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Original Article

Rapamycin reverses paraquat-induced acute lung injury in a rat model through inhibition of NFκB activation

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Abstract: Objective: To evaluate the role of rapamycin (RAPA) in paraquat (PQ)-induced acute lung injury. Methods: Lung tissues were stained with HE and lung histology was observed. Mortality rate, and neutrophil and leukocyte count in blood and bronchoalveolar lavage fluid (BALF) were recorded. Protein content in BALF was determined by Coomassie blue staining. Malondialdehyde (MDA) content, glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity in blood were determined by thiobarbituric acid (TBA) assay, pyrogallol autoxidation method, and modified Haefman method, respectively. The NF-κB activity was measured by gel electrophoretic mobility shift assay (EMSA). Carbon dioxide partial pressure ($PaCO_2$), partial pressure of oxygen (PaO_2) and pH values were measured by automated blood gas analyzer. Results: HE staining results demonstrated RAPA alleviated pathological changes of acute alveolitis in SD rats. Trend of protein content in BALF was PQ group > RAPA treatment group > control group ($P < 0.05$). Neutrophil and leukocyte count in RAPA treatment group was significantly lower than PQ group at 3, 5, and 7 days after injection ($P < 0.05$). Trend of MDA content was RAPA treatment group > PQ group > control group ($P < 0.05$). Trend of GSH-Px and SOD activity was control group > RAPA treatment group > PQ group ($P < 0.05$). Compared with PQ group, PaO_2 in RAPA treatment group was markedly higher and $PaCO_2$ was lower ($P < 0.05$). Conclusion: PQ-induced acute lung injury was effectively reversed with RAPA, through inhibition of NF-κB activation.

Keywords: Rapamycin, paraquat, acute lung injury, paraquat-induced acute lung injury rat model, malondialdehyde, glutathione peroxidase, superoxide dismutase, NF-κB

Introduction

Paraquat (PQ), also known as methyl viologen or gramoxone, is a member of nitrogen-containing heterocyclic organic compounds that have significant application in agriculture [1]. PQ, a quartenary nitrogen herbicide, used as a quick-action and non-specific contact weed killer in agriculture, but is extremely toxic to humans when exposed through direct contact or through bioaccumulation in food chain [2]. Globally, 250,000 to 370,000 people die from pesticide poisoning each year, and more than 90% of individuals with acute poisoning attempted suicide by intentionally ingesting PQ [3]. PQ may also cause chronic health problems through chronic oral, skin contact and respiratory exposure. In lung tissues, PQ causes pulmonary damage ranging from acute respiratory distress to progressive lung fibrosis [4-6]. Clinically, PQ poisoning is classified into 3 degrees: mild poisoning (oral irritation and gastric upset); moderate poisoning (acute renal failure, hepatitis followed by pulmonary fibrosis and death after 2-3 weeks); and fulminant poisoning (multiple organ failure and cardiogenic shock causing death within hours to a few days after ingestion) [7, 8]. Management strategies for PQ poisoning include extracorporeal elimination, immunosuppressants and antioxidants [9, 10]. However, there is no sufficient evidence indicating good clinical outcomes, and mortality rates remain very high despite current treatment methods [11, 12].

Previous studies demonstrated that pro-inflammatory and fibrotic cytokines are involved in lung injury caused by PQ poisoning, along with activation of signaling pathways that regulate cell apoptosis, survival and proliferation [13-17]. One such signaling pathway involves the mammalian target of rapamycin (mTOR), a serine/threonine kinase that belongs to the phosphoinositide kinase-related kinase (PIKK) family. The mTOR is highly conserved and ubiquitously expressed protein that controls cell

Table 1. Comparison of mortality rate among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection

Group	1 d (n = 12)	Mortality rate	3 d (n = 12)	Mortality rate	5 d (n = 12)	Mortality rate	7 d (n = 12)	Mortality rate
Control group	0	0	0	0	0	0	0	0
PQ group	4	33.3%	6	50.0%	6	50.0%	10	83.3%
RAPA treatment group	2	16.7%	4	33.3%	3	25.0%	6	50%
χ^2	4.80		7.75		8.00		17.10	
P	0.09		0.02		0.02		< 0.01	

Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection.

growth, proliferation and survival, downstream of the phosphatidylinasitol 3-kinase (PI3K)/protein kinase B pathway [18-20]. mTOR senses growth and metabolic signals, such as ATP, oxygen, amino acids, glucose, and reactive oxidant species, and exerts anabolic effects by stimulating protein synthesis and ribosomal biogenesis, enhancing cell proliferation, and promoting cell survival [21]. Rapamycin (RAPA) is a highly specific and potent inhibitor of mTOR, which displays anti-proliferative and immunosuppressive properties [22-24]. RAPA and its analogs, through mTOR suppression, can inhibit critical phosphorylation events associated with epithelial-mesenchymal transition in hepatocyte, with potential relevance to suppressing liver fibrosis [25]. Accordingly, administration of mTOR inhibitors in experimental liver fibrosis animal model shows good inhibition of the diseases state [26, 27]. In this study, we constructed an acute lung injury model by exposing experimental rats to PQ. This study evaluates the protective effect of RAPA in suppressing PQ-induced acute lung injury, to provide an understanding of the underlying mechanisms of PQ-induced lung injury and to test the efficiency of RAPA treatment in PQ poisoning.

