# Nrf2 overexpression protects against paraquat-induced A549 cell injury primarily by upregulating P-glycoprotein and reducing intracellular paraquat accumulation

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**Abstract.** Paraquat (PQ) intoxication causes thousands of mortalities every year, worldwide. Its pulmonary-targeted accumulation and the acute lung injury it subsequently causes, remain a challenge for detoxification treatment. A previous study has demonstrated that the upregulation of nuclear factor erythroid-2 related factor 2 (Nrf2) prevents PQ toxicity in cell line and murine models. As Nrf2 target genes include a group of membrane transporters, the current study assessed the protective mechanism exerted by Nrf2 against PQ toxicity and intracellular PQ accumulation via its effects on P-glycoprotein (P-gp), a downstream transporter of Nrf2. Adenovirus vectors containing the Nrf2 gene were transfected into A549 cells. Cell proliferation was assessed by Cell Counting Kit-8. The levels of LDH, MDA, SOD, TNF-α, IL-6 levels were detected using their respective ELISA kits. In addition, the levels of Nrf2 and P-gp protein expression were detected by western blot analysis. The concentration of PQ was measured by HPLC. The results revealed that overexpressed Nrf2 significantly increased P-gp protein levels, decreased the intracellular accumulation of PQ and attenuated PQ-induced toxicity. However, the protective effects of Nrf2 overexpression on PQ-challenged A549 cells were abrogated following cyclosporine A treatment, a competitive inhibitor of P-gp, which also increased intracellular PQ levels. These data indicated that Nrf2 gene overexpression prevented PQ toxicity in A549 cells, potentially via the upregulation of P-gp activity and the inhibition of intracellular PQ accumulation. Thus, Nrf2 and P-gp may serve as potential therapeutic targets for the treatment of PQ-induced injury.

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*Key words:* paraquat, nuclear factor erythroid-2 related factor 2, p-glycoprotein, transport, cyclosporine A

## Introduction

Paraquat (*N,N*'-dimethyl-4,4'-bipyridinium dichloride; PQ) is one of the most commonly used herbicides worldwide (1,2). However, long-term accumulation or acute intoxication of PQ may cause organ injury, resulting in a mortality rate of >60-70% (3,4). Following oral administration, PQ is absorbed through the gastrointestinal tract within 1-2 h and accumulates in various organs, including the lung, liver, kidney and central nervous system (5). PQ primarily amasses within alveolar epithelial and bronchiolar Clara cells of the lung, and results in acute lung injury and acute respiratory distress syndrome, which is the leading cause of mortality in patients with PQ poisoning (6). Although the exact pathogenic mechanism of PQ remains largely unknown, PQ-induced pulmonary-specific accumulation, excessive oxidative stress, inflammatory injury and an imbalance in the deposition of the extracellular matrix, are major contributors to lung injury following PQ intoxication (7-9). However, pulmonary-targeted accumulation, which results in acute lung injury, remains a challenge for detoxification treatment.

P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter family, is vital for many cellular processes that require the transport of chemicals across the cell membrane (10,11). P-gp hyperactivity causes drugs to be pumped out of cells, resulting in chemotherapeutic agent and antimicrobial drug resistance (12,13). Previous studies have demonstrated that P-gp may be closely associated with the removal of PQ from cells (14,15). In corroboration with this association, P-gp levels are significantly increased in rat alveolar type II cells following PQ exposure (16) and a high level of P-gp has been revealed to alleviate PQ toxicity in human epithelial colon cancer cells and to significantly reduce rat lung tissue PQ concentration (17,18). However, these protective effects were reversed following treatment with a specific

inhibitor of P-gp, cyclosporine A (CsA) (18). Therefore, P-gp appears to serve an important role in PQ transport across cells and upregulating the activity of P-gp may be an effective measure to attenuate the intracellular accumulation of PQ and thus, its toxicity.

