etc. in the body [Toyokuni and Akatsuka, 2007]. In recent years, the regulatory effect of NRF2-ARE on the activities of downstream drug transporters received growing attention. ATP-binding cassette (ABC) family proteins are important renal tubular transporters for excretion of toxins, with ABCB1 (ATP-binding cassette, sub-family B [MDR/TAP], member 1; also known as MDR1 [multidrug resistance protein 1] and P-gp [P-glycoprotein]), ABC1 (ATP-binding cassette, sub-family C [CFTR/MRP], member 1; also known as MRP1 [multidrug resistance-associated protein 1]), and ABCG2 (ATP-binding cassette, sub-family G [WHITE], member 2) as the most important representatives of the family, the expression of which is under the regulation of NRF2-ARE pathway [Suttana et al., 2010; Ishikawa et al., 2013; Ji et al., 2013]. These transporters can promote the pumping of toxins or drugs out of the cells, playing a key role in many aspects such as toxin excretion, drug metabolism, tumor drug resistance, etc. [Klaassen and Aleksunes, 2010]. The activity of ABC transporters may, thus, affect the PQ excretion in renal tubular cells and play an important role in PQ pathogenesis. Therefore, the present study further explored whether the reversal of PQ toxicity by CRF is associated with ABC pathways.

**MATERIALS AND METHODS**

**CELL CULTURE**

HK-2, a proximal tubular cell line derived from normal human kidney, was obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in nutrient mixture F12 supplemented with penicillin G (100 U/mL), streptomycin (100 μg/mL), and 10% fetal calf serum in a humidified incubator with 5% CO₂ atmosphere at 37°C.

**HPLC**

PQ was dissolved in deionized water, prepared into 1.0 mg/mL stock solution, and stored in 4°C refrigerator for further use. To extract the sample, 0.5 mL media were centrifuged at 12,000 r/min, and the supernatants were directly used for sample injection. For cell samples, the cells were collected and added with 0.2 mL RIPA cell lysis buffer. After complete lysis of the cells, the lysates were added with 1 mL of chromatography mobile phase and centrifuged at 12,000 r/min. The sample was then filtered through an organic solvent-compatible 0.45-μm syringe filter and used for injection. The instrumental analysis was performed using Agilent 1100 series HPLC system, equipped with DAD detector; the detection wavelength was 256 nm, and the column used was Agilent 20RBAX-SB-Aq (5 μm, 4.6 × 250 mm); the mobile phase was 1% acetonitrile and 99% aqueous trifluoroacetic acid (0.1%), at a flow rate of 0.6 mL/min, and the injection volume was 20 μL.

**MTT ASSAY**

The growth-inhibitory effect of PQ and the protective effects of CF were measured using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay. HK-2 cells (6 × 10⁴ cells/well) were seeded in 96-well tissue culture microplate for 24 h, and then the cells were exposed to different treatments. After that, 25 μL of MTT solution (5 mg/mL in phosphate buffered saline) was added into each well and incubated for 4 h at 37°C. The metabolized MTT product was dissolved in DMSO and quantified by measuring the optical density at 570 nm on a microplate reader (Dynex Technologies, USA).

**FLOW CYTOMETRIC ANALYSIS**

After exposure to different treatments of PQ and CF, HK-2 cells were washed with PBS and fixed in 75% ethanol overnight at −20°C. The fixed cells were stained with PI (1.21 mg/mL Tris, 700 U/mL RNase, 50.1 μg/mL PI, pH 8.0) for 4 h in darkness. Red PI
fluorescence was measured with Coulter Epics XL flow cytometer (Coulter, Germany) using CXP software. In each sample, 10,000 events for apoptosis detection and cell cycle analysis were measured. Data were analyzed with the Expo32 analysis tool. DNA histogram was represented by the proportion of cells in G0/G1, S, G2/M phases. Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in the cell cycle pattern.

ANNEXIN-V–FLUOS STAINING ASSAY
Annexin-V–FLUOS staining assay was used to detect the plasma membrane alterations in cells treated with PQ. Briefly, the cells treated with PSP-SeNPs for 24 h were stained with annexin-V–FLUOS (2 μg/mL) in binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, pH 7.4) for 15 min. The slides were then washed with PBS and directly observed by fluorescence microscopy (Bio-Rad Radiance 2100 MP Scanning System).