Materials and methods

Animals and treatments

Male Sprague-Dawley (SD) rats (n = 144; approximate body weight, 240-330 g) were purchased from the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). All SD rats were housed in stainless-steel cages with free access to food and water, and were kept at room temperature exposed to alternate cycles of 12-hour light and darkness. The rats were randomly divided into 3 groups: control group (n = 48): SD rats were intraperitoneally injected with 1.25 ml/kg saline solution once daily. PQ group (n = 48): SD rats were intraperitoneally injected with 15 ml/kg 2% PQ solution once daily. RAPA treatment group (n = 48): PQ-poisoned SD rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg RAPA once daily. The intraperitoneal injections were administered at the same time daily. The survival of the SD rats in each group was observed with 4 observation periods at days-1, 3, 5, and 7, after initiation of the peritoneal injections. Twelve rats in each group were sacrificed under anesthesia at 1, 3, 5, and 7 days after intraperitoneal injection. All animal procedures were in accordance with National Institutes of Health (NIH) guidelines [28], and all experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the First Affiliated Hospital of China Medical University.

Tissue preparation and hematoxylin-eosin (HE) staining

Lung tissues removed from SD rats immediately after execution at 1, 3, 5, and 7 days were rinsed with ice-cold saline solution to remove excess blood. All lung tissues were fixed with paraformaldehyde (10%), embedded in paraffin, and sliced at thickness of 5 μm. Then all slices were stained with HE and observed under an optical microscope.

Protein content determination and cell count in bronchoalveolar lavage fluid (BALF)

SD rats in the control group, PQ group and RAPA treatment group were intratracheally injected with 4 ml, 3 ml, and 3 ml saline solution (4°C) 3 times, immediately after sacrificing

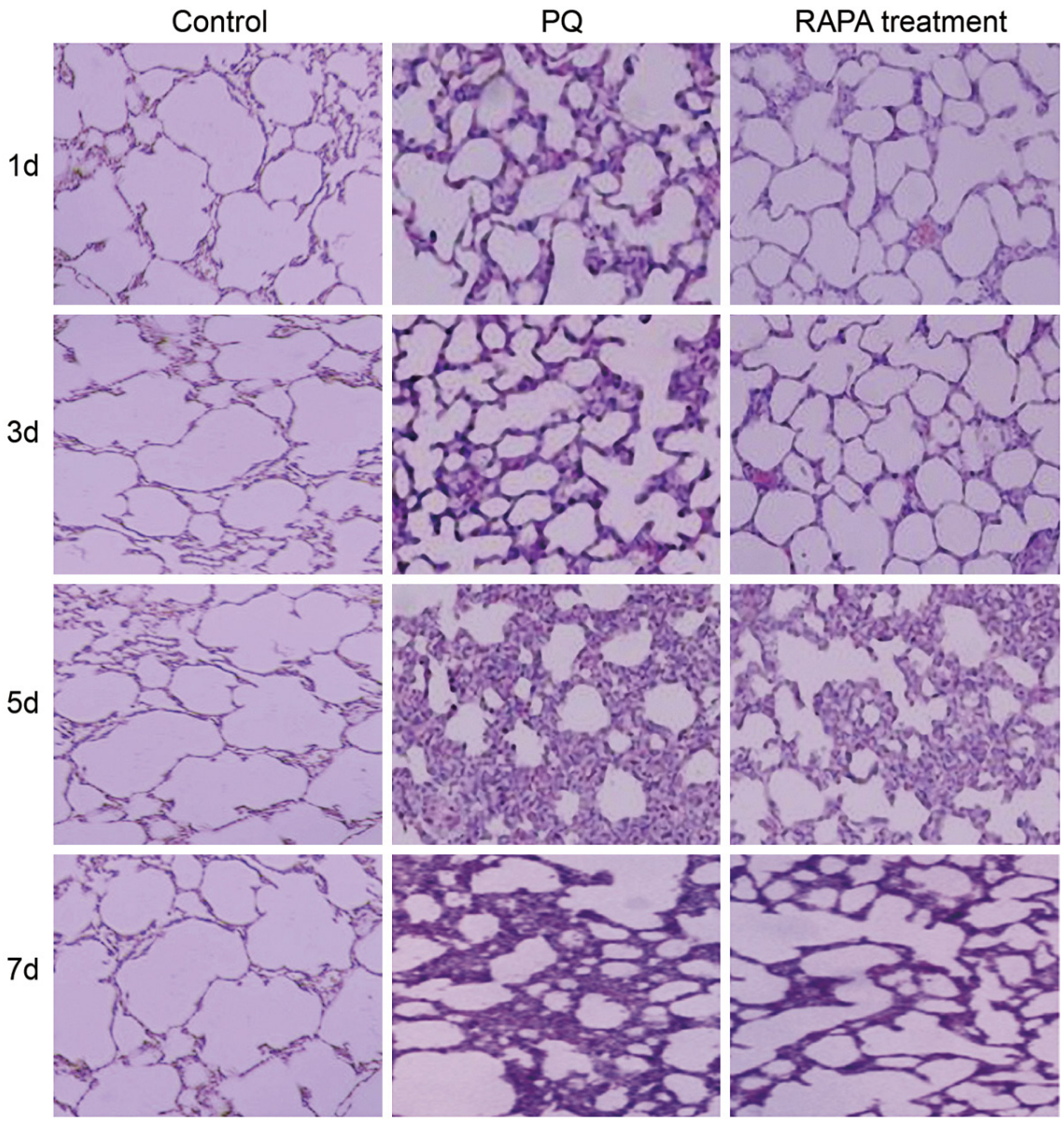

Figure 1. Dynamic pathological changes in rat lung tissues observed after hematoxylin-eosin staining and visualized under an optical microscope (× 200). Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection.

