Lysine is a Potential Antidote for Paraquat Poisoning Induced Pulmonary Fibrosis

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Abstract

Background and Purpose Pulmonary fibrosis (PF) is an irreversible lung disease with little efficient treatments. Epithelial-mesenchymal transition (EMT) in alveolar type II (AT II) cells is an initial process for PF in response to multiple insults. We previously identified paraquat (PQ), inducing acute lung injury and PF, as an agonist for STIM1, promoting STIM1-TRPC1 association for intracellular calcium burden and thus EMT. Here, we investigated the strategy targeting STIM1-TRPC1 association and excessive calcium influx in treating PF. Experimental approach Co-immunoprecipitation was for STIM1-TRPC1 association analysis. Western blotting and Real-time quantitative reverse transcription PCR (QPCR) were for EMT analysis. Calcium imaging, flow cytometry, and luciferase report assay were for analyzing calcium signaling. ELISA, histomorphology, and PQ-poisoned mice or cynomolgus model were for evaluating the efficacy of lysine in treating PF. Retrospective analysis was for analyzing the correlation between the severity of PQ poisoning and blood calcium levels. Key Results Lysine treatment significantly reduces PQ-raised STIM1-TRPC1 association, excessive calcium influx, and thus EMT in AT II cells. As a result, lysine treatment strikingly decreases the mortality of PQ-poisoned mice, with a fully recovery of PQ-induced PF. Immune cells activation, largely occurred accompanied with epithelial damages and PF, are almost normalized with lysine treatment in PQ-poisoned cynomolgus model. A negative correlation between the blood calcium levels and the prognosis were observed in PQ-poisoned patients. Conclusions and Implications These results demonstrate lysine as a potential antidote for PQ-induced PF and provide evidence for maintaining calcium homeostasis as a potential strategy for treating PF.

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Abstract

Background and Purpose

Pulmonary fibrosis (PF) is an irreversible lung disease with little efficient treatments. Epithelial-mesenchymal transition (EMT) in alveolar type II (AT II) cells is an initial process for PF in response to multiple insults. We previously identified paraquat (PQ), inducing acute lung injury and PF, as an agonist for STIM1, promoting STIM1-TRPC1 association for intracellular calcium burden and thus EMT. Here, we investigated the strategy targeting STIM1-TRPC1 association and excessive calcium influx in treating PF.

Experimental approach

Co-immunoprecipitation was for STIM1-TRPC1 association analysis. Western blotting and Real-time quantitative reverse transcription PCR (QPCR) were for EMT analysis. Calcium imaging, flow cytometry, and luciferase report assay were for analyzing calcium signaling. ELISA, histomorphology, and PQ-poisoned mice or cynomolgus model were for evaluating the efficacy of lysine in treating PF. Retrospective analysis was for analyzing the correlation between the severity of PQ poisoning and blood calcium levels.

Key Results

Lysine treatment significantly reduces PQ-raised STIM1-TRPC1 association, excessive calcium influx, and thus EMT in AT II cells. As a result, lysine treatment strikingly decreases the mortality of PQ-poisoned mice, with a fully recovery of PQ-induced PF. Immune cells activation, largely occurred accompanied with epithelial damages and PF, are almost normalized with lysine treatment in PQ-poisoned cynomolgus model. A negative correlation between the blood calcium levels and the prognosis were observed in PQ-poisoned patients.

Conclusions and Implications

These results demonstrate lysine as a potential antidote for PQ-induced PF and provide evidence for maintaining calcium homeostasis as a potential strategy for treating PF.

Bulleted Point Summary

What is already known

EMT in AT II cells is an initial process for PF in response to multiple insults, with multiple molecular targets identified in fibrotic lung diseases in mice model. However, no relevant effective clinic strategies have been developed.

We once identified PQ targets STIM1 to promote STIM1-mediated calcium influx and EMT for PF.

What does this study add

Lysine, a clinical utilized drug, suppresses PQ-driven EMT process, reduces the PQ- raised mortality rate, and reverses PQ-induced PF progression.

Maintaining intracellular calcium homeostasis would be a potential strategy to recover PF progression.

What is the clinical significance

Lysine is a potential safe drug for treating PQ-induced PF in clinic.

1. Introduction

Pulmonary fibrosis is a mostly irreversible lung disease resulted from acute lung injury, including severe infections such as COVID-19, radiation, chemotherapy, toxins, COPD, etc(George, Wells & Jenkins, 2020; Meziani et al., 2018; Wijsenbeek, Suzuki & Maher, 2022; Wilson & Wynn, 2009). The incidence of PF is not easy to estimate. Whereas, idiopathic pulmonary fibrosis (IPF), a specific chronic and progressive PF, was once classed as a rare disease but with an incidence rate continuously increased these years(Hutchinson, Fogarty, Hubbard & McKeever, 2015; Navaratnam, Fogarty, Glendening, McKeever & Hubbard, 2013). Importantly, the average survival year of IPF is around 3-5 years after diagnosis(Vancheri, Failla, Crimi & Raghu, 2010). However, the approved treatments for PF are limited or with low efficacy, including pirfenidone and nintedanib(Johannson, Chaudhuri, Adegunsoye & Wolters, 2021). Therefore, it is still required efforts to elucidate important mechanisms for developing efficient strategies to treat PF progression.

With years' efforts, it has been now well recognized that the progression of PF shares three common phases, including injury, inflammation, and tissue repair and contraction(Wilson & Wynn, 2009). In brief, the lung injury caused by multiple factors mentioned above would lead to inflammation for replacing the damaged epithelial or endothelial cells to maintain barrier function and integrity, from which several cytokines are produced, leading to EMT in AT II cells for tissue repair but also initiates fibrosis by synthesizing ECM components like fibronectin and collagen type 1, etc.(Kim et al., 2006; Kim et al., 2009). EMT has been reported to be an important process for fibrosis in multiple lung diseases, except IPF with controversial conclusions(Gauldie, 2002; Strieter, 2002). Recently, a single cell-RNA Seq analysis identified that EMT process in AT II cells does require for IPF progression(Xu et al., 2016). These studies together emphasize the importance of EMT in promotion of pulmonary fibrosis. Till now, multiple molecular targets modulating

EMT have been reported to be effective in fibrotic lung diseases in mice model, however, no relevant effective clinic strategies so far have been reported(Bartis, Mise, Mahida, Eickelberg & Thickett, 2014).