WESTERN BLOT ANALYSIS
Treated HK-2 cells were harvested and collect as cell pellets, which were lysed in lysis buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) on ice for 1 h. After centrifugation at 14,000g for 15 min, supernatants were collected and measured for protein concentration by using bicinchoninic acid assay (Sigma) according to the manufacturer’s instructions. The samples were mixed with sample buffer, boiled for 5 min and stored at −80°C until use. Treated HK-2 cells were washed twice with PBS and lysed in lysis buffer (50 mM Tris–Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) on ice for 1 h, and centrifuged at 12,000g for 10 min at 4°C. Total protein, as determined by the Bio–Rad protein assay, was mixed with 2× loading buffer (Sigma), and pre-heated at 95°C for 5 min. The samples were separated on SDS denaturing 12% polyacrylamide gel and electrophoretically transferred to PVDF membranes. The membranes were then blocked with 5% non-fat milk, washed, and probed with primary antibodies purchased from Cell Signaling Technology at 1:1,000 dilutions overnight at 4°C. After washing, the membranes were incubated with corresponding secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology) and visualized by Pierce ECL Western blotting substrate.

TRANSFECTION OF HK-2 CELLS WITH ABCC1 SIRNA
The siRNA test was conducted using ABCC1 (Locus ID 4363) Trilencer-27 Human SiRNA Kit (OriGene). One day prior to transfection, the cells were plated onto 6-well plate at a density of 1–4 × 10⁵/well, with each well containing 2 mL DMEM (with FBS and antibiotics). After 24 h, when the cells reached a 70–90% confluence, transfection was performed using X-tremeGENE siRNA transfection reagent (Roche) according to the manufacturer’s instructions. Briefly, siRNA was added into the well at a ratio of 5:1; after culturing at 37°C for 20–24 h, the media containing transfection complex were replaced with fresh culture medium containing FBS. After another 3–8 h, the culture was used for subsequent post-transfection detections.

STATISTICAL ANALYSIS
Experiments were carried out at least in triplicate and results were expressed as mean ± SD. Differences between two groups were analyzed by two-tailed Student’s t-test, whereas those between three or more groups were analyzed by one-way analysis of variance (ANOVA). Difference with P < 0.05 (*) or P < 0.01 (**) was considered statistically significant.

RESULTS

THE UPTAKE OF PQ BY HK-2 CELLS
As shown in Figure 1A, the chromatogram of PQ standard evidently displays sharp characteristic peaks of PQ standard sample, indicating a successful sample separation and good detection effect. To analyze PQ content, we first established a PQ standard curve, i.e., six concentrations of PQ were analyzed by HPLC, and linear regression was conducted based on the calculated peak areas. The R² value (R² = 0.9999) demonstrated a linear correlation and good reliability of the method (Fig. 1B). We next examined the accumulation of PQ in cells from the aspects of dose and time course, and the results showed that with either the increase of PQ addition or the time prolonging, the actual concentrations of PQ in the medium and HK-2 cells increased gradually (Fig. 2).

CYTOTOXICITY OF PQ AND THE PROTECTIVE EFFECT OF CF
Our previous studies found that CF can reduce PQ-induced cytotoxicity in a concentration-dependent manner [Hong et al.,

![Image](323x114 to 550x366)

**Fig. 1.** HPLC chromatograms of PQ. (A) Standard of PQ added. The used HPLC conditions are described in Materials and Methods. (B) PQ standard curve. Six concentrations of PQ were analyzed by HPLC, and linear regression was conducted based on the calculated peak areas. PQ, paraquat.
Here, we further confirmed that after simultaneous addition of both PQ and CF, HPLC detection indicated that CF can concentration dependently reduce the accumulation of PQ in HK-2 cells (Fig. 3A). Meanwhile, the results of MTT assay showed that with the increase in CF concentration, the viability of HK-2 cells increased too; the effect of CF alone on the viability of HK-2 cells was not significant (Fig. 3B), indicating that CF can effectively suppress the accumulation of PQ in HK-2 cells and thereby reduce its cytotoxic effects.

**EFFECTS OF PQ ON ABC PROTEIN EXPRESSION AND THE REVERSING EFFECT OF CF**

CF can affect PQ accumulation in the cells, and the ABC family members are important renal tubular transporters for excretion of
toxins. Therefore, we specifically studied the relationship of PQ and CF with these transporter proteins. ABCB1, ABCC1, and ABCG2 are the most important representatives of ABC family. Western blot results showed that while PQ significantly inhibited the expression of ABCC1, it did not significantly influence ABCB1 level; there was no expression of ABCG2 in HK-2 cells (Fig. 4A and B). Our results also indicated that CF preconditioning can increase the expression of ABCC1, thereby abolishing the inhibitory effect of PQ on ABCC1 expression (Fig. 4C and D). Therefore, in HK-2 cells, PQ might have inhibited its own transport via suppressing ABCC1 expression and thereby increased its own accumulation in the cells. To the contrast, CF can promote the transport of PQ via enhancing ABCC1 expression and thereby reduce the intracellular accumulation of PQ and consequently its cytotoxic effects.

