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Original Article

Protective effects of sodium salicylate on paraquat poisoning

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Abstract: Objectives: This study aims to investigate the effect of sodium salicylate on lungs of rats with paraquat poisoning. Methods: Rats were divided into 3 groups: control group, paraquat poisoning group, and sodium salicylate intervention group, and 14 day and 28 day were selected as observation time points. The arterial blood gas in rat of each group was observed. HE staining was used to observe the morphological changes in rat lung tissue. QRT-PCR was used to detect the mRNA expression changes of TNF-α and IL-6 in lung tissue and blood. Western blot was used to detect protein changes of TNF-α and IL-6 in lung tissue, and ELISA was used to detect the protein changes of TNF-α and IL-6 in blood. Results: Compared with paraquat poisoning group, oxygen partial pressure in sodium salicylate intervention group was significantly increased, and carbon dioxide partial pressure was significantly decreased ($P<0.05$). HE staining showed that pathological changes were significant in rat lung tissue in paraquat poisoning group, while sodium salicylate intervention can reverse the changes to some extent. Compared with the control group, expressions of TNF-α and IL-6 in lung tissues and blood were significantly increased both in mRNA level and in protein level in prarquat poisoning group ($P<0.05$). Actually intervention by sodium salicylate reduced the expression of TNF-α and IL-6 in lung tissues and blood.Conclusion: Sodium salicylate can alleviate the lung injury induced by paraquat poisoning, which may be associated with the decreased expression of TNF-α and IL-6.

Keywords: Paraquat, sodium salicylate, TNF-α, IL-6

Introduction

Paraquat (PQ) is generally made into dichloride or methyl disulfide, and the main formulation is 20% aqueous solution. PQ continues to be used as an herbicide since it was registered first time in 1962 [1], which has become the second herbicides in the world [2]. PQ belongs to bipyridine-based compounds, which will damage most of the internal organs after swallowing, especially the heart, liver, lung, kidney [3-5]. With the widespread use, it has become one herbicide with the highest mortality rate for human acute poisoning. It was reported that PQ had a high mortality rate [6], which presented a huge challenge for medical workers. Actually there is still no specific antidote for PQ, so developing drugs for treatment PQ poisoning has become an urgent task.

Among damaged organ by PQ poisoning, early inflammatory characteristics is obvious in lung tissue: damaged alveolar epithelial cells, alveolar hemorrhage and edema, inflammatory cell infiltration, irreversible fibrosis in alveolar and pulmonary interstitial cells [7]. Early treatment protocols usually include anti-inflammatory drugs, and many anti-inflammatory drugs have been considered to treat PQ poisoning. Anti-inflammatory therapy has become an important means for early treatment of PQ poisoning. Meanwhile, some key genes with largely different expression in occurrence and development of inflammation have become candidate targets for gene therapy.

In this study, the selected sodium salicylate is one classic antipyretic analgesic, anti-inflammatory, and anti-rheumatic drug. In 1971, some researchers firstly proposed that NSAIDs (including salicylates) could play anti-inflammatory roles by inhibiting prostaglandin [8]. In the mid-1990s, studies showed that the anti-inflammatory of salicylates was closely related to NF-κB [9]. There were rare reports about whether sodium salicylate can relieve or treat PQ poisoning.

Table 1. Primer sequences of qRT-PCR

Gene	Primer sequence (5'-3')
TNFα	Forward primer: CTCCGGGCTCAGAATTTCC
	Reverse primer: CGCAATCCAGGCCACTACTT
IL-6	Forward primer: CCACCAGGAACGAAAGTCAAC
	Reverse primer: GGCAGTGGCTGTCAACAACA
GAPDH	Forward primer: GTGAAGGTGCGGAGTCAACG
	Reverse primer: TGAGGTCAATGAAGGGGTC

In this study, we established a rat model of PQ poisoning and observed whether sodium salicylate could protect the lung injury induced by PQ poisoning. We also explored the associated mechanisms about how sodium salicylate alleviated PQ poisoning. Our findings can provide theoretical basis for PQ poisoning treatment.