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a transcription factor that belongs to the cap 'n' collar family of basic leucine zipper proteins, which is tightly regulated by Kelch-like ECH-associated protein 1 (Keap1) (19). The Keap1-Nrf2 pathway, including its targeted cytoprotective protein expression, is the fundamental mechanism for cellular defence against oxidative and electrophilic stress (20,21). Various natural compounds, including cycloartenyl ferulate and resveratrol, inhibit PQ-induced oxidative stress and apoptosis by activating the Nrf2 pathway (22,23). A previous study demonstrated that Nrf2 overexpression attenuated PQ toxicity in A549 cells and mice by activating heme oxygenase-1 and NAD(P)H: Quinone oxidoreductase 1 (24). The primary focus of research on Nrf2 targets has been on detoxifying/antioxidant enzymes; however, several ABC transporters are Nrf2 targets (25). Furthermore, previous studies have revealed that the activation of Nrf2 is necessary to increase P-gp activity (26-28). Therefore, the current study hypothesized that Nrf2 may prevent organ injury in PQ poisoning by increasing P-gp activation and reducing intracellular PQ concentration.

The present study revealed that the Nrf2 gene was overexpressed in the A549 cell line. Subsequent to this result, the role of P-gp activation in PQ-challenged A549 cells was determined. It was hypothesized that Nrf2 and P-gp activity may be potential targets for the prevention of organ injury in PQ poisoning.

## Materials and methods

*Materials and reagents*. PQ and the adenoviral (AD) system, including plasmids containing the predesigned human Nrf2 gene and fetal bovine serum (FBS), were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). RPMI-1640 medium was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham MA, USA). A549 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The Cell Counting Kit-8 (CCK-8) was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Lactate dehydrogenase (LDH), superoxide dismutase (SOD), malondialdehyde (MDA), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) detection kits were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Antibodies against Nrf2 and P-gp were obtained from Abcam (Cambridge, UK). GFP was purchased from Thermo Fisher Scientific, Inc. CsA (cat. no. 59865-13-3) was purchased from Sigma-Aldrich; Merck, KGaA.

*Transfection and transfection efficiency analysis*. A549 cells were grown in a monolayer culture in RPMI-1640 medium supplemented with 10% FBS at 37˚C in a humidified atmosphere with 5% $CO_2$. Following this incubation, cells ($4x10^4$ cells/well) were inoculated onto a 96-well culture plate and left to grow in the logarithmic stage for 24 h at 37˚C. A549 cells were transfected with adenoviral vectors containing either AD-Nrf2 or AD-(GFP) (cat. no. PEP033; Thermo Fisher Scientific, Inc.), at a multiplicity of infection of 50. Control cells were cultured in RPMI-1640 medium only. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and maintained at 37˚C in a 5% $CO_2$-humidified incubator for 24 h. A fluorescence microscope (magnification, x100; Olympus CKX41SF; Olympus Corporation, Tokyo, Japan) was used to determine the percentage of GFP synthesizing cells. The ratio of cells that emitted green fluorescence in the same field of view was regarded as the transfection efficiency. Nrf2 overexpression was also confirmed via western blotting.

*Cell culture and treatments*. The current study included five groups: A control group, a PQ group, a PQ + AD-Nrf2 group, a PQ + AD-Nrf2 + CsA group and a control + CsA group. A549 cells were grown in a monolayer culture in RPMI-1640 medium supplemented with 10% FBS and maintained at 37˚C in a 5% $CO_2$-humidified incubator. A549 cells ($4x10^4$ cells/well) were seeded onto a 96-well culture plate. Following logarithmic stage growth for 24 h at 37˚C, A549 cells were transfected with AD-Nrf2 and preincubated with CsA at a concentration of 12 μg/ml or vehicle control (0.1% ethanol). Cells were subsequently incubated in RPMI-1640 medium supplemented with 10% FBS and cultured at 37˚C in a 5% $CO_2$-humidified incubator for 24 h and the experimental groups were administered 10 μl PQ ($1x10^3$ mol/l). Following further incubation for 24 h at 37˚C, cells were harvested and used for subsequent experimentation.

*CCK-8 assay*. Cell survival was assessed using a CCK-8 kit, according to the manufacturer's protocol and viable cell density was adjusted to $4x10^4$ cells/ml. A549 cells ($4x10^4$ cells/well) were seeded onto a 96-well culture plate and cultured in RPMI-1640 medium supplemented with 10% FBS and maintained at 37˚C in a 5% $CO_2$-humidified incubator for 24 h. Growth medium was replaced with serum-free medium and cells were cultured for a further 24 h. Subsequently, the supernatant was discarded and 10 μl CCK-8 solution was added to each well. Following incubation for 1 h at 37˚C, absorbance was measured at 450 nm using a Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

*LDH release assay*. The LDH release was examined using a LDH release assay, according to the manufacturer's protocol. The release of LDH was expressed as a percentage of the total LDH quantity in cells treated with 2% Triton X-100.