Several animal models have been established to understand the PF progression, from which PQ poisoning-induced acute lung injury is widely recognized to induce PF in a short period. Other models, like bleomycin-induced PF, require a relatively long period (O'Dwyer & Moore, 2018), which contains EMT, endothelial-to-mesenchymal transition, fibroblast-to-myofibroblast transition (FMT), etc(Moss, Ryter & Rosas, 2022). Considering others and our previous studies all identify EMT as a key process in PQ-driven PF(Chowdhury, Zielonka, Kalyanaraman, Hartley, Murphy & Avadhani, 2020; Su, Cong, Bi & Gao, 2018; Yamada, Aki, Unuma, Funakoshi & Uemura, 2015), PQ-induced acute lung injury would be an ideal model to identify potential strategies targeting EMT for treating PF. PQ has been well recognized to produce ROS that leads to severe cellular damage, EMT, and thus pulmonary fibrosis(Zheng, Goncalves, Abiko, Li, Kumagai & Aschner, 2020), which oxidative stress has been well characterized as a central signal for multiple factors-raised PF. Interestingly, clinical treatments to eradicate ROS production are not sufficient to treat PQ-induced PF and following high mortality (Dinis-Oliveira, Duarte, Sanchez-Navarro, Remiao, Bastos & Carvalho, 2008; Subbiah & Tiwari, 2021), indicating that other important mechanisms are still required to be elucidated. Recent studies have identified several other potential mechanisms and relevant new strategies for treating PQ poisoning, however, the efficacy is limited, yet exhibiting attenuation of the symptom (Subbiah & Tiwari, 2021), including metformin treatment only slightly reducing PQ induced pulmonary fibrosis(Wu et al., 2019) and maintaining an around 30% survival rate in PQ-poisoned mice model (Algire et al., 2012), rapamycin treatment exhibiting an increased survival rate on day 2 but almost losing the efficacy on day 3 in PQ-poisoned zebrafish model (Feng, Bian, Zhang, Wang & Chen, 2019). Similarly, rapamycin attenuates but not reverses PQ-induced pulmonary fibrosis in animal models(Tai et al., 2020; Xu et al., 2017). These observations raised us to identify the molecular target for PQ-induced EMT. Our previous work has successfully revealed that STIM1 is another important molecular target of PQ for EMT in AT II cells(Yang et al., 2022). STIM1 is an essential component for extracellular calcium entry by association with either ORAI1 or TRP family members(Soboloff, Rothberg, Madesh & Gill, 2012). We found that PQ targeting STIM1 promotes the exposure of the poly-lysine (K) region in the C-terminal of STIM1, which facilitates the association between STIM1 and TRPC1 for extracellular calcium entry and intracellular calcium increase(Yang et al., 2022). Note, it has been reported that intracellular calcium burden is required for EMT in multiple cancer cells for tumor metastasis. Therefore, we speculated that intracellular calcium increase is an important signal for PF, targeting intracellular calcium burden would be a potential strategy to treat PF(Wei et al., 2021). Herein, we utilized PQ-induced EMT and PF model with combination of information from mice model, cynomolgus model, and PQ poisoned patients, to examine the precise restriction of intracellular calcium burden as a potential strategy to treat PF.

2. Materials and methods

2.1 Cell Culture

A549 cells were cultured in Ham's F-12K Medium (BasalMedia, L450KJ) supplemented with 10% fetal bovine serum (GIBCO, 42Q1095K), penicillin (100 IU/ml, Genom, GNM15140) and streptomycin (100 μg/ml, Genom, GNM15140). MLE-12 cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, AWcell-0005) supplemented with 2% fetal bovine serum (GIBCO, 42Q1095K), 1% GlutaMAX-1 (AWcell-0900), 10 mM HEPES buffer solution (GIBCO, 15630-080), 1% ITS (AWcell-4507), 10 nM hydrocortisone (AWcell-8450), 10 nM β-estradiol (AWcell-8140), penicillin (100 IU/ml, Genom, GNM15140) and streptomycin (100 μg/ml, Genom, GNM15140). These cell lines were grown at 37°C in a 5% carbon dioxide incubator and were passaged following trypsinization.

2.2 Mice

Six-week-old male C57BL/6 mice were randomly and evenly divided into groups as indicated in specific experiments. The mice were intraperitoneally injected with PQ (50 mg/kg, Sigma-Aldrich, 36541) for one time, whereas vehicle control, SKF (10 mg/kg, MedChemExpress, HY-100001) or lysine (10 mg/kg, Sangon

Biotech, A602769) was intraperitoneally injected every day for 3 days, or as indicated. The weight, daily food and water uptake of the treated mice were recorded every day. Mice were sacrificed on day 4 or as indicated, and lung lobes were removed, fixed in 4% paraformaldehyde, dehydrated, and subjected to paraffin embedding. Lung microsections (5 μm) were stained with hematoxylin & eosin (H&E) and Masson's trichrome to visualize fibrotic lesions. Cheek blood samples were prepared and the serum was isolated by centrifugation with 2000 rpm for 5 min. The serum was monitored in Servicebio (Wuhan, China) for blood levels of calcium, ALT, AST, and creatinine. All the animal studies were approved by the Ethics Committee of Shanghai General Hospital.

2.3 PQ-poisoned cynomolgus model

The PQ-poisoned cynomolgus model was performed at Medicilon Inc. (Shanghai, China). Briefly, five-year-old male cynomolgus monkeys were orally administrated with 12.5 mg/kg PQ once, together with vehicle or 40 mg/kg lysine by intravenous injection every day for three days. Blood was collected by femoral venipuncture as indicated. The population of immune cells was measured by laboratory blood tests and the blood calcium levels were monitored by serum biochemistry. For CRP analysis, serum was prepared by the centrifugation of collected blood with 3500 rpm for 10 min at room temperature. The prepared serum was then subjected to CRP ELISA analysis according to the manufacturer's instructions.

2.4 Hydroxyproline assay

To estimate the collagen synthesis in the lungs, we measured the amount of hydroxyproline via ELISA analysis (Shanghai Yubo Biological Technology). Briefly, lung tissues were homogenized in 1 ml phosphate-buffered saline (PBS) and centrifuged at 4000 rpm for 15 min. The supernatant was collected and BCA measurement was performed to examine the total amount of proteins in lung tissues. Hydroxyproline content was measured according to the Manufacturer's instructions and determined by a colorimetric assay using a spectrophotometer at a wavelength of 450 nm. Data were present as μg hydroxyproline /μg of lung tissue.

2.5 Clinical Samples

We retrospectively reviewed a part of patients with paraquat poisoning (n = 18) at Qilu Hospital of Shandong University and normal healthy volunteers (n = 22) at Shanghai General Hospital. Overall, the biochemical analysis of electrolytes and the blood creatinine levels previously recorded were analyzed. The concentrations of PQ in blood were measured by High-Performance Liquid Chromatography. All the studies were approved by the Ethics Committee of Shanghai General Hospital and Qilu Hospital.