at 1, 3, 5, and 7 days. BALF was retrieved and the volume was recorded. Retrieved BALF from 3 times of intratracheal injection was mixed well and centrifuged at 1500 rpm at 4°C for 5 min (centrifugal radius, 13.5 cm). Protein content was determined from the supernatant with the Coomassie blue staining. Cell pellet was resuspended in 0.3 ml saline solution. The total number of neutrophils and leukocytes were counted using hemocytometer. Mean values and percentage of neutrophils in BALF was calculated.

Determination of malondialdehyde (MDA) content, glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity and neutrophil and leukocyte count in blood

A single intraperitoneal injection of sodium pentobarbital (40 mg/kg, 1%) was given to the

Table 2. Comparison of protein content in bronchoalveolar lavage fluid among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection (μg/L, $\bar{x} \pm s$)

Group	1 d	3 d	5 d	7 d	F^*	P^*
Control group	46.7 ± 8.19	51.88 ± 14.60	53.67 ± 9.72	47.76 ± 10.96	0.034	0.991
PQ group	144.61 ± 19.80[a]	151.18 ± 39.24[a]	154.71 ± 28.63[a]	153.28 ± 33.26[a]	0.053	0.984
RAPA treatment group	113.55 ± 12.68[a,b]	124.02 ± 16.30[a,b]	108.08 ± 17.03[a,b]	106.19 ± 17.40[a,b]	0.032	0.992

Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection; [a]compared with control group, $P < 0.001$; [b]compared with PQ group, $P < 0.001$; *compared at 4 different time points in the same group, $P > 0.05$.

Table 3. Comparison of leukocyte count and neutrophils percentage in blood and bronchoalveolar lavage fluid among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection ($\bar{x} \pm s$)

Group	Time	n	Blood		BALF	
			Leukocyte count (× 10^9/L)	Neutrophils percentage (%)	leukocyte count (× 10^9/L)	Neutrophils percentage (%)
Control group	1d	12	2.91 ± 1.21	13.75 ± 5.52	0.18 ± 0.03	21.25 ± 2.35
	3 d	12	2.52 ± 1.18	15.69 ± 5.22	0.18 ± 0.02	21.05 ± 2.46
	5 d	12	2.74 ± 1.43	16.94 ± 5.84	0.17 ± 0.01	21.53 ± 2.41
	7 d	12	2.15 ± 1.13	15.50 ± 6.15	0.17 ± 0.04	20.89 ± 2.46
PQ group	1 d	12	7.95 ± 2.59[a]	41.50 ± 10.20[a]	0.32 ± 0.04[a]	41.15 ± 9.68[a]
	3 d	12	9.02 ± 4.00[a]	51.78 ± 8.58[a]	0.39 ± 0.12[a]	42.37 ± 10.25[a]
	5 d	12	8.54 ± 3.10[a]	55.42 ± 9.83[a]	0.48 ± 0.05[a]	44.70 ± 9.69[a]
	7 d	12	8.57 ± 2.97[a]	57.67±9.68[a]	0.40 ± 0.05[a]	40.98 ± 4.76[a]
RAPA treatment group	1 d	12	6.46 ± 2.58[a]	40.62 ± 7.28[a]	0.32 ± 0.06[a]	40.45 ± 6.44[a]
	3 d	12	6.10 ± 2.41[ab]	36.40 ± 8.43[ab]	0.32 ± 0.05[a]	38.13 ± 6.78[a]
	5 d	12	5.65 ± 1.93[ab]	35.10 ± 8.44[ab]	0.29 ± 0.06[ab]	34.10 ± 6.23[ab]
	7 d	10	4.98 ± 1.75[ab]	34.15 ± 8.30[ab]	0.29 ± 0.01[ab]	32.49 ± 6.61[ab]

BALF, bronchoalveolar lavage fluid; Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection; [a]compared with control group, $P < 0.05$; [ab]compared with PQ group, $P < 0.05$.

SD rats in the control group, PQ group and RAPA treatment group. The abdominal cavity was opened, and blood was collected from the inferior vena cava, centrifuged at 3000 rpm for 5 min, separated from serum, and stored at -70°C. The neutrophil and leukocyte count in blood was obtained using the hemocytometer. Mean values and percentage of neutrophils in blood was calculated. The content of MDA and the activity of SOD were determined with thiobarbituric acid (TBA) assay and the pyrogallol autoxidation method, respectively. The GSH-Px activity was determined by the modified Haefman method. Procedures were performed according to manufacturer's instructions.