Methods and materials

Establishment of PQ poisoning rat model

Totally 18 rats in 8-10 weeks Wister rat (SLAC Laboratory Animal Company, Shanghai, China) were used in this study, and the body weight is about 250-300 g. Before formal experiments, all rats were adaptively fed for one week with free access to food and drinking water. During experiments, 3R principles of animal welfare were followed. Rats were divided into 3 groups: control group (0.9% NaCl), PQ poisoning group (PQ group) and NaSAL intervention group (PQ+NaSAL group), and each group has 6 rats. In control group, 0.005 mL/g normal saline was given to rat by gavage, and equal volume of saline was given by intraperitoneal injected after 2 h. For PQ group, according to the body weight, each rat was given 80 mg/kg PQ by intragastric infusion, and after 2 h, intraperitoneal injection of an equal volume of saline. For PQ+NaSAL group, PQ was given similarly to PQ group, and intraperitoneal injection of an equal volume of NaSAL after 2 h. After PQ poisoning, animals were killed by the observation time points, and all animal experiments were finished at 13:00 to 15:00. All animal experiments were conducted according to the ethical guidelines of Shanghai Ninth People's Hospital.

Sample collection

After PQ poisoning 14 d and 28 d, 3 rats in each group were used to collect blood by abdominal aortic puncture, and then isolated serum was reserved at -80°C. Then after 10% chloral hydrate intraperitoneal injection for anesthesia, the abdominal cavity was open, and blood was collected from abdominal aorta for arterial blood gas analysis. Then rats were killed and entire lung was cut for observation. The lung was divided into left and right lungs, which were washed three times with sterile saline. Left lung was fixed in 4% formaldehyde for 1 week and embedded by paraffin, and right lung tissue was reserved at -80°C for other detection.

The partial pressure of oxygen and carbon dioxide in abdominal aorta blood were analyzed by Blood Gas Analysis System, and all operation procedures were strictly based on the instructions.

HE staining

Xylene was used to strip paraffin of rat lung tissue sections for observing the morphological changes, then through high concentration to low concentration of alcohol and distilled water, acidic water and ammonia for a few seconds. After rinsing by distilled water for 1 h, sections were immerged into distilled water for a moment. Then 70% and 90% alcohol were used for dehydration for each 10 min. And the alcoholic eosin stain was used for 2-3 min. After dehydration by alcohol, transparence by xylene, seal by gum drops, and each stained section was sealed by coverslip. When the gum dried a little, tag was pasted. The morphological changes of lung cells were observed under microscope.

QRT-PCR

Trizol (Invitrogen, CA, USA) reagent was used to extract total RNA and cDNA was reverse transcribed by oligo (dT) method. The primers of TNF-α, IL-6, and GAPDH were designed by prism2.0 software and provided by ABI Company, and the sequences were shown in **Table 1**. The SYBR Green PCR method was used and GAPDH was used as internal reference. The reaction conditions were following: 50°C for 2 min, 95°C for 10 min, and followed by 40 cycles at 95°C for 15 s, 60°C for 60 s. The relative expression of each gene was calculated by the $2^{-\Delta\Delta T}$ method.

Western blot

Total proteins were extracted and the protein concentration was detected by BCA method. After boiled for 5 min with loading buffer, 20 ug proteins were loaded into 10% SDS-PAGE and

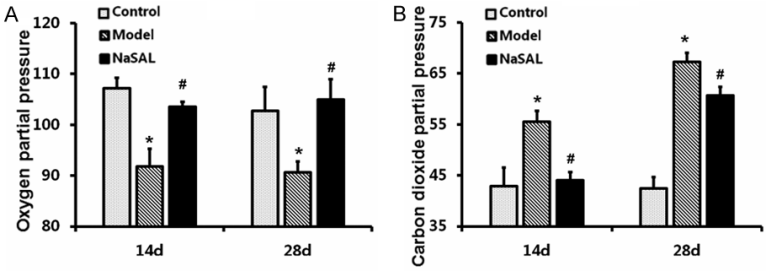

Figure 1. Changes of blood gases in aorta abdominalis. A. Compared with control group, the partial pressure of oxygen was decreased in PQ group at 14 d and 28 d (*, P<0.05). And it was increased significantly in PQ+NaSAL group (#, P<0.05). B. Compared with control group, the partial pressure of carbon dioxide was increased in PQ group at 14 d and 28 d (*, P<0.05). And there was no significant changes in PQ+NaSAL group (P>0.05).

then transferred to PVDF membrane at 100V constant voltage at ice incubation. And 5% skim milk was used for blocking at room temperature 1 h. The primary antibodies of TNF-α (Abcam, USA), IL-6 (Abcam, USA), and β-actin (Abcam, USA) were rabbit anti-rat. After incubation with antibody overnight at °C, the secondary antibody (HRP-conjugated goat anti-rabbit IgG) was added to incubate at room temperature for 1 h. Finally, the membrane was developed by enhanced chemiluminescence reagent. The developed film was scanned and analyzed by Image lab 3.0 software (BIO-RAD, CA, USA). The relative expression intensity was calculated by the ratio of grey intensity between interest proteins and β-actin.