*MDA, SOD, TNF-α and IL-6 detection*. The cells were centrifuged at 14,000 x g for 15 min at 4˚C and the supernatant was transferred to centrifuge tubes. The levels of MDA (cat. no. A003-1), SOD (cat. no. A001-3), TNF-α (cat. no. H052) and IL-6 (cat. no. H007) in cell supernatant was detected using their respective ELISA kits, according to the manufacturer's protocol. Samples were analyzed using a spectrophotometer. TNF-α, IL-6 and SOD activities were expressed in pg/ml and MDA levels were expressed as nmol/ml.

*Western blot analysis*. Total protein was extracted from A549 cells using radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China) and

incubated on ice for 30 min. Cell lysates were centrifuged for 20 min at 16,000 x g at 4˚C. Total protein was quantified using a bicinchoninic acid assay and 20 $\mu$g protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.) and blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary antibodies against Nrf2 (1:1,000; cat. no. ab62352), P-gp (1:1,000; cat. no. ab170904) or GAPDH (1:5,000; cat. no. ab8245; all Abcam, Cambridge, UK) antibodies overnight at 4˚C. Membranes were washed with Tris-buffered saline Tween® 20. Following primary incubation, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2,000; cat. no. 14708) or mouse (1:2,000; cat. no. 14709; both Cell Signaling Technology, Inc., Danvers, MA, USA) secondary antibodies for 2 h at room temperature. Protein bands were visualized using the chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Quantitative analysis was performed using Image J software (Version 1.8.0_172; National Institutes of Health, Bethesda, MD, USA). GAPDH was utilized as the loading control.

*High performance liquid chromatography (HPLC) analysis*. Transfected A549 cells exposed to PQ were harvested. Following repeated freezing and thawing, cells were centrifuged at 16,000 x g for 5 min at 4˚C. Cell supernatant (200 $\mu$l) was subsequently collected and treated with an equivalent volume of acetonitrile (200 $\mu$l; cat. no. A0793; TCI Development Co., Ltd., Shanghai, China). Following centrifugation at 12,000 x g for 15 min at 4˚C, 200 $\mu$l cell supernatant was filtered through an organic solvent-compatible 0.45-$\mu$m syringe filter (cat. no. F512545; Sangon Biotech Co., Ltd, Shanghai, China) and 20 $\mu$l was injected into an Agilent 1100 series HPLC system (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a diode-array UV detector, on-line degasser, autosampler, thermostat-columned compartment and quaternary pump. Samples were eluted isocratically with an isocratic elution composed of 4% mobile phase A (acetonitrile) and 96% mobile phase B (20 mM sodium dihydrogen phosphate, 0.4 mM sodium heptanesulfonate and adjusted to pH 2.3) at a flow rate of 0.6 ml/min at 30˚C. Separation was performed using an Agilent Zorbax-SB-Aq column (internal diameter, 4.6 mm; length, 250 mm; Agilent Technologies, Inc.) and results were detected at a wavelength of 256 nm.

*Statistical analysis*. Experiments were performed at least three times and data were presented as the mean ± standard deviation. Differences between three or more groups were analyzed using one-way analysis of variance followed by the least significant difference test. P<0.05 was considered to indicate a statistically significant result.

## Results

*Transfection efficiency analysis*. The results of transfection efficacy analysis verified that the control group did not express green fluorescence (Fig. 1A). From the green fluorescence observed under a magnification of x100, AD vector transfection efficiency was determined to be >90% (data not shown). The results of western blotting revealed that Nrf2 expression in cells transfected with AD-Nrf2 was significantly higher compared with the control group (P<0.001; Fig. 1B), which demonstrated that the cell model that was abundant for Nrf2 expression, and that the use of AD vectors was successful.