2.6 Quantitative RT-PCR

A549 cells were treated with PQ in a dose dependent manner, as indicated, with or without 5 mM lysine, for 24h. The cells were lysed in TRIZOL (Vazyme Biotech Co, R701-02) and total RNA was extracted and reverse transcribed into cDNA by HiScript III RT SuperMix kit (Vazyme Biotech Co, R323). The mRNA expression of E-cadherin and Vimentin was determined by semi-quantification with ChamQ SYBR Color qPCR Master Mix (Vazyme Biotech Co, R323, Q411-02) in QuantStudio 7 Flex. Data were normalized to Actin and calculated by $2^{[-\text{ (Ct target gene-Ct Actin)}]}$. The sequence of the PCR primers was previously reported(Yang et al., 2022).

2.7 Western blot

Cells were harvested and the protein lysates were separated by 10% SDS-PAGE gels and transferred onto a nitrocellulose filter membrane (NC) membrane (Pall,66485), blocked with 5% non-fat milk in phosphate buffered solution with 0.1% Tween 20 (PBST). Primary antibodies were incubated at 4 overnight, including Anti-STIM1 (Cell Signaling Technology, 5668, 1:1000), Anti-ORAI1 (Santa Cruz, sc-377281, 1:500), Anti-TRPC1 (Proteintech, 19482, 1:1000), Anti-E-cadherin (Cell Signaling Technology, 3195S, 1:1000), Anti-Vimentin (Cell Signaling Technology, 5741S, 1:1000), and Anti-GAPDH (Proteintech, 60004-1-1g, 1:10000).

2.8 Immunoprecipitation

A549 cells were treated with 800 μM PQ with or without 5 mM lysine for 0, 15, or 30 min and lysed in RIPA buffer (Beyotime, P0013D) with addition of proteasome inhibitor (Roche, 04693124001). The lysates were quantified by Bicinchoninic acid (BCA) method and incubated with STIM1 antibody (1 μl/sample) overnight, followed by incubation with 20 μl protein A/G beads (Santa Cruz, SC-2003) for another 3h at 4°C. The immune complexes were washed three times with PBS and subjected to Western Blot. Specific antibodies were used for STIM1 (Cell Signaling Technology, 5668, 1:1000), ORAI1 (Santa Cruz, sc-377281, 1:500), and TRPC1 (Proteintech, 19482, 1:1000).

2.9 Reporter gene assay

A549 cells were transfected with NFAT-luciferase/Renilla plasmids by using polyjet transfection reagent (Signagen, SL100688). The transfected cells were pretreated w/wo lysine in a dose dependent manner for 2h, followed by 800 μM PQ stimulation for additional 24h. The amount of NFAT-luc or Renilla was measured by using the dual-luciferase Kit Assay Reporter System (Vazyme Biotech Co, DL101-01) and examined by using a luminometer (Varioskan Flash, Thermo Fisher, MA, USA). The data were present as the ratio of the NFAT-luc luminescence to Renilla luminescence.

2.10 Flow cytometric analysis

A549 cells or MLE-12 cells were seeded in a twelve-well plate with a density of 2×10^5 cells/well in complete growth medium. Following with or without 5 mM lysine treatment, the cells were exposed to PQ in a dose dependent manner, as indicated, for 24h, washed twice with PBS and incubated with 2 μM Fluo-3/AM (Beyotime, S1056) diluted in Hanks' Balanced Salt Solution (Beyotime, C0218) for 30 min at 37°C in the dark. The stained cells were then dissociated with trypsin, resuspended with PBS and collected to detect the presence of $[Ca^{2+}]_i$ using a FACScan flow cytometer (BD LSRFortessa, NJ, USA).

2.11 Single-cell Ca^{2+} measurements

A549 cells were plated in 35 mm glass bottom dishes. After attachment, the cells were incubated with 2 μM Fura-2 AM (Beyotime, S1052) diluted in Ca^{2+} free HBSS for 30 min. The stained cells were sequentially treated with 800 μM PQ, 2 mM $CaCl_2$, and lysine in a dose dependent manner, as indicated, for measurements of calcium fluxes. Fura-2 (340/380) filter set (pE-340fura, CoolLED, UK), a 20 × 0.3 N A. objective lens, and a Photometrics Iris9 camera was used to capture images at a frequency of 1 image pair every 2 s. Relative fluorescence ratio at wavelengths of 340 nM and 380 nM (F340/F380) was measured by Visview and utilized for the assessment of cytoplasmic calcium levels.

2.12 GC-MS Metabolomics Analysis

Human blood from PQ poisoning patients were obtained at Qilu Hospital of Shandong University. Plasma was isolated by Ficoll-density centrifugation and collected to carry out the GC-MS Metabolomics Analysis (Oebiotech, Shanghai).

2.13 Statistical analysis

Data are represented as mean ± SD or mean ± SEM as indicated. The comparisons between any two groups were analyzed by two-tailed one type Student's *t-test* or in experiment with multiple comparisons were performed using one-way or two-way ANOVA followed by Bonferroni post-tests, as indicated. Pearson correlation coefficient was performed to analyze the correlation between electrolytes and PQ in blood. Kaplan-Meier analysis was performed to analyze the survival rate after PQ poisoning. *P < 0.05, **P < 0.01, ***P < 0.001.

3. Results

3.1 SKF treatment exhibits poor efficacy on treating PQ poisoning induced pulmonary fibrosis

We recently identified STIM1 as another molecular target of PQ, which results in intracellular calcium burden and EMT in AT II cells(Yang et al., 2022). Hence, we assumed that inhibition of calcium influx mediated by STIM1 would be a potential strategy for treating PQ toxicity and pulmonary fibrosis. We therefore examined

the efficacy of SKF96365 (SKF), a SOCE inhibitor and a TRP channel blocker, in treating PQ-poisoned mice. As shown in Fig. 1A, consistent with previous reports, PQ significantly induces the production of hydroxyprolin, a product of collagen degradation acting as one of the parameters for reflecting the severity of pulmonary fibrosis (Spagnolo et al., 2020), which was impressively reduced with SKF co-treatment (Fig. 1A). However, SKF treatment did not improve the survival rate of PQ poisoning. All the mice either in the PQ group or the PQ + SKF group died with a similar rate (data not shown). Moreover, we further monitored the morphological change of lungs in the survived mice by H&E staining and Masson staining, and found severe pulmonary fibrosis in the PQ-poisoned mice either with or without SKF co-treatment (Fig. 1B). Note, it seemed that SKF alone would lead to sort of pulmonary fibrosis, indicating that both intracellular calcium burden or calcium deficiency would be harmful to lung homeostasis. Consistently, PQ-poisoned mice exhibited gradual weight loss following the progression of the disease, which was not well alleviated by SKF, yet SKF treatment showed a temporary remission in day 1 (Fig. 1C). Taken together, these observations suggested that SKF was not sufficient to reverse PQ toxicity and pulmonary fibrosis, yet with a reduction in hydroxyproline production.