ELISA

Blood specimen was centrifuged at 3000 rpm for 10 min to separate serum and red blood cells. Each 50 ul standard samples of different concentration from ELISA Kit (Abcam, USA) were added into different wells on the ELISA plate, and 10 ul sample was added into each well (40 ul diluted sample was added into different well too). Except for blank well, each well was added by 100 ul HRP labeled antibody and covered with microplate sealers, then incubated 1 h. After washed 5 times, 50 ul substrate A and 50 ul substrate B were added into each well. After incubated in 37°C for 15 min, 50 ul stop solution was added into each well. The OD value was measured at a wavelength of 450 nm within 15 min.

Statistical analysis

All the data were analyzed by SPSS 18.0 software and shown as the $\bar{x}\pm S$. Normality test was used to estimate the data. For multiple groups, one-way-ANOVA were used. When homogeneity of variance, LSD and SNK method were applied, while Tamhane's T2 or Dunner's T3 methods were used when heterogeneity. P< 0.05 was considered as statistically significant and P< 0.01 was considered as highly significant.

Results

Changes of abdominal arterial blood gases

To investigate the changes of blood gases by PQ poisoning, we detected the changes of blood gases in abdominal artery. Compared to control group, oxygen partial pressure was continually decreased, and reached lowest at 28 d, which was significantly lower than control group (P<0.05). In PQ+NaSAL group, oxygen partial pressure was significantly increased (P<0.05). For carbon dioxide partial pressure, it was significantly increased in PQ group when compared with control group (P<0.05), while there was significant difference between PQ group and PQ+NaSAL group (P<0.05). The details were shown in **Figure 1**, which indicated that the lung ventilation function was improved in PQ+NaSAL intervention group when compared to PQ poisoning group.

Morphological changes of lung tissue in rats

To study the injury in lung tissue by PQ poisoning, we observed the morphological changes of lung tissue in rats of different groups. After poisoning by PQ for 14 d, fibroblast proliferation was obvious, alveolar septum was widened, and a little collagen was deposited and patchy fibrosis. There was extensive inflammatory cell infiltration in lung tissues. Compared with control group, fibrosis area was diffused, large numbers of alveolar structures were collapsed and destroyed, much collagen was deposited, alveolar septa and alveolar part was occupied by much collagen and fibrin, and compensatory excessive expansion of alveolar space was occasionally observed after 28 d. In PQ+NaSAL intervention group, the pathological changes were similar to PQ group, but the injury degree was less. The inflammatory cell infiltration in

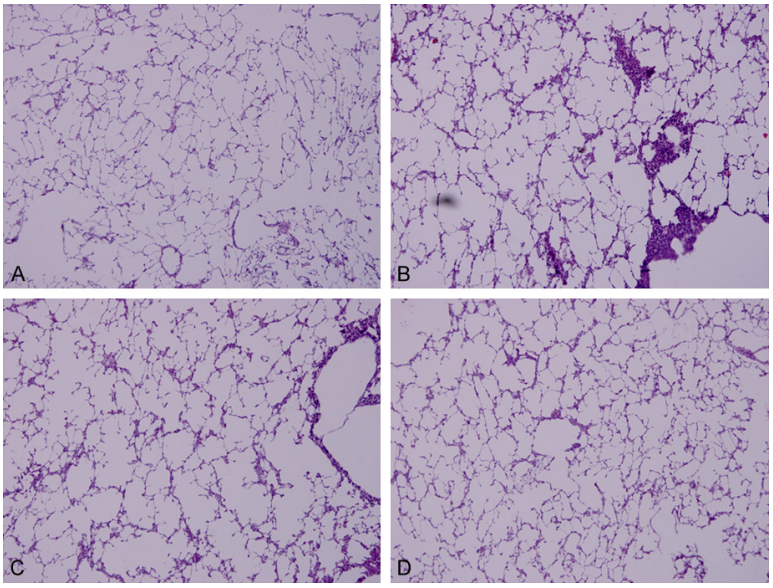

Figure 2. Morphological changes of lung tissues in PQ poisoning model and PQ+NaSAL group by HE staining. The pathological changes were shown. (A) Control group, (B) PQ group, (C) PQ+NaSAL group at 14 d, and (D) PQ+NaSAL 28 d.

lung tissues decreased. In 14 d and 28 d, a few alveolar was collapse, and collagen deposition was observed, but around normal lung tissue was also observed (as shown in **Figure 2**). The results indicated that NaSAL can relieve the morphological changes of lung induced by PQ.