*Cell viability*. The results demonstrated that PQ exposure significantly reduced A549 cell viability (P<0.001; Fig. 2A) and that PQ-induced cytotoxicity was alleviated following Nrf2 treatment (P<0.001; Fig. 2A). However, treatment with CsA significantly reversed the protective effects of Nrf2 on cell viability following PQ exposure (P<0.01; Fig. 2A). No significant differences were identified between the control and the control + CsA group.

*LDH activity*. The LDH activity of A549 cells was assessed to determine PQ-induced cell injury. The LDH activity of PQ-exposed cells was significantly increased when compared with the control group (P<0.001; Fig. 2B). However, treatment with Nrf2 reversed the PQ induced increase of LDH activity (P<0.01; Fig. 2B). Furthermore, LDH activity significantly increased in cells of the PQ + AD-Nrf2 + CsA-treated group compared with those of the PQ + AD-Nrf2 group (P<0.01; Fig. 2B). No significant differences were identified between the control and the control + CsA group.

*SOD and MDA*. Compared with the control group, a significant reduction in SOD activity following PQ exposure was observed (P<0.001; Fig. 3A). However, treatment with AD-Nrf2 reversed this effect (P<0.01; Fig. 3A). The SOD activity of the PQ + AD-Nrf2 + CsA-treated group was also significantly decreased compared with those cells exposed to PQ + AD-Nrf2 treatment (P<0.01; Fig. 3A).

Cells of the PQ group exhibited a significant increase in MDA concentration when compared with the control (P<0.001; Fig. 3B). Whereas, the MDA concentration of cells treated with PQ + AD-Nrf2 was significantly reduced when compared with the PQ group (P<0.001; Fig. 3B). However, treatment with CsA reversed the protective effect of AD-Nrf2 (P<0.001; Fig. 3B). No significant differences were identified in SOD activity or MDA concentration between the control and the control + CsA group.

*TNF-α and IL-6*. When compared with the control group, significant increases in TNF-α and IL-6 were observed in the PQ group (P<0.001; Fig. 3C and D). AD-Nrf2 treatment reversed these increases (TNF-α, P<0.001; IL-6, P<0.01; Fig. 3C and D) and the levels of TNF-α and IL-6 in the PQ + AD-Nrf2 + CsA-treated group were significantly increased compared with cells exposed to PQ + AD-Nrf2 (P<0.01; Fig. 3C and D). No significant differences were identified in the levels of TNF-α or IL-6 expression between the control and the control + CsA group.

*P-gp protein expression*. Treatment with PQ induced a significant decrease of P-gp protein levels when compared with the control group (Fig. 4A). However, P-gp protein expression increased ~1.9-fold in the AD-Nrf2-treated group compared with the PQ group (P<0.001; Fig. 4B). Treatment with CsA was demonstrated to reverse the increase in P-gp expression in the PQ + AD-Nrf2 + CsA group compared with the