3.2 Lysine normalizes PQ-induced STIM1-TRPC1 association and intracellular calcium burden

The above observations suggested that a precise reduction of PQ-induced intracellular calcium burden but not calcium influx depletion might be beneficial for treating PQ-raised pulmonary fibrosis. Our previous work revealed that PQ targets STIM1, which facilitates the exposure of poly-K region in STIM1 and ultimately leads to an increase of intracellular calcium levels. Specifically, the STIM1-TRPC1 axis mediates PQ toxicity, from which the poly-K region in STIM1 and the D639&D640 residues in TRPC1 are mainly required for STIM1-TRPC1 interaction in response to PQ stimulation(Yang et al., 2022). We therefore hypothesized that addition of exogenous lysine would compete with the poly-K region for binding to D639&D640 residues and results in a blockage of PQ-raised STIM1-TRPC1 association. We then monitored whether lysine would affect the STIM1-TRPC1 association enhanced by PQ stimulation. As shown in Fig. 2A, PQ-increased STIM1-TRPC1 association was suppressed by lysine co-treatment in a time-dependent manner. Secondly, we found that PQ-induced NFAT luciferase production, a well-established downstream of calcium signaling, was also largely reduced by administration of lysine in a dose dependent manner, with a normalization to basal level when cells treated with high amount of lysine (Fig. 2B). Furthermore, we examined the effect of lysine on PQ-induced extracellular calcium entry and found that PQ-raised extracellular calcium influx was dramatically declined with lysine treatment in a dose dependent manner (Fig. 2C). As a result, PQ-induced intracellular calcium burden was significantly reduced with lysine treatment both in A549 cells and MLE-12 cells, two type of AT II cells (Fig. 2D&2E). Taken together, these results together suggest that lysine largely suppresses PQ-raised STIM1-TRPC1 association and thus intracellular calcium burden.

3.3 Lysine inhibits PQ-induced EMT process in AT II cells

We and others both found that PQ-induced EMT is critical for pulmonary fibrosis. Our previous work further suggested that PQ-promoted STIM1-TRPC1 association and intracellular calcium burden are essential for EMT. We therefore wondered whether PQ-induced EMT would be reversed by lysine treatment. PQ treatment significantly reduced the expression of E-Cadherin, the epithelial marker, while enhanced the expression of Vimentin, the mesenchymal marker. Lysine treatment significantly alleviated the reduction of E-Cadherin and normalized the upregulated Vimentin in PQ- treated A549 cells (Fig. 2F). Similar results were observed in MLE-12 cells (Fig. 2G). Intriguingly, we have also examined the efficacy of lysine in STIM1-overexpressed A549 cells. Though lysine still inhibited PQ-induced EMT process (Fig. 2H), such inhibition seemed not to be as striking as those observed in normal A549 cells (Fig. 2F), indicating that cells with STIM1 overexpression were much sensitive to PQ stimulation and the efficacy of lysine could be ameliorated probably due to additional STIM1 targeted by PQ. We further confirmed the protective effect of lysine in PQ-induced EMT by analyzing the mRNA expression of E-Cadherin and Vimentin (Fig. 2I&2J). Note, we have repeatedly observed that lysine alone enhanced Vimentin expression in the protein level but not the mRNA level, indicating that lysine might affect Vimentin stability. Nevertheless, these results together suggest that lysine is sufficient to suppress PQ-induced EMT, indicating that lysine is a potential antidote

for PQ poisoning and pulmonary fibrosis by reducing STIM1-TRPC1 activation.

3.4 Lysine largely suppresses PQ-induced pulmonary fibrosis and mortality

To clarify whether lysine is sufficient to normalize PQ-raised pulmonary fibrosis, we then verified the therapeutic effects of lysine *in vivo* . PQ poisoning raised a continuous weight loss in mice, whereas administration of lysine impressively recovered and normalized the weight loss after long time treatment (Fig. 3A). Importantly, all the PQ-poisoned mice exhibited consistent trend of weight loss, whereas administration of lysine in PQ-poisoned mice exhibited individual difference, which some of the mice were instantly resistant to PQ poisoning with lysine treatment, others exhibited a reduction of weight loss and were ultimately recovered (Fig. 3B), indicating that a continuous treatment of lysine would be beneficial for neutralizing the toxicity of PQ. Nevertheless, treatment with lysine in PQ-poisoned mice largely increased the survival rate, which PQ-poisoned mice all died at day 7 whereas 62.5% mice were survived with lysine treatment (Fig. 3C). Consistently, the hydroxyproline levels increased by PQ were significantly alleviated by co-administration with lysine (Fig. 3D). The pulmonary fibrosis and alveolitis induced by PQ were amazingly healed, accompanied with an increased food intake and water intake in lysine treated group (Fig. 3E, Supplemental Fig. 1A-C). Consistently, PQ-induced damages in liver and kidney were largely recovered by lysine treatment, indicated by ALT, AST, or Creatine levels in blood (Fig. 3F-3H). Observations of lysine in treating PQ-poisoned mice model raised the potency of lysine in anti-PQ poisoning and PQ-raised pulmonary fibrosis in humans. Indeed, lysine in plasma was largely reduced in PQ-poisoned patients (Fig. 3I), making administration of lysine be reasonable for PQ-poisoned patients. Taken together, these results strongly suggested that lysine is the antidote for PQ poisoning and would be a potential strategy for PQ-raised pulmonary fibrosis.