Expression changes of TNF-α and IL-6 by qRT-PCR

To explore the potential mechanisms about how NaSAL relieved the injury by PQ, we used qRT-PCR to detect the expression changes of TNF-α and IL-6in lung tissues and blood. As shown in **Figure 3**, the expressions of TNF-α and IL-6 in mRNA level were significantly increased in lung tissues of PQ group in 14 d and 28 d ($P<0.01$), which was consistent to reported results. After intervention by salicylate, the expressions of TNF-α and IL-6 in mRNA level were significantly decreased ($P<0.01$), which indicated that salicylate might relieve damage by PQ through regulating the expression of TNF-α and IL-6 inflammatory genes.

Expression changes of TNF-α and IL-6 proteins by Western blot

To further validate that salicylate regulated some inflammatory genes, we applied Western blot to detect the expression changes of TNF-α and IL-6 proteins in lung tissues. Compared with control group, the expressions of TNF-α and IL-6 were significantly increased ($P<0.05$), while the expressions were decreased significantly ($P<0.05$), as shown in **Figure 4**. The results confirmed that salicylate can regulate the expressions of TNF-α and IL-6 not only in mRNA level, but in protein level.

Expression changes of TNF-α and IL-6 proteins in blood by Elisa

We also detected the expression changes of TNF-α and IL-6 proteins in blood by Elisa assay. Compared with control group, the expressions of TNF-α and IL-6 were significantly increased ($P<0.05$), while the expressions were decreased significantly ($P<0.05$) in blood, as shown in **Figure 5**. The results further validated that salicylate may remit the PQ damage in lung through regulating the expressions of inflammatory genes such as TNF-α and IL-6.

Discussion

In this study, we observed the morphological changes of lung tissues in rats with PQ poisoning. The expression of some inflammatory genes such as TNF-α and IL-6 were detected in mRNA level and protein level in different groups. We discussed the potential mechanisms about how sodium salicylate remitted the lung damage caused by PQ.

In this study, our results showed that several damage signs were observed after paraquat poisoning, such as reduced oxygen partial pressure, increased partial pressure of carbon dioxide, fibroblast proliferation, widened alveolar septum, patchy fibrosis, and collagen deposition. All these pathological changed indicated that the establishment of rat model for PQ poisoning was successful, which was the basis to explore the molecular mechanisms.

One of the features of PQ poisoning is accompanied with strong inflammatory response, and