Electrophoretic mobility gel shift assay (EMSA) for nuclear factor (NF)-κB activity in lung tissues

Lung tissue (100 mg) was obtained from SD rats of control group, PQ group and RAPA treatment group. Nucleoprotein was extracted, and protein concentration was determined by Coomassie blue kit in accordance with manufacturer's instructions. The EMSA detection was performed with [γ-32P] ATP and T4 polynucleotide kinase end labeling NF-κB probe (5'-AGTTGAGGGGACTTTCCCAGG-3'), and the end-labeled oligonucleotides were purified by using the ethanol precipitation method. After

Table 4. Comparison of malondialdehyde content, glutathione peroxidase and superoxide dismutase activity in blood among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection ($\bar{x} \pm s$)

Group	Time (d)	n	MDA (nmol/ml)	SOD (U/ml)	GSH-Px (U/ml)
Control group	1	12	4.39 ± 0.65	3186.3 ± 424.0	1445.2 ± 137.5
	3	12	4.02 ± 0.70	3190.0 ± 455.2	1495.7 ± 165.2
	5	12	4.14 ± 0.67	3317.3 ± 378.3	1487.4 ± 142.2
	7	12	4.09 ± 0.78	3384.0 ± 537.2	1545.5 ± 170.0
PQ group	1	12	6.55 ± 1.24[a]	2578.3 ± 457.3[a]	1054.0 ± 152.2[a]
	3	12	8.89 ± 1.35[a]	2637.9 ± 596.0[a]	812.0 ± 108.0[a]
	5	12	8.63 ± 1.40[a]	2653.5 ± 612.5[a]	1197.7 ± 178.2[a]
	7	12	8.11 ± 1.17[a]	2714.3 ± 671.0[a]	1247.8 ± 156.2[a]
RAPA treatment group	1	12	5.67 ± 1.34[ab]	2842.7 ± 557.8[a]	1193.3 ± 135.0[ab]
	3	12	5.51 ± 1.10[ab]	3695.7 ± 448.2[ab]	1315.6 ± 87.0[ab]
	5	12	5.24 ± 0.89[ab]	3742.4 ± 512.2[ab]	1340.5 ± 194.0[ab]
	7	12	4.92 ± 0.85[ab]	3912.2 ± 375.7[ab]	1456.5 ± 185.0[ab]

MDA, malondialdehyde; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection; [a]compared with control group, $P < 0.05$; [ab]compared with PQ group, $P < 0.05$.

the binding reaction between labeled NF-κB probes and extracted nucleoprotein (10 μg) at room temperature, the samples were electrophoresed using polyacrylamide gel (6%). The gel was vacuum dried at 60°C for 75 min, and autoradiography of the dried gel was performed at 8°C. The electrophoretic images and optical density of amplified bands were semi-quantitative analyzed by using Gel-Pro software (Version 4.5). Relative NF-κB activity = the area of electrophoretic bands × fluorescence intensity. Negative control, positive control, competitive inhibitor binding and non-competitive inhibitor binding were used to determine the specificity of NF-κB DNA-binding complex.

Western blot for P-p38 protein level

Lung tissue (100 mg) was obtained from SD rats of control group, PQ group and RAPA treatment group. After washed by saline solution, precooled homogenization buffer [Tris-buffered saline (TBS): phenylmethylsulfonyl fluoride (PMSF) = 20:1; Sheng Long Co., Shanghai, China] was added with 10 μl/g. Then, the tissue samples were homogenized with a high-speed disperser and centrifuged at 12,000 × g at 4°C for 15 min. Protein content was determined from the supernatant with the Coomassie brilliant blue G250 (Bo Gu Co., Shanghai, China). Cytoplasmic protein (150 μg) was obtain from each sample, and added with 150 μg sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Cheng Bin Co., Shanghai, China) with the concentration double of potent concentration, heated in a boiling water bath for 5 min, and electrophoresised in wells containing pre-prepared 80 g/L SDS-PAGE after natural cooling. The protein gel samples were then transferred onto nitrocellulose membrane. After the membrane was blocked by blocking solution, mouse monoclonal anti-p-p38 MAPK (D-8; 1:1000) antibodies (Abace Co., Beijing, China) and HRP-labeled goat anti-mouse IgG (1:1000) secondary antibodies (Sunshine Bio. Co., Nanjing, China) were added successively for incubation. The membrane was then washed with TBS/0.05% Tween-20 (TTBS; Boster Biotech Co., Wuhan, China) 3 times for 15 min per time, added with electrochemiluminescence (ECL) detection reagents (chemiluminescence substrate; Vazyme biotech co., ltd., Nanjing, China) to react for 3-5 min. The membrane was exposed in the cassette for 1 min and the exposed film was developed. The developed binds were scanned into computer, and semi-quantified with the Gel-Pro Analyzer gel analysis software program. The protein levels were represented with the ratio of arbitrary unit (AU, Darea Ddensity, AU = the area of bands × fluorescence intensity).