3.5 Lysine alleviates PQ-induced inflammatory responses

Lysine was once considered in clinic for preventing or treating cold sores due to herpes simplex with little side effects (Chi, Wang, Delamere, Wojnarowska, Peters & Kanjirath, 2015). Lysine has also been proved to prevent and treat brain trauma and brain ischemia (Kondoh, Kameishi, Mallick, Ono & Torii, 2010). Furthermore, lysine has been believed to be beneficial for the immune response. Considering systemic inflammation is also involved in PQ-raised EMT and pulmonary fibrosis (Dinis-Oliveira, Duarte, Sanchez-Navarro, Remiao, Bastos & Carvalho, 2008), we then analyzed the populations of immune cells in a PQ-poisoned cynomolgus model treated w/wo lysine. Neutrophils and monocytes are two immune populations reflecting PQ poisoning-induced inflammatory responses (Cao et al., 2019; Zhao, Song, Gao, Feng & Li, 2019). Our results show that PQ poisoning increased the number and percentage of neutrophils and monocytes while lysine treatment remarkably reduced such increasement (Fig. 4A&B). In contrast, similar as other reports(Bhardwaj & Saxena, 2014), a reduction of reticulocytes was observed in PQ-poisoned cynomolgus, while lysine treatment even slightly increased the reticulocytes (Fig. 4C). Intriguingly, compared to the vehicle control, we have observed an increase of lymphocytes and WBC in PQ-poisoned cynomolgus treated with lysine (Fig. 4D&E). Considering the C-reactive protein (CRP) was largely reduced in lysine-treated cynomolgus (Fig. 4F), the increased number of WBC and lymphocytes could be the results of nutritional functions by lysine(Bouyeh, 2012) that benefits immune homeostasis. Taken together, these results together indicate that lysine treatment would also maintain immune homeostasis during PQ poisoning.

3.6 Blood calcium levels are negatively correlated with the severity of PQ poisoning in patients

As lysine suppresses PQ-induced pulmonary fibrosis by reducing STIM1-TRPC1 axis increased intracellular calcium levels and EMT process, we considered intracellular calcium homeostasis was critical for maintaining normal pulmonary functions. To further confirmed the importance of calcium signal during PQ-raised pulmonary fibrosis, we turned our sights into the clinical records. By carefully analyzing the laboratory blood tests, we found that the blood calcium levels of PQ-poisoned patients were significantly decreased compared to healthy volunteers. Further analysis revealed a negative correlation between the calcium levels and the PQ concentrations in blood (Fig. 5A&B). Importantly, no significant correlation was observed in potassium, sodium, or chlorine (Fig. 5C-E). We further tracked the records of each patient with little poisoning. These patients exhibited continuous reduction of blood calcium levels during the malignant progression indicated

by the increased blood creatinine levels. Intriguingly, the blood calcium levels were restored, yet not fully, when patients were recovered from PQ poisoning indicated by the reduction of blood creatinine levels into normal range (Fig. 5F). Whereas, patients suffering high amount of PQ poisoning died accompanied with extremely high creatinine levels and low blood calcium levels (Fig. 5G). The reduction of blood calcium levels in PQ-poisoned patients could be due to multiple reasons, from which one of the possibilities could be due to the large usage of extracellular calcium pools by STIM1-mediated calcium entry as we previously reported (Yang et al., 2022). To address this possibility, we analyzed the blood calcium levels in two PQ-poisoned animal models with or without lysine treatment, and found that PQ-poisoned mice exhibited a significant reduction of blood calcium levels, which was dramatically restored by lysine treatment (Fig. 5H). Consistent with the observations in PQ-poisoned mice model, lysine treatment impressively alleviated the reduction of blood calcium levels in the PQ-poisoned cynomolgus model (Fig. 5I). Interestingly, PQ-poisoned group exhibited a recovery of blood calcium levels during daytime in the second and third days, which might be ascribed to a feedback regulation of blood calcium levels due to calcium absorption in the gut and calcium reabsorption in the kidney. Nevertheless, these results indicated the potential functions of calcium signaling in modulation of the pathogenesis in PQ-raised toxicity and pulmonary fibrosis.

4. Discussion and Conclusions

Interstitial lung disease (ILD) accompanied with pulmonary fibrosis is a progressive disease with high morbidity and early mortality. Treatment of ILD associated PF includes immunomodulatory or antifibrotic drugs, which antifibrotic drugs are mainly utilized in IPF(Johannson, Chaudhuri, Adegunsoye & Wolters, 2021). Unfortunately, immunomodulatory therapies, including azathioprine(Idiopathic Pulmonary Fibrosis Clinical Research, Raghu, Anstrom, King, Lasky & Martinez, 2012), cyclophosphamide(Hoyles et al., 2006), methotrexate(Dawson, Quah, Earnshaw, Amoasii, Mudawi & Spencer, 2021; Johannson, Chaudhuri, Adegunsoye & Wolters, 2021), etc., exhibit efficacy only in a certain subset of patients or with controversial conclusions. Therefore, immunosuppressant therapies reach limited confidence in treating ILD associated PF. Multiple antifibrotic strategies were examined in treating IPF, including bosentan, interferon gamma, etanercept, imatanib, everolimus, ambrisentan, macitentan, and warfarin, but reach a negative outcome(Idiopathic Pulmonary Fibrosis Clinical Research, Raghu, Anstrom, King, Lasky & Martinez, 2012; Lederer & Martinez, 2018; Raghu et al., 2015). Pirfenidone and nintedanib were reported to be efficient in alleviating IPF progression, but the mortality benefit is less robust(Johannson, Chaudhuri, Adegunsoye & Wolters, 2021; Nathan et al., 2017; Nathan et al., 2019). These observations together raise that there is still an urgent need to develop potential universal strategies to treat PF with high efficacy. Several novel therapies were designed to reduce the production of TGF-β1 or the activation of TGF-β signaling(Johannson, Chaudhuri, Adegunsoye & Wolters, 2021). TGF-β signaling is both required for EMT and FMT during PF(Bartis, Mise, Mahida, Eickelberg & Thickett, 2014; Moss, Ryter & Rosas, 2022). Others and our studies all identified the importance of EMT in promotion of PF progression, which the continuous epithelial damage due to multiple types of insults results in EMT in AT II cells and thus PF progression. Therefore, EMT is a shared mechanism that could be an ideal target for developing strategies to treat PF. Here, we utilized PQ poisoning-raised EMT and PF model to identify the importance of intracellular calcium homeostasis in maintaining AT II cell fates. Specifically, either intracellular calcium burden or deficiency would result in PF. And, by combination of PQ-poisoned mice model, cynomolgus model, and clinical information from PQ poisoned patients, we identified that lysine, a utilized clinical drug, as the antidote to treat PQ poisoning by normalizing PQ-raised STIM1 association with TRPC1 for excessive intracellular calcium burden in AT II cells, leading to a remarkable reduction of PQ-induced EMT and PF, further raising that strategies maintaining intracellular calcium homeostasis would be beneficial for treating PF, which requires further efforts to elucidate.