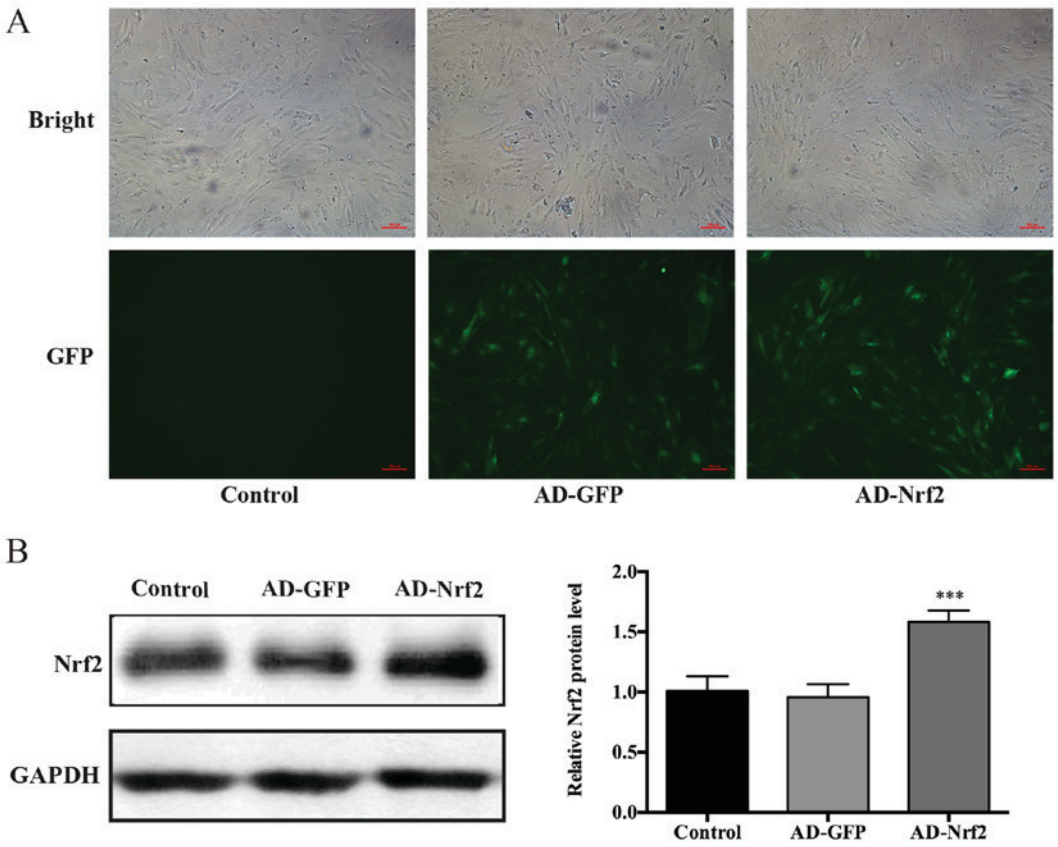


Figure 1. Transfection efficiency analysis. (A) Following 24 h transfection, A549 cells were observed using a fluorescence microscope. Scale bar=100 $\mu$m. (B) Representative western blotting images presenting Nrf2 protein expression. Data are presented as the mean ± standard deviation (n=4). ***P<0.001 vs. with the control group. Bright, bright field; Nrf2, nuclear factor erythroid-2 related factor 2; AD, adenovirus.

PQ + AD-Nrf2 group (P<0.001; Fig. 4B). No significant differences were identified in the level of P-gp protein expression in the control group compared with the control + CsA group.

*PQ concentration*. The concentration of PQ in the PQ-treated group was 3.14±0.14 mg/l. Cells that received PQ + AD-Nrf2 treatment exhibited a significant decrease in PQ concentration compared with the PQ group (2.53±0.15; P<0.001; Fig. 5). Furthermore, cells of the PQ + AD-Nrf2 + CsA group significantly reversed the effect of AD-Nrf2 treatment, resulting in a PQ concentration of 2.85±0.22 mg/l (P<0.01; Fig. 5).

## Discussion

PQ intoxication and the subsequent selective accumulation of PQ molecules results in multi-organ failure and severe pulmonary injury when in the lung (29). The current study successfully constructed Nrf2-overexpressed A549 cells that exhibited resistance to PQ toxicity. Nrf2 treatment was revealed to upregulate P-gp activity and subsequently reduce the intracellular accumulation of PQ and cell injury. However, these results were reversed following treatment with the specific inhibitor of P-gp (CsA). To the best of our knowledge, this is the first study to assess the role of Nrf2 treatment on P-gp expression and PQ concentration in PQ exposed cells, which may serve as potential therapeutic targets for PQ detoxification.

Nrf2 is a key transcription factor that has been demonstrated to be an effective target for the prevention or treatment of various human diseases, which include cardiovascular diseases, neurodegenerative diseases, neuropsychiatric disorders and cancer (30-34). Previous studies have revealed that the upregulation of Nrf2 is critical for cytoprotection against various types of cell injury, which include hydrogen peroxide-induced oxidative stress and inflammation (35-37). In another previous study, mifepristone-induced Nrf2 gene overexpression in the lungs ameliorated PQ-induced injury by activating the Nrf2-antioxidant response element (ARE) pathway (38). In addition, silent information regulator 2-related enzyme 1 was demonstrated to trigger the Nrf2/ARE antioxidant pathway and protect against lung injury induced by PQ poisoning (39). These studies have revealed the important role of Nrf2 in the prevention of PQ toxicity. Consistent with these results, the Nrf2 gene-transfected A549 cells of the current study presented resistance to PQ toxicity, as evidenced by an elevated cell viability, a decreased LDH activity, an improved oxidative stress response and an attenuation of inflammation, with decreased TNF-α and IL-6 levels following PQ challenge.