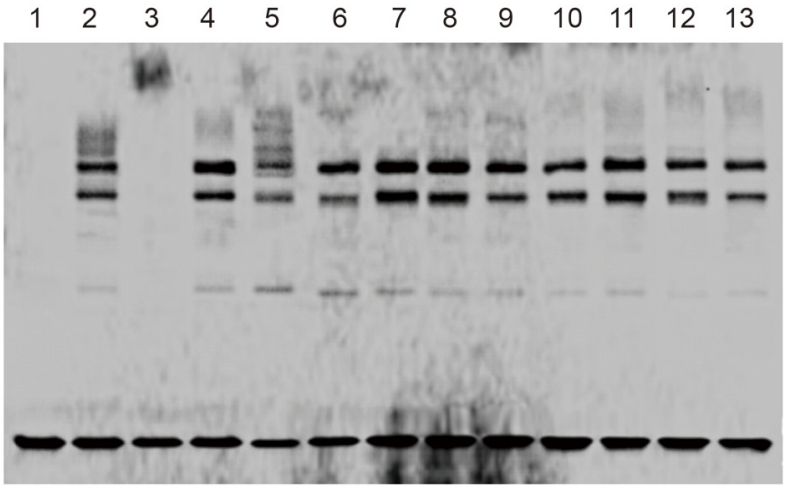

Figure 2. Comparison of protein content among bronchoalveolar lavage fluid in control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection. Activity of NF-κB in the lung tissues of control group, paraquat group and rapamycin treatment group by electrophoretic mobility gel shift assay. 1, negative control group; 2, positive control group; 3, competitive inhibitor binding group; 4, non-competitive inhibitor binding group; 5, control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; 6, 7, 8, 9, PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution after 1, 3, 5 and 7 days, respectively; 10, 11, 12, 13, RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin after 1, 3, 5 and 7 days, respectively.

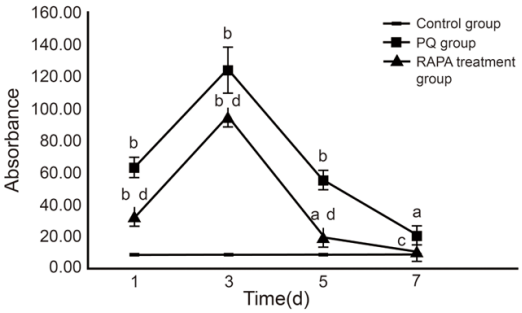

Figure 3. Comparison of dynamic change in NF-κB activity in the lung tissues among control group, paraquat group and rapamycin treatment group at 1, 3, 5 and 7 days after intraperitoneal injection. Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; a, compared with control group, $P < 0.05$; b, compared with control group, $P < 0.05$; c, compared with PQ group, $P < 0.05$; d, compared with PQ group, $P < 0.01$.

Arterial blood gas analysis and lung wet-dry weight ratio (W/D)

Carotid arterial blood was obtained from SD rats in the control group, PQ group and RAPA treatment group immediately after execution at 1, 3, 5, and 7 days. Carbon dioxide partial pressure ($PaCO_2$), partial pressure of oxygen (PaO_2) and pH value was measured by using the automated blood gas analyzer. Right upper lobe was obtained and the wet weight was measured. The obtained right upper lobe was dried in a convection oven until constant weight; the dry weight was measured, and the W/D was calculated.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Measurement data were presented as means ± standard deviation (SD, $\bar{x} \pm s$). The statistical comparison between two groups was conducted using the *t*-test; and the statistical comparison among multiple groups using the analysis of variance (ANOVA). Count data were presented as ratio or rate, and verified with the χ^2 test. *P* values < 0.05 were considered statistically significant.

Results

Mortality rate of SD rats

The survival of SD rats was recorded at 1, 3, 5, and 7 days after intraperitoneal injection, as shown in **Table 1**. There was no mortality in the control group. The mortality rate in the PQ group at 1, 3, 5, and 7 days after intraperitoneal injection was 33.3%, 50.0%, 50.0% and 83.3%, respectively, and the mortality rate in RAPA treatment group at 1, 3, 5, and 7 days after intraperitoneal injection was 16.7%, 33.3%, 25.0%, and 50%, respectively. The differences in the mortality rates between the

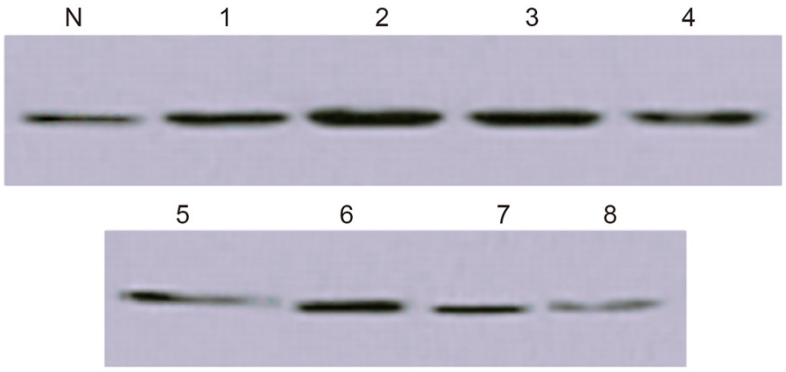

Figure 4. Dynamic changes in phosphorylation of p38MAPK protein in lung tissues before and after treatment with rapamycin at 1, 3, 5, and 7 days after intraperitoneal injection. N, control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; 1, 2, 3, 4, PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution for 1, 3, 5, and 7 days; 5, 6, 7, 8, RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin for 1, 3, 5, and 7 days.

control group, PQ group and RAPA treatment groups at 3, 5, and 7 days after intraperitoneal injections were statistically significant (all $P < 0.05$).