AT II cells are well characterized to differentiate into AT I cells during pulmonary injury for maintaining the function of pulmonary gas exchange(Desai, Brownfield & Krasnow, 2014). A failure of AT II cells differentiation into AT I cells have been reported to develop fibrosis in mice(Wu et al., 2020). Several signaling have been revealed to be required for AT II cell proliferation(Aspal & Zemans, 2020), however, it is remained elusive for AT II-to-AT I differentiation. WNT, Notch, BMP, and TGFβ all have been reported to participate in AT II-to-AT I differentiation, while the signaling networks of these signals inside cells are

not well elucidated(Aspal & Zemans, 2020). Calcium ions, important second messengers and exhibit almost omnipotent functions, have been well recognized to participate in the above signaling cascades(Berridge, Bootman & Roderick, 2003; Gooch, Gorin, Zhang & Abboud, 2004; Kuhl, Sheldahl, Park, Miller & Moon, 2000; Song et al., 2020). Our results further revealed the importance of intracellular calcium homeostasis in maintaining AT II cell fates, which either calcium overload or deficiency would lead to EMT and fibrosis, whether dynamic calcium signaling is required to maintain the stemness of AT II to differentiate into AT I remains to be further explored. Note, intracellular calcium signaling would be dynamically modulated by fluxes from both extracellular calcium pools and calcium stores inside cells(Berridge, Bootman & Roderick, 2003). Further efforts should be made to clarify the major routes for intracellular calcium signaling and relevant mechanisms in modulation of the stemness of AT II cells.

PQ is an efficient herbicide widely utilized worldwide. However, PQ is also extremely toxic to mammals by promotion of ROS production, inducing EMT in AT II cells, leading to pulmonary fibrosis and high mortality(Subbiah & Tiwari, 2021). Till now, no efficient antidote has been developed to treat PQ toxicity, making the utilization of PQ in agriculture production be restricted in multiple countries(Dinis-Oliveira, Duarte, Sanchez-Navarro, Remiao, Bastos & Carvalho, 2008). Strategies including charcoal perfusion, reduction of oxidative stress, and anti-inflammation therapies have been well established to treat PQ poisoning in clinic, however, the efficacy is limited and mortality of PQ-poisoned patients is hard to alleviated(Okonek, Hofmann & Henningsen, 1976). Recently, Qian et al., reported a target strategy to neutralize PQ poisoning, however, with no safety evaluation be examined (Qian et al., 2021). Our previous study identified STIM1 as an important molecular target of PQ, here we further developed lysine, a safe approach already be utilized in clinic, as a potential antidote to treat PQ poisoning by restriction of STIM1 association with TRPC for excessive extracellular calcium influx. Further studies are required to examine the efficacy of lysine in treating PQ-poisoned patients either solely or with the combination of well-established strategies utilized in clinic, especially the charcoal perfusion.

In conclusion, our previous study found that PQ targets the STIM1-TRPC1 axis for extracellular calcium entry following with intracellular Ca^{2+} overload in pulmonary epithelial cells and thus results in pulmonary fibrosis. Here we identified lysine as an antidote for PQ poisoning-induced EMT and PF progression, from which we further emphasized the importance of calcium signals for modulation of AT II cells differentiation for fibrosis, providing potential molecular targets and safe strategies for treating PF progression.

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Author Contribution: Z. Y. and W. Y. generated the concept, designed the experiments, analyzed the data, and wrote the manuscript. W. Y. and Z. Y. conducted the key experiments. X. M., Y. Z., X. Ma., Z. C., M. W. and W. D. performed the experiments and analyzed the results. R.T., Z. Y. and R. W. interpreted the results and supervised the study. Y. T., X. J., and R.T. collected and provided human samples. R. T. analyzed the clinical information. All the authors approved the final manuscript.

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Competing Interests' Statement: The authors declare that they have no conflicts of interest with the contents of this article.

Figure Legends

Figure 1. SKF plays little roles in ameliorating PQ poisoning in mice. (A) ELISA analysis of hydroxyproline in PQ-poisoned lungs treated w/wo SKF for 3 days. Mean ± s.d., ***P < 0.001; Two-Way ANOVA.**(B)** HE staining and Masson staining of PQ-poisoned lungs treated w/wo SKF for 3 days. Images are representative of more than three mice in each group. **(C)** Daily weight change of PQ-poisoned mice treated w/wo SKF for 3 days. Mean ± s.d..

Figure 1

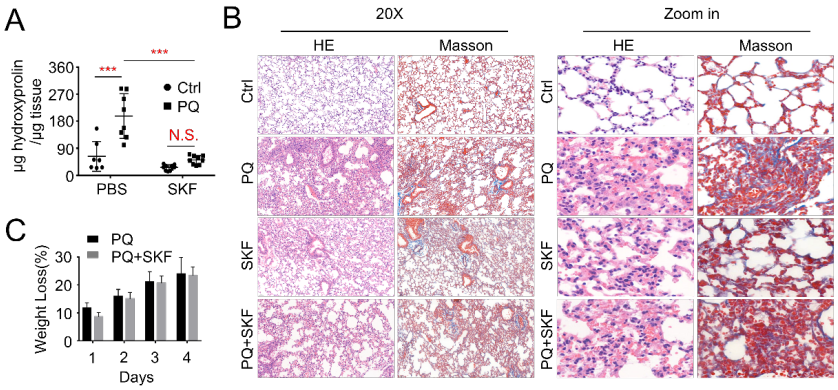


Figure 2. Lysine suppresses PQ induced intracellular calcium burden and EMT. (A) Co–IP analysis of STIM1 association with ORAI1 or TRPC1 in A549 cells treated with 800 μM PQ for 0, 15, 30 min, together w/wo 5 mM lysine as indicated. Images are representative of three experiments. **(B)** NFAT luciferase expression in A549 cells treated w/wo 800 μM PQ, together with 0-, 1-, 2.5-, or 5-fold lysine as in the culture medium as indicated, for 24 hours. Mean ± s.d., ***P < 0.001; One-Way ANOVA. n = 3. N.S., not significant.**(C)** Calcium imaging of A549 cells stimulated with 800 μM PQ, followed by 2 mM $CaCl_2$, and 100 μM, 200 μM, or 400 μM lysine as indicated. Mean ± sem. **(D-E)** FACS analysis of intracellular calcium levels by Fluo-3 staining in A549 cells (D) and MLE 12 cells (E). Cells were treated w/wo PQ in a dose dependent manner as indicated, together w/wo 5 mM lysine, for 24 hours. Mean ± s.d., ***P < 0.001, *P < 0.05; unpaired two–tailed Student's*t–test* . n = 3. **(F-H)** WB analysis of the expression of E-Cadherin and Vimentin, makers of EMT, in A549 cells (F), MLE-12 cells (G), or A549 cells stably expressed STIM1 (H) treated with PQ in a dose dependent manner, as indicated, together w/wo 5 mM lysine for 24 hours.**(I-J)** RT-PCR analysis to detect the expression of*E-Cadherin, or Vimentin* in A549 cells (I) or MLE-12 cells (J) treated with PQ in a dose dependent manner, as indicated, together w/wo 5 mM lysine for 18 hours.