In the present study, it was demonstrated that the overexpressed Nrf2 gene also decreased intracellular PQ concentrations, which indicated that certain membrane transporters may be involved in Nrf2-induced cytoprotection against PQ toxicity. Not including phase-II detoxification enzymes, the activities of Nrf2-regulated phase-III drug transporters represent a cellular mechanism that protects against

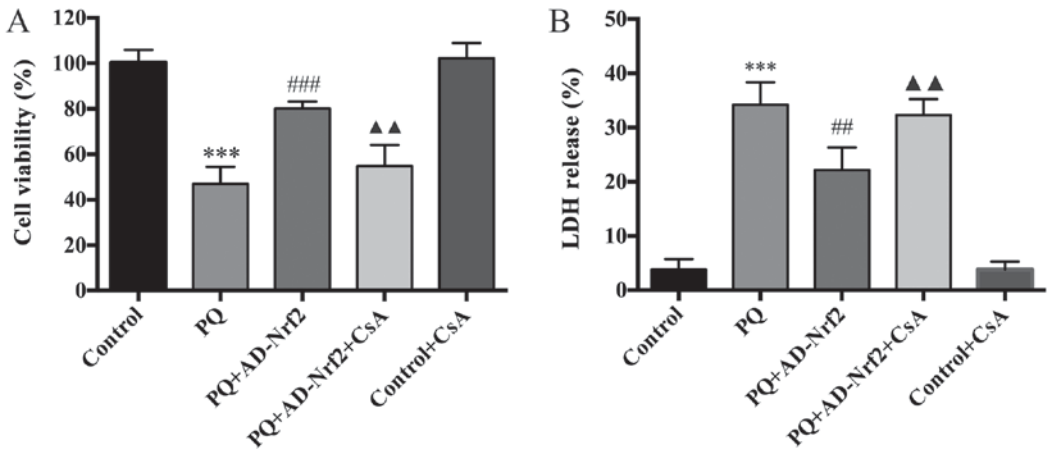


Figure 2. Effect of Nrf2 and CsA on A549 cell viability and LDH activity. (A) Cell viability and (B) LDH activity were assessed. Data are expressed as the mean ± standard deviation (n=4). ***P<0.001 vs. the control group; ###P<0.001 and ##P<0.01 vs. the PQ group; ▲▲P<0.01 vs. the PQ + AD-Nrf2 group. Nrf2, nuclear factor erythroid-2 related factor 2; CsA, cyclosporine A; LDH, lactate dehydrogenase; PQ, paraquat; AD, adenovirus.