Pathological changes

HE staining results are displayed in **Figure 1**. In control group, HE staining results showed clear alveolar structure and thin alveolar wall. In addition, no widening or hyperemia was observed in alveolar septum and no symptom of inflammatory cell infiltration and bleeding was detected. In PQ group, alveolitis was clearly noticeable and pulmonary interstitial and alveolar edema progressed most rapidly. The alveolar space was filled with homogeneous edema fluid, free neutrophils and macrophages. Vascular endothelial cell injury, diffuse pulmonary hemorrhage, alveolar collapse, and formation of hyaline membrane occurred at 1 and 3 days after injection with PQ. By the fifth day after injection with PQ, a heavy infiltration of inflammatory cells, mainly neutrophils and macrophages and few lymphocytes, occurred in alveolar space, accompanied with edema and hyporrhea. At seventh day after injection with PQ, inflammatory lesion was alleviated, but edema and hyporrhea remained and proliferation of fibroblasts and tissues appeared. The alveolar septum was significantly expanded. In RAPA treatment group, pulmonary lesions were similar to the PQ group, but significantly milder. It was observed that the lung was slightly enlarged; ecchymosis and blutene chloaide appeared in part of the lung. The surface of lung tissues was uneven locally, with a scatter of small white nodules at the late stage. The HE staining results demonstrated RAPA alleviated pathological changes of acute alveolitis in SD rats and possibly inhibited of collagen deposition.

Protein content change in BALF

The protein content in BALF was determined with the Coomassie blue staining at 1, 3, 5, and 7 days after intraperitoneal injection (**Table 2**). At each time point, the protein content in BALF of both PQ group and RAPA treatment group was significantly higher than the control group (all $P < 0.001$). The protein content in BALF of the RAPA treatment group was obviously lower than PQ group (all $P < 0.001$). The protein content in BALF was similar at different time points within each group, including the control group, the PQ group and the RAPA treatment group (all $P > 0.05$).

Leukocyte count and neutrophils percentage in blood and BALF

The number of leukocytes and the percentage of neutrophils in blood and BALF were determined at 1, 3, 5, and 7 days after intraperitoneal injection (**Table 3**). At each time point, the number of leukocytes and the percentage of neutrophils in both blood and BALF of the PQ group and RAPA treatment group were markedly higher, compared with the control group (all $P < 0.001$). At 3, 5, and 7 days after intraperitoneal injection, the number of leukocytes and the percentage of neutrophils in blood of the RAPA treatment group were significantly lower than PQ group (all $P < 0.005$). At 1 day after intraperitoneal injection, no statistical difference in the number of leukocytes and the percentage of neutrophils in blood was found between the RAPA treatment group and the PQ group (both $P > 0.005$). In addition, the number

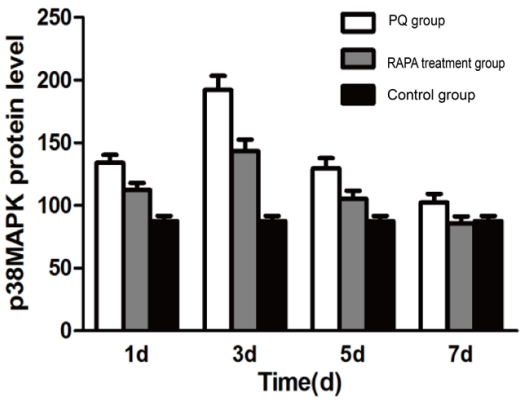

Figure 5. Comparison of phosphorylated p38MAPK protein level among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection. Control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin.

of leukocytes and the percentage of neutrophils in BALF of the RAPA treatment group were lower than the PQ group at 5 and 7 days after intraperitoneal injection, respectively (all $P < 0.005$). At 1 and 3 days after intraperitoneal injection, there was no statistical difference in the number of leukocytes and the percentage of neutrophils in BALF between the RAPA treatment group and the PQ group (all $P > 0.005$).

MDA content, GSH-Px and SOD activity in plasma

The content of MDA and the activity of SOD and GSH-Px were determined using thiobarbituric acid (TBA) assay, pyrogallol autoxidation method, and modified Haefman method, respectively, at different time points (**Table 4**). At each time point, the MDA content in plasma of PQ group was higher than control group and RAPA treatment group (all $P < 0.05$). At each time point, the activity of SOD in plasma of the PQ group was observably lower than the control group (all $P < 0.05$). At 3, 5, and 7 days after intraperitoneal injection, the activity of SOD in plasma of RAPA treatment group was higher than the control group (all $P < 0.05$). Compared with the PQ group, the activity of SOD in plasma of the RAPA treatment group was evidently higher at 3, 5, and 7 days after intraperitoneal injection (all $P < 0.05$). At 1 day after intraperitoneal injection, the difference in the activity of SOD in plasma between the PQ group and the RAPA treatment group showed no statistical significance ($P > 0.05$). At each time point, the activity of GSH-Px in plasma of the PQ group and the RAPA treatment group was lower than control group (all $P < 0.05$). The activity of GSH-Px in plasma of the RAPA treatment group was higher than the PQ group at the same time point (all $P < 0.05$).