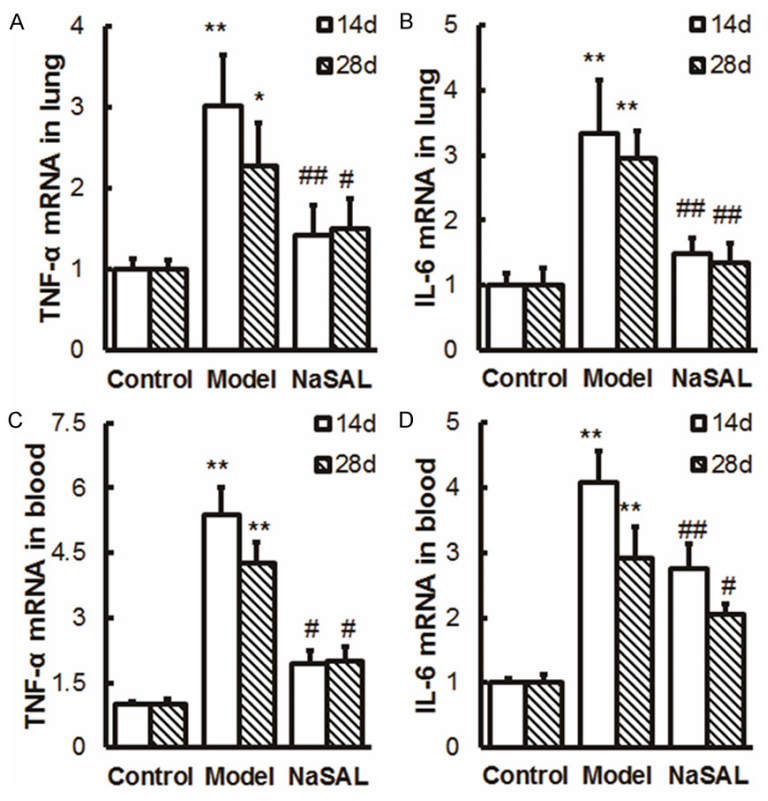

Figure 3. Expressions of TNF-α and IL-6 in lung tissues and blood by qRT-PCR. A. Compared with control group, TNF-α mRNA expression was highly significantly increased in PQ group in lung tissues at 14 d (**, P<0.01), while the difference was also significant at 28 d (*, P<0.05). Compared to PQ group, TNF-α expression was highly significantly decreased in PQ+NaSAL invention group at 14 d (##, P<0.01), while the difference was also significant at 28 d (#, P<0.05). B. Compared with control group, IL-6 mRNA expression was highly significantly increased in PQ group in lung tissues at 14 d (**, P<0.01), while the difference was also highly significant at 28 d (**, P<0.01). Compared to PQ group, IL-6 mRNA expression was highly significantly decreased in PQ+NaSAL invention group at 14 d (##, P<0.01), while the difference was also highly significant at 28 d (##, P<0.01). C. Compared with control group, TNF-α mRNA expression was highly significantly increased in PQ group in blood at 14 d (**, P<0.01), while the difference was also highly significant at 28 d (**, P<0.01). Compared to PQ group, TNF-α expression was significantly decreased in PQ+NaSAL invention group at 14 d (#, P<0.05), while the difference was also significant at 28 d (#, P<0.05). D. Compared with control group, IL-6 mRNA expression was highly significantly increased in PQ group in blood at 14 d (**, P<0.01), while the difference was also highly significant at 28 d (**, P<0.01). Compared to PQ group, IL-6 expression was highly significantly decreased in PQ+NaSAL invention group at 14 d (##, P<0.01), while the difference was also significant at 28 d (#, P<0.05).

TNF-α andIL-6 were two widely studied inflammatory genes. TNF-α is one of the molecules that were involved in the various stages of the early immune response and inflammation, and it can regulate biological activity of NF-κB pathway through activating NF-κB gene [10, 11]. In this study, we found that expressions of TNF-α mRNA and protein were increased in lung tissue and blood after PQ poisoning, which indicated that the potential mechanisms of lung damage by PQ were associated with the activation of NF-κB pathway by TNF-α. In inflammation, IL-6 may promote thrombosis through inducing C-reactive protein and fibrinogen [12]. Through binding to IL-6 antibody, increased IL-6 can cause the occurrence of inflammatory diseases, such as rheumatoid arthritis, Crohn's disease [13]. In rheumatoid arthritis, IL-6 can stimulate T lymphocytes, B lymphocytes to secrete inflammatory mediators, promote maturation of B lymphocytes, and increase the effects of IL-1β and TNF-α [14, 15]. In inflammation, IL-6 plays roles as chemotaxis to other inflammatory cells, such as neutral lymphocytes and monocytes macrophages [16], which indicates that IL-6 plays important roles in inflammation. In this study, we found that IL-6 was up-regulated in lung tissues and blood in PQ poisoning models, which was consistent to early inflammation in injury lung. IL-6 and TNF-α can be regarded as key target to treat early damage in lung by PQ poisoning as IL-6 and TNF-α play important roles in inflammatory response.

Sodium salicylate used in this study is a classic drug of salicylic acid, which was reported to be associated to in roles of TNF-α, IL-6 and NF-κB in a variety of inflammation-related diseases. In Parkinson's disease, sodium salicylate can effectively decrease the expressions of IL-6, IL-1β, and TNF-α [17]. In melanoma, sodium salicylate can influence metastasis, invasion, and proliferation through decreasing the transcription activities of TNF-α and NF-κB [18]. In myelodysplastic syndrome and acute myeloge-