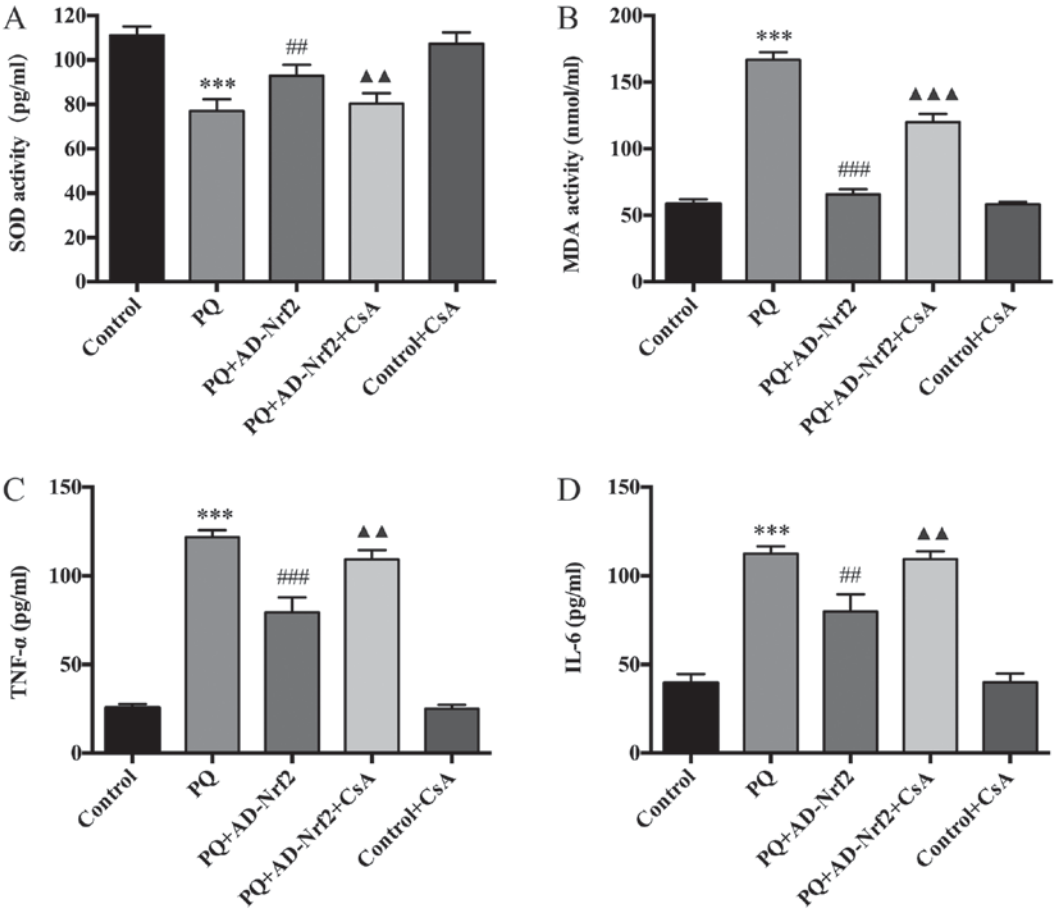


Figure 3. Effect of Nrf2 and CsA on oxidative stress and inflammation. (A) SOD activity, (B) MDA content, (C) TNF-α protein expression and (D) IL-6 protein expression were determined. Data are presented as the mean ± standard deviation (n=4). ***P<0.001 vs. the control group; ###P<0.001 and ##P<0.01 vs. the PQ group; ▲▲▲P<0.001 and ▲▲P<0.01 vs. the PQ + AD-Nrf2 group. Nrf2, nuclear factor erythroid-2 related factor 2; CsA, cyclosporine A; SOD, superoxide dismutase; MDA, malondialdehyde; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; PQ, paraquat; AD, adenovirus.

xenobiotics (40). Maher *et al* (41) revealed that the activation of the Nrf2 pathway may stimulate the coordinated induction of hepatic multi-drug resistance-associated proteins, which are ATP-dependent efflux transporters that serve an important role in cellular defense against various xenobiotics. The activities of other membrane transporters, including organic cation transporters, are also induced by Nrf2 (42). Since P-gp has been extensively studied and demonstrated to be closely associated with PQ excretion (17,43,44), the present study further assessed whether P-gp served a role in the Nrf2-induced inhibition of PQ accumulation and cytoprotection in PQ-challenged A549 cells.