Change of NF-κB activity in lung tissues

The NF-κB activation in lung tissues was determined by EMSA, as shown in **Figures 2** and **3**. The activity of NF-κB in the control group did not change and remained at low basal level. NF-κB activity in lung tissues of the PQ group enhanced at 1 day after intraperitoneal injection and reached its peak at 3 days after intraperitoneal injection. A gradual decreasing trend, but still relatively high activity was observed at 5 days after intraperitoneal injection, and rapidly decreased to control group level by 7 days after intraperitoneal injection. A pattern of NF-κB activity similar to the PQ group was observed in lung tissues of RAPA treatment group. At 1, 3 and 5 days after intraperitoneal injection, the activity of NF-κB in PQ group was higher than RAPA treatment group, whose NF-κB activity in-turn was significantly higher than the control group (all $P < 0.05$).

Change of phosphorylation of p38MAPK in lung tissues

The phosphorylation of p38MAPK in the lung tissues of the control group was few (87.51 ± 4.25). The protein levels of p38MAPK in the PQ group at 1, 3, 5 and 7 days after intraperitoneal injection were 134.15 ± 6.15, 192.15 ± 11.26, 129.45 ± 8.45 and 102.45 ± 6.89, respectively. In the RAPA treatment group, p38MAPK protein levels were 112.45 ± 5.62, 143.15 ± 9.45, 105.26 ± 6.48 and 85.79 ± 5.48, respectively, at 1, 3, 5 and 7 days after intraperitoneal injection. Compared with the control group, the phosphorylated p38MAPK protein levels in the lung tissues of the PQ group enhanced obviously at 1 day after intraperitoneal injection; reached its highest point at 3 days after intraperitoneal injection; and decreased at 5 and 7 days after intraperitoneal injection which were still higher than the levels in the control group ($P < 0.05$). Compared with the PQ group, the phosphorylation of p38MAPK in the lung tis-

Table 5. Comparison of changes in carbon dioxide partial pressure, partial pressure of oxygen, pH value and lung wet-dry weight ratio among control group, paraquat group and rapamycin treatment group at 1, 3, 5, and 7 days after intraperitoneal injection ($\bar{x} \pm s$)

Group	Time (d)	n	pH value	$PaCO_2$ (kPa)	PaO_2 (kPa)	W/D (%)
Control group	1	12	7.12 ± 0.05	3.53 ± 0.60	12.10 ± 1.32	4.32 ± 0.26
	3	12	7.20 ± 0.05	4.74 ± 0.41	12.32 ± 1.01	4.28 ± 0.32
	5	12	7.22 ± 0.04	4.51 ± 0.32	12.10 ± 0.97	4.41 ± 0.29
	7	12	7.21 ± 0.03	4.61 ± 0.24	12.02 ± 0.87	4.24 ± 0.22
PQ group	1	12	7.19 ± 0.05	3.95 ± 0.71[a]	7.80 ± 0.81[a]	6.18 ± 0.21[ab]
	3	12	7.21 ± 0.04	6.73 ± 0.64[a]	6.88 ± 0.72[a]	6.25 ± 0.32[ab]
	5	12	7.24 ± 0.03	7.14 ± 0.51[a]	7.02 ± 0.93[a]	6.33 ± 0.26[ab]
	7	12	7.26 ± 0.05	6.95 ± 0.82[a]	6.98 ± 1.12[a]	6.46 ± 0.33[ab]
RAPA treatment group	1	12	7.20 ± 0.05	3.95 ± 0.13[a]	8.54 ± 0.81[ab]	5.01 ± 0.24[ab]
	3	12	7.21 ± 0.08	5.16 ± 0.51[ab]	8.26 ± 1.02[ab]	5.18 ± 0.30[ab]
	5	12	7.18 ± 0.03	5.22 ± 0.42[ab]	8.11 ± 0.97[ab]	5.07 ± 0.25[ab]
	7	12	7.21 ± 0.05	5.92 ± 0.51[ab]	8.35 ± 0.75[ab]	5.02 ± 0.27[ab]

$PaCO_2$, carbon dioxide partial pressure; PaO_2, partial pressure of oxygen; W/D, lung wet-dry weight ratio; control group, Sprague-Dawley rats were intraperitoneally injected with 1.25 ml/kg saline solution; PQ group, Sprague-Dawley rats were intraperitoneally injected with 15 ml/kg 2% paraquat solution; RAPA treatment group, paraquat-poisoned Sprague-Dawley rats were immediately intraperitoneally injected with 1.25 ml/kg saline solution containing 4 mg/kg rapamycin; 1 d, 3 d, 5 d, 7 d, 1, 3, 5, and 7 days after intraperitoneal injection; [a]compared with control group, $P < 0.05$; [ab]compared with PQ group, $P < 0.05$.

sues of the RAPA treatment group attenuated evidently at 1, 3, 5 and 7 days after intraperitoneal injection ($P < 0.05$). Compared with the control group, the phosphorylated p38MAPK protein levels in the lung tissues of the RAPA treatment group increased observably at 1, 3, and 5 days after intraperitoneal injection ($P < 0.05$) (**Figures 4**, **5**).