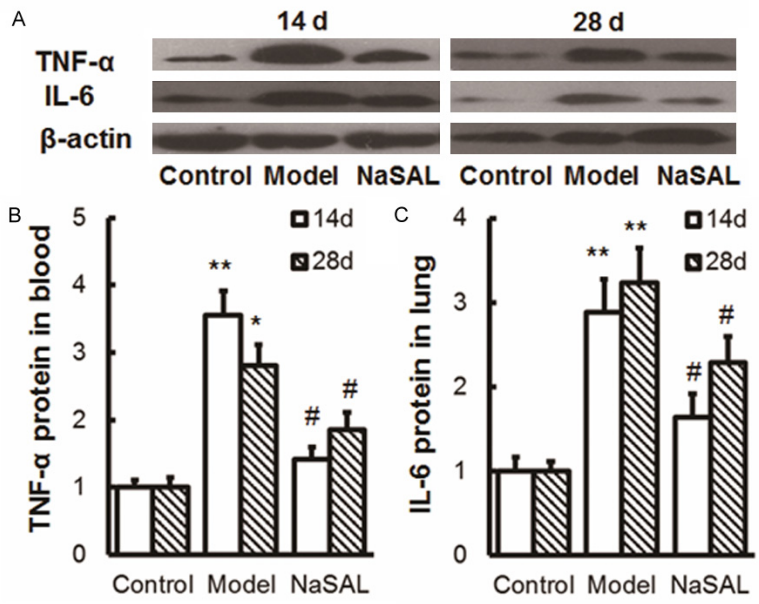

Figure 4. Expressions of TNF-α and IL-6 proteins in lung tissues by Western blot. A. Western blot results of TNF-α and IL-6 proteins among different groups at 14 d and 28 d respectively. B. Compared with control group, TNF-α protein expression was highly significantly increased in PQ group in lung tissues at 14 d (**, P<0.01), while the difference was also significant at 28 d (*, P<0.05). Compared to PQ group, TNF-α expression was significantly decreased in PQ+NaSAL invention group at 14 d (#, P<0.05), while the difference was also significant at 28 d (#, P<0.05). C. Compared with control group, IL-6 protein expression was highly significantly increased in PQ group in lung tissues at 14 d (**, P<0.01), while the difference was also highly significant at 28 d (**, P<0.01). Compared to PQ group, IL-6 expression was significantly decreased in PQ+NaSAL invention group at 14 d (#, P<0.05), while the difference was also significant at 28 d (#, P<0.05).

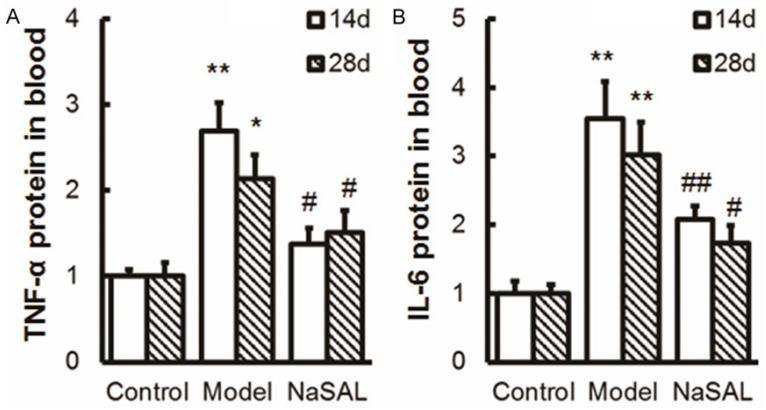

Figure 5. Expressions of TNF-α and IL-6 proteins in blood by Elisa. A. Compared with control group, TNF-α expression was highly significantly increased in PQ group in blood at 14 d (**, P<0.01), while the difference was also significant at 28 d (*, P<0.05). Compared to PQ group, TNF-α expression was significantly decreased in PQ+NaSAL invention group at 14 d (#, P<0.05), while the difference was also significant at 28 d (#, P<0.05). B. Compared with control group, IL-6 protein expression highly significantly increased in PQ group in blood at 14 d (**, P<0.01), while the difference was also highly significant at 28 d (**, P<0.01). Compared to PQ group, IL-6 protein expression highly significantly decreased in PQ+NaSAL invention group at 14 d (##, P<0.01), while the difference was also significant at 28 d (#, P<0.05).

nous leukemia, usage of sodium salicylate can safely and effectively decrease NF-κB expression [19]. In this study, as we expected, sodium salicylate intervention significantly reduce expressions of TNF-α and IL-6 in lung tissue and blood. And accordingly, decreased partial pressure of oxygen and increased partial pressure of carbon dioxide was improved, and rat lung ventilation function recoveries to a certain degree. At the same time, pathological damages were alleviated through morphological observation. All these results indicated that sodium salicylate can be used to alleviate the lung damage by PQ poisoning.

From results in this study, one of potential mechanism of lung damage by PQ poisoning was increased expressions of TNF-α and IL-6 in lung tissue and blood, and sodium salicylate can repress this up-regulation. As regulated drug for inflammatory gene like TNF-α and IL-6, sodium salicylate may be a novel candidate to treat the lung damage induced by PQ poisoning.

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Disclosure of conflict of interest

None.

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