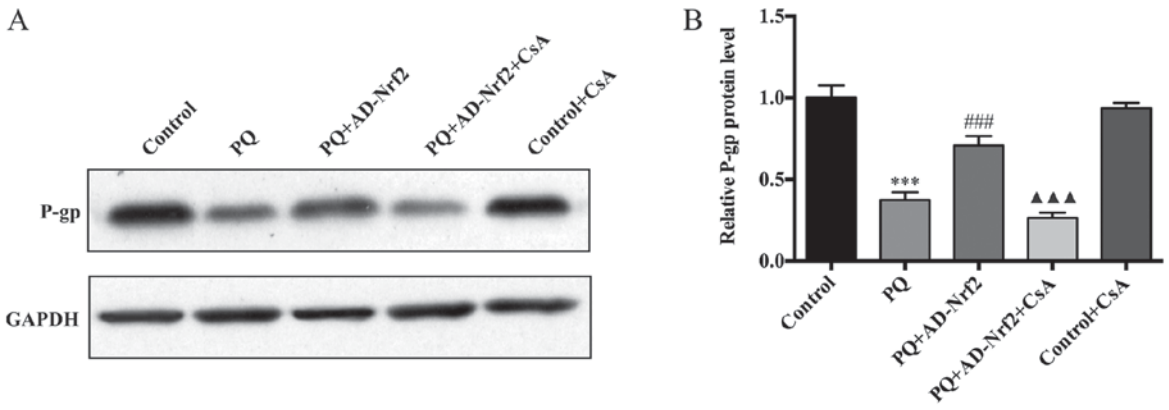


Figure 4. Effect of Nrf2 and CsA on the expression of P-gp. (A) Representative western blotting images presenting P-gp and GAPDH expression. (B) Quantified P-gp protein levels from western blotting data. Data are presented as the mean ± standard deviation (n=4). ***P<0.001 vs. the control group; ###P<0.001 vs. the PQ group; ▲▲▲P<0.001 vs. the PQ + AD-Nrf2 group. Nrf2, nuclear factor erythroid-2 related factor 2; CsA, cyclosporine A; P-gp, P-glycoprotein; PQ, paraquat; AD, adenovirus.

In the current study, P-gp protein levels significantly increased following Nrf2 overexpression in A549 cells. Upregulated P-gp was also demonstrated to reduce intracellular PQ concentrations, which was ameliorated following treatment with the specific inhibitor of P-gp (CsA). Furthermore, Nrf2-induced cytoprotection against oxidative stress and inflammation was inhibited following CsA treatment. These results indicate that P-gp serves a vital role in PQ transport and in the protective effects of Nrf2 against PQ toxicity. The current study corroborated with several previous studies (38,39). It has been demonstrated that an increased P-gp protein level exerts protective effects against PQ-induced toxicity *in vitro* and *in vivo*, by inhibiting the accumulation of intracellular PQ, indicating that PQ may be a P-gp substrate (18). Several other studies have revealed that a decrease of P-gp activity at the blood-brain barrier leads to the increased accumulation of neurotoxicants in the brain (45-47).

The present study had several limitations. Experiments of the current study were performed *in vitro*, meaning that the influences of Nrf2 and P-gp *in vivo* are unknown. In addition, Nrf2 overexpression in PQ poisoning may also activate other various key efflux transporters excluding P-gp, which include multidrug resistance-associated proteins and organic anion transporting polypeptide 2. Thus, additional studies are required to assess Nrf2 and the role of other transporters *in vitro* and *in vivo*.

In summary, the current study demonstrated that the Nrf2-mediated increase of P-gp is an important theoretical pathway against PQ toxicity, which reduces intracellular PQ concentrations. Therefore, the present study provides a novel therapeutic target for PQ accumulation and toxicity within the lung.

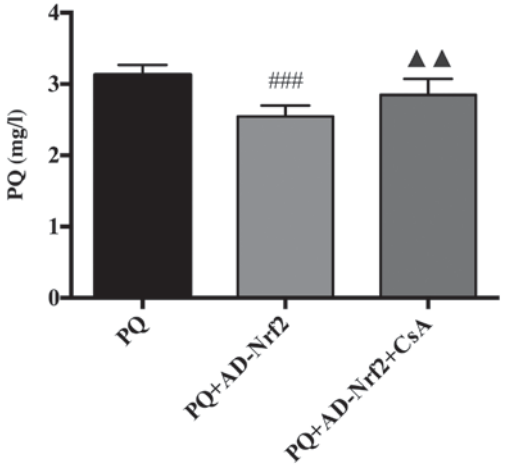


Figure 5. Effect of Nrf2 and CsA on the concentration of PQ. Data are presented as the mean ± standard deviation (n=4). ###P<0.001 vs. the PQ group; ▲▲P<0.01 vs. the PQ + AD-Nrf2 group. Nrf2, nuclear factor erythroid-2 related factor 2; CsA, cyclosporine A; PQ, paraquat; AD, adenovirus.

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Not applicable.

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**Availability of data and materials**

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

**Authors' contributions**

GLH and ZQL designed the study. BW prepared the manuscript, participated in experimental design and guided students to complete experiments and statistical anlaysis. HXL, JL, YJG, YHT, ZJC and LFH collected the data. GJZ analyzed the data.

**Ethics approval and consent to participate**

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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