THE ROLE OF ABCC1 IN THE INHIBITORY EFFECT OF CF ON PQ ACCUMULATION IN CELLS

Our study was then deepened to verify the importance of ABCC1 in reversal of PQ toxicity by CF. First, the inhibitory effect of ABCC1 siRNA was tested, and the results showed that both 10 and 20 nM siRNA effectively inhibited the expression of ABCC1 in cells (Fig. 5A and B), and the addition of ABCC1 siRNA effectively inhibited CF-induced upregulation of ABCC1 protein (Fig. 5C and D). After the addition of PQ and CF as well as the transfection with ABCC1 siRNA simultaneously, HPLC detection of PQ contents in HK-2 cells showed that after suppressing the expression of ABCC1, even a higher concentration of CF failed to reduce the amount of PQ accumulation in the cells (Fig. 3A), suggesting that while CF can effectively attenuate the toxic effect of accumulated PQ in HK-2 cells in a concentration-dependent manner, ABCC1 siRNA can elevate the intracellular content of PQ, demonstrating that ABCC1 indeed plays an important role in CF reversal of PQ toxicity.

CF ANTAGONIZES PQ-INDUCED APOPTOSIS WITH THE INVOLVEMENT OF ABCC1

We further detected the role of ABCC1 in the antagonizing effect of CF on PQ-induced apoptosis. PI-flow cytometric analysis was employed to examine the effects of PQ on the cell cycle distribution and occurrence of apoptotic cell death. The results revealed that while PQ significantly induced the appearance of sub-G1 apoptotic peak, CF significantly inhibited apoptosis (Fig. 6). CF alone did not affect the cell cycle distribution, suggesting its good safety. Furthermore, TUNEL-DAPI co-staining assay was used to confirm the cell apoptosis by examination of apoptotic DNA fragmentation, an important biochemical feature of apoptosis. In the assay, the green fluorescence represents cells with DNA damage, and blue color indicates the nuclei. Overlapping of both colors indicate DNA damage occurring in the nuclear region. As shown in Figure 7, PQ significantly induced the occurrence of DNA fragmentation in cells, whereas CF effectively inhibited the adverse effect of PQ. The results also showed that ABCC1 siRNA effectively abolished the protective effect of CF on PQ-induced apoptosis, demonstrating the importance contribution of ABCC1 to the protective effect of CF against PQ-induced apoptosis.

DISCUSSION

Since firstly reported in 1966, PQ poisoning has become a clinical difficulty for its high mortality and lack of effective specific

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**Fig. 4.** The expression of ABC family proteins in HK-2 cells. (A and B) The cells were treated with PQ in a dose for 24 h, and the expression levels of ABCC1 and ABCB1 were analyzed by Western blot. (C and D) The cells treated with PQ (800 μM) and CF (80 μM) for 24 h, and the expression levels of ABCC1 and ABCB1 were analyzed by Western blot. Equal protein loading was confirmed by analysis of β-actin in the protein extracts. This experiment was repeated twice with similar results, *P < 0.01.
treatment. Kidney is not only one of the major target organs of PQ poisoning, but also the main pathway of PQ excretion. Clinical studies confirmed that renal injury was positively correlated with PQ poisoning-caused mortality [Wunnapuk et al., 2013]. Thus, PQ-induced renal toxicity currently becomes a research hotspot in the field, and searching for drugs with renal protective effect is of a great practical significance.

Recent studies have shown that CF has various bioactivities including antioxidant, lipid-lowering, and antitumorigenic activities [Kaneda et al., 2007; Nagasaka et al., 2007; Islam et al., 2008]. For example, Nagasaka et al. [2007] have recently reported that phytosterol ferulates isolated from rice bran inhibit NF-kB activity in macrophages. And Mizushina et al. [2013] found that Cycloartenyl trans-ferulate, a component of the bran byproduct of sake-brewing rice, could inhibit mammalian DNA polymerase and suppress inflammation. Given the multi-mechanisms underlying the cell-protective effect of CF, our previous studies established a model of PQ-induced tubular epithelial (HK-2) cell injury to observe the protective effect of CF on HK-2 cells. Our results showed that CF can concentration dependently reduce PQ-induced cytotoxicity and HK-2 cell apoptosis, and inhibit PQ-induced caspase activation, poly(ADP-ribose) polymerase (PARP) cleavage, mitochondrial dysfunction, and ROS generation via regulating Bcl-2 (B-cell lymphoma 2) family members; moreover, CF at concentrations lower than 160 μM showed no cytotoxic side effects [Hong et al., 2013], suggesting that CF can well antagonize PQ-induced nephrotoxicity and may become a safe and effective renal protective drug. Its effectiveness and underlying mechanisms warrant further explorations.