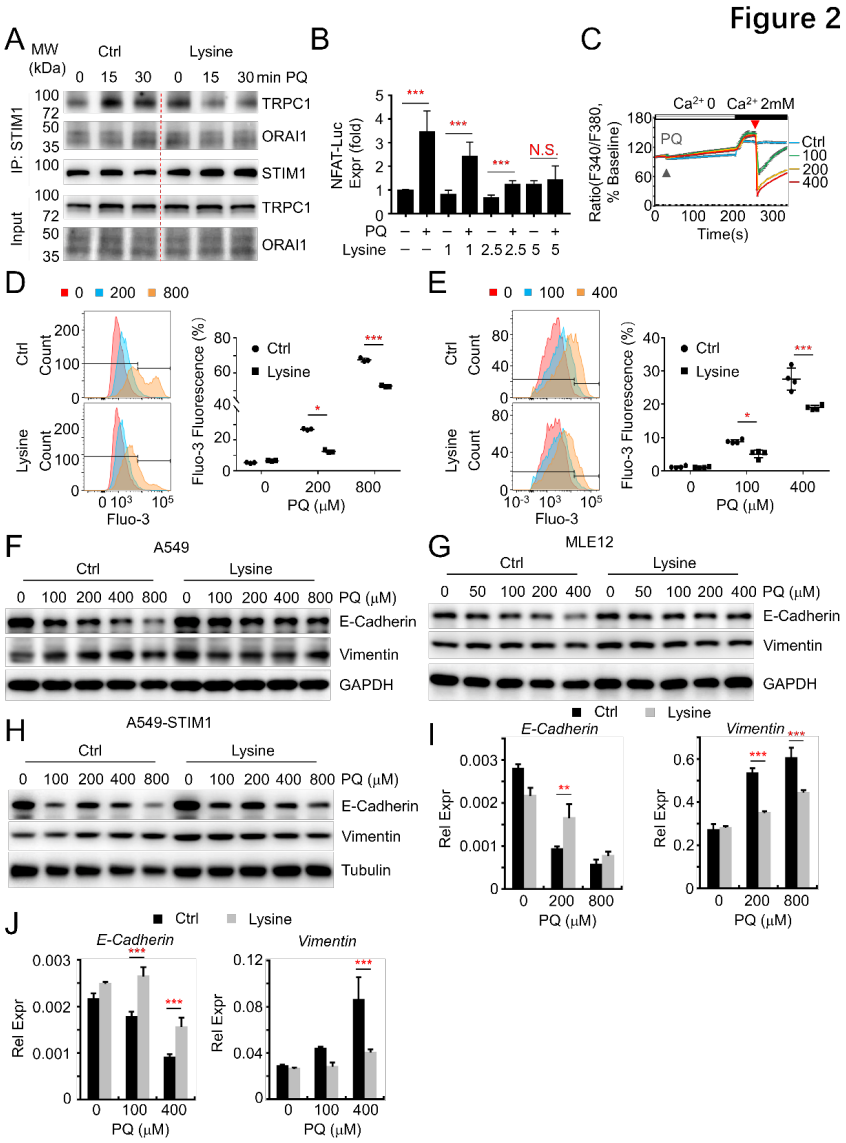


Figure 3. Lysine is an efficient antidote for PQ poisoning. **(A-B)** Daily weight change of PQ-poisoned mice treated w/wo lysine for 14 days. Mean ± s.d., *P < 0.05; Two-Way ANOVA. n = 8 in each group. **(C)** Kaplan-Meier analysis of survival rate in PQ-poisoned mice treated w/wo lysine. n = 8 initially in each group.**(D)** ELISA analysis of hydroxyproline in PQ-poisoned lungs treated w/wo lysine for 3 days. Mean ± s.d., **P < 0.01, ***P < 0.001; Two-Way ANOVA. N.S., not significant. **(E)** HE staining and Masson staining of PQ-poisoned lungs treated w/wo lysine for 3 days. Images are representative of more than three mice in each group. **(F-H)** Levels of serum biochemical parameters, including ALT (F), AST (G), and Creatinine (H) in PQ-poisoned mice treated w/wo lysine for 3 days. **(I)** Amino acid metabolic profiles measurement in plasma from healthy volunteers (n = 6) or PQ-poisoned patients (n = 7). Mean ± s.d., *P < 0.05; unpaired two–tailed Student's *t–test* .