Arterial blood gas analysis results and W/D

The results of arterial blood gas analysis and calculated W/D are shown in **Table 5**. At each time point, $PaCO_2$ and W/D in the RAPA treatment group were higher than control group (both $P < 0.05$), and PaO_2 in RAPA treatment group was lower than the control group ($P < 0.05$). Compared with the PQ group, W/D in RAPA treatment group was lower, while PaO_2 in the RAPA treatment group was markedly higher (both $P < 0.05$). Additionally, $PaCO_2$ in the RAPA treatment group was lower than PQ group at 3, 5 and 7 days after intraperitoneal injection (all $P < 0.05$). No statistical significance was found in the difference in pH value between the control group, PQ group and RAPA treatment group (all $P > 0.05$).

Discussion

PQ is highly toxic to humans and induces extensive pulmonary injury, including pulmonary edema, hypoxemia, respiratory failure, and pulmonary fibrosis [29-31]. Generally, acute lung injury is diffuse heterogeneous lung injury characterized by hypoxemia, non-cardiogenic pulmonary edema, low lung compliance and widespread capillary leakage [32]. In clinic, acute lung injury induced by PQ is complex process that involves a variety of peroxidase enzyme, such as GSH-PX and SOD, and peroxide, such as MDA [33]. The mTOR pathway is a sensor of energy status in the cell and is a major signaling hub, regulating several aspects of mRNA translation and protein synthesis, in a phosphorylation-dependent manner [34-36]. The mTOR signal pathway may increase the negative effects of acute lung injury caused by PQ poisoning and RAPA may be a novel therapeutic strategy [37, 38]. Accordingly, RAPA treatment has potent anti-inflammatory, anti-proliferative, and anti-angiogenic properties [39, 40]. Based on the evidenced functions of RAPA, we conducted this present study to further explore the underlying relationships between RAPA and PQ-induced acute lung injury.

In the present study, our main results demonstrate that RAPA can reduce acute lung injury, decreased protein synthesis and increased apoptosis of leukocytes and neutrophils in BALF in rats exposed to PQ. The dramatic reduction in leukocytes and neutrophils, which are markers of inflammation, in BALF caused by

RAPA correlates with reversal of acute lung injury, and is consistent with the improved survival of PQ-induced acute lung injury rats in our study [41]. In addition, the activation of the proinflammatory and antiapoptotic transcription factor NF-κB was attenuated. These results imply that inactivation of mTOR by RAPA may sensitize the proapoptotic responses, inhibition of protein synthesis and the inhibition of NF-κB signaling pathway. Previously, it was shown that mTOR is involved in activation of NF-κB signaling pathway [42]. Dan et al. have reported that mTOR signaling pathway interacts with IKK and is involved in Akt-dependent regulation of NF-κB activation [43]. Therefore, it is plausible that RAPA inhibition of mTOR caused repression of NF-κB activation observed in our study. Further, Hou et al. showed that inhibition of mTOR by RAPA repress PQ-induced inflammatory cytokine production, leading reduction of leukocytes and neutrophils populations in BALF and blood, lowering in protein synthesis, and decreased phosphorylation of NF-κB in neutrophils [44].

In our study, GSH-Px and SOD activity in blood increased and the content of MDA in blood decreased upon intraperitoneal injection of RAPA in PQ-induced acute lung injury rats. A previous study detected increased production of ischemia and markers such as MDA, indicating lipid peroxidation in the lung tissue during acute lung injury. By contrast, decreased activity of antioxidative enzymes, GSH-Px and SOD, was observed in lung tissue as a result of acute lung injury [33]. During acute lung injury induced by PQ, GSH-Px and SOD activities are likely to be perturbed as a result of overproduction of oxygen-derived radicals [45]. The pulmonary parenchyma is very vulnerable to free oxygen radicals, which cause oxidative damage to biomembranes, lipids, proteins and DNA, leading to lung dysfunction and cell death [33]. We found that the MDA content was down-regulated after RAPA administration, in addition, the hallmark of injury protection and repair, SOD and GSH-Px activity, were up-regulated after administrated of RAPA, suggesting that RAPA decreases MDA content and increases SOD and GSH-Px activity. However, the potential mechanisms through which RAPA plays a role in the regulation of MDA content and SOD and GSH-Px activity are still unclear. One explanation of the important results is supposed to be that inhibition of mTOR caused by RAPA leading to repression of NF-κB activation might play a crucial role in removing free oxygen radicals, resulting in protection against acute lung injury.

In addition, we found that PQ-induced acute lung injury rats intraperitoneally injected with RAPA had lower $PaCO_2$ as well as higher PaO_2, as compared the rats in the PQ group, suggesting that RAPA also plays a role in the regulation of $PaCO_2$ and PaO_2. With respect to gas exchange, the impact of increasing flow on ventilation and oxygenation occurred independent of tracheal pressure generation alone [46]. With increasing PaO_2 and decreasing $PaCO_2$ progressive change occur, resulting from intraperitoneal injection of RAPA, demonstrating that $PaCO_2$ and PaO_2 improved in a somewhat flow-dependent manner. Therefore, our results revealed RAPA might have functions in the improvement of hypoxemia in acute lung injury induced by PQ.

In conclusion, we demonstrated that PQ-induced acute lung injury could be effectively reduced by RAPA treatment and the pathological changes of acute alveolitis can be alleviated. Thus RAPA treatment could be significantly beneficial in PQ poisoning induced acute lung injury.

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Disclosure of conflict of interest

None.

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