Our further studies also found that CF decreased the accumulation of PQ in HK-2 cells in a concentration-dependent manner, thereby alleviating the cytotoxic effects of PQ. Reduced intracellular accumulation of PQ suggested that it was transported out of the cells, the process of which was often regulated by a number of transporter proteins. Therefore, CF might have affected the expression of some transporter proteins and thereby reduced the intracellular accumulation of PQ. Renal excretion is a major route to clear drugs and their metabolites out of the body, including passive glomerular filtration and secretion and reabsorption of renal tubules and collecting ducts. The process of both secretion and reabsorption involves multiple drug-transporter proteins widely distributed on the renal tubules [Chen et al., 2007; Li et al., 2011; Lacher et al., 2014]. Renal transporters include 2 families: ABC and soluble carrier (SLC). Our previous studies also found that CF antagonizing PQ toxicity was related to NRF2 pathway [Hong et al., 2013], and recent studies showed that some drug transporters, including ABCB1, ABCC1, and ABCG2, are regulated by NRF2 [Kimura et al., 2006; Tanaka et al., 2008; Song et al., 2009; Cole, 2014]. For example, Song et al. [2009] reported that 15-Deoxy-delta 12, 14-prostaglandin J2-induced upregulation of multidrug resistance-associated protein 1 via Nrf2 activation in human breast cancer cells. Studies on the above transporters found that HK-2 cells did not express ABCG2, and both CF and PQ did not significantly affect the expression of ABCB1; however, while PQ downregulated the expression of ABCC1, CF increased ABCC1 expression and thereby reversed PQ-induced ABCC1 suppression. The studies of Lacher et al. [2014] indicate that paraquat is not a P-gp substrate. And the results of Tanaka et al. [2008] suggest that activation of Nrf2 protects mouse kidneys from FeNTA-induced oxidative stress damage by coordinately up-regulating the expression of cytoprotective genes (including Mrp1). The above results are consistent
with our findings. Therefore, in HK-2 cells, CF may antagonize PQ toxicity via ABCC1.

Furthermore, we used RNAi technology to verify the specific role of ABCC1 in CF antagonizing PQ toxicity and showed that the addition of ABCC1 siRNA effectively inhibited CF-induced upregulation of ABCC1 expression, and the suppressed ABCC1 expression abolished the inhibitory effect of CF on the amount of PQ accumulation in cells, indicating that CF indeed regulated PQ accumulation in the cells via modulating ABCC1. Further flow cytometric analysis of the role of ABCC1 in CF antagonizing PQ-induced apoptosis showed that PQ significantly induced the appearance of sub-G1 apoptotic peak, whereas CF significantly inhibited cell apoptosis; moreover, CF alone did not affect the distribution of cell cycle. Meanwhile, TUNEL-DAPI double-staining detected that PQ significantly induced the occurrence of DNA fragmentation in cells, which, however, was effectively suppressed by CF. Our results also showed that ABCC1 siRNA effectively inhibited the protective effect of CF on PQ-induced apoptosis, further confirming that CF indeed antagonizes PQ toxicity in HK-2 cells via ABCC1. Kimura et al. [2006] also found that Interferon-γ played protective roles in sodium arsenite-induced renal
injury by up-regulating intrarenal multidrug resistance-associated protein 1 expression. And El Azreq et al. [2012] indicate for the first time that collagen/β1 integrin/ERK signaling up-regulates the expression and function of ABCC1 and suggest that its activation could represent an important pathway in cancer chemoresistance. Therefore, beside the regulation of ABC family proteins, other mechanisms may be involved in the protective effects of CF on PQ. For instance, CF could reverse PQ-induced cytotoxicity in HK-2 cells via regulation of NRF-2 signaling pathways and other ROS-mediated signaling. Taken together, CF could effectively reverse the PQ-induced apoptotic cell death via inhibition of PQ accumulation and regulation of ABCC1 expression and other signaling pathways, which provides a new strategy for treatment of PQ poisoning and renal toxicity.

ACKNOWLEDGMENTS

This study was partly supported by the grant of key construction academic subject of Zhejiang Province (2012-XK-A28), the key discipline construction project of colleges and universities in Zhejiang Province, Natural Science Foundation of Zhejiang...
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