Figure 3

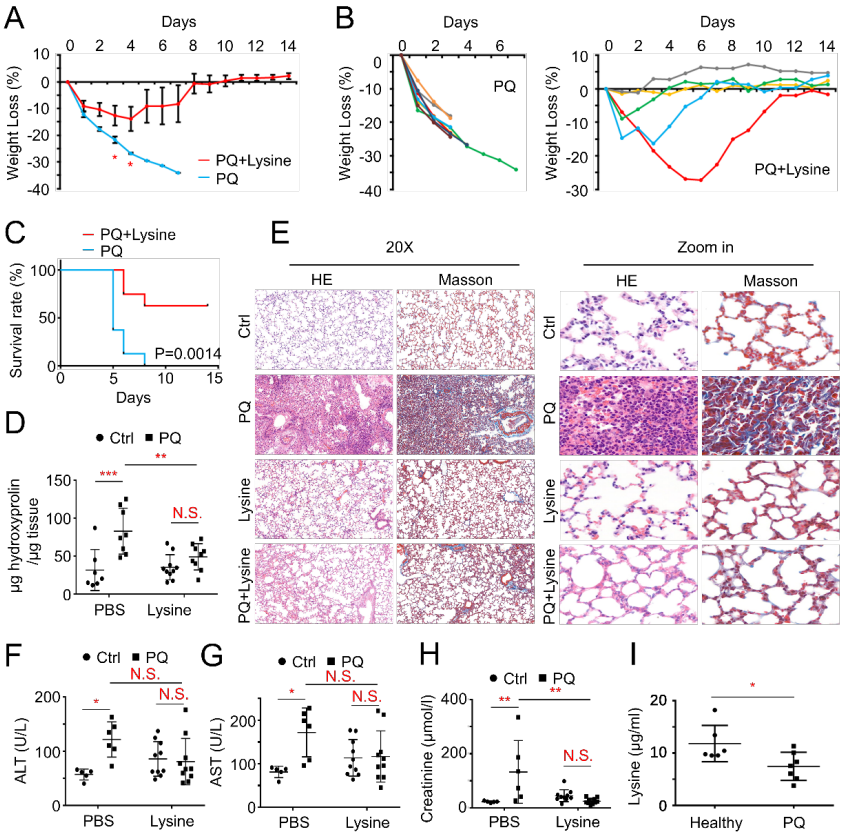


Figure 4. Lysine treatment attenuates PQ-raised immune responses. (A-E) The number or percentage of neutrophils (A), monocytes (B), reticulocytes (C), lymphocytes (D), or WBC (E) analyzed from laboratory examination of blood in PQ-poisoned cynomolgus monkey treated w/wo lysine for 3 days. Arrows indicate lysine treatment.**(F)** ELISA analysis of serum C-reactive protein (CRP) in PQ-poisoned cynomolgus monkey treated w/wo lysine. Each dot represents the result of measurement in different time point. Mean ± s.d., **P < 0.01; unpaired two–tailed Student's *t–test* .

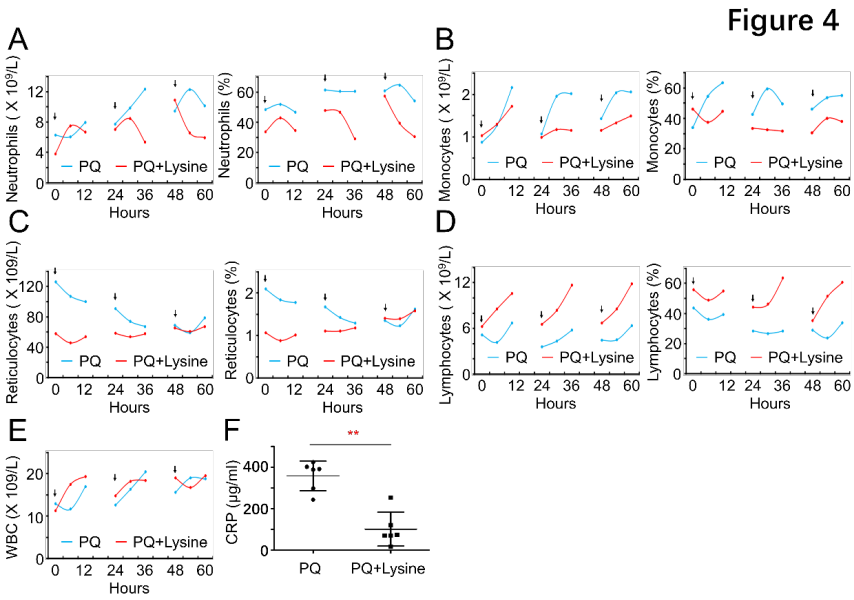
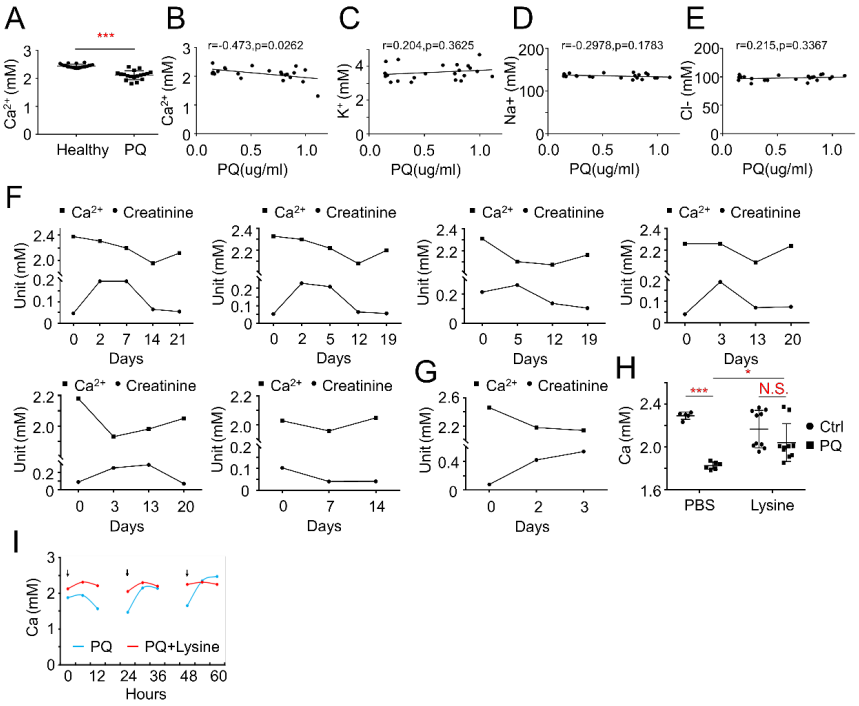


Figure 5. Blood calcium levels are negatively correlated with the severity of PQ poisoning. **(A-B)** A retrospective analysis of the correlation between PQ and blood calcium levels. (A) Mean ± s.d., ***P < 0.001; unpaired two–tailed Student's t–test. n = 22 in healthy group, n= 18 in PQ group. (B) Pearson correlation coefficient. n = 22. **(C-E)** The correlation between PQ and levels of blood potassium (K^+, C), sodium (Na^+, D) or chloride (Cl^-, E). **(F&G)** Analysis of blood calcium levels and blood creatinine levels in each PQ-poisoned patient during disease progression. **(H)** Levels of serum calcium levels in PQ-poisoned mice treated w/wo lysine for 3 days. **(I)**Levels of blood calcium in PQ-poisoned cynomolgus monkey treated w/wo lysine for 3 days. Arrows indicate lysine treatment.

Figure 5



Supplemental Figure Legends

Figure S1. Lysine is a potential antidote for PQ poisoning. (A-B) Daily food intake (A) and daily water intake (B) were recorded in control (Ctrl) or PQ-poisoned mice treated w/wo lysine for 3 days. Mean ± s.d., *P < 0.05, **P < 0.01, ***P < 0.001; Two-way ANOVA. **(C)** Daily weight change of PQ-poisoned mice treated w/wo lysine for 3 days. Mean ± s.d., *P < 0.05; Two-Way ANOVA. n = 5 in each group. **(D)** Representative images of lung tissue from control (Ctrl) or PQ-poisoned mice treated w/wo lysine for 3 days.

Figure S1