

Original Article

Salidroside alleviates paraquat-induced rat acute lung injury by repressing TGF- β 1 expression

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Abstract: Objective: This study was designed to investigate the protective effects of salidroside (SDS) via suppressing the expression of transforming growth factor- β 1 (TGF- β 1) in rat acute lung injury (ALI) induced by paraquat (PQ) and to explore the potential molecular mechanisms. Methods: A total of 90 male rats (190-210 g) were randomly and evenly divided into 9 groups: control group, PQ groups (4 groups), and PQ + SDS groups (4 groups). The rats in control group were treated with equal volume of saline intraperitoneally. The rats in PQ groups were exposed to PQ solution (20 mg/kg) by gastric gavage for 1, 6, 24, and 72 hours, respectively. The rats in PQ + SDS groups were intraperitoneally injected once with SDS (10 mg/kg) every 12 hours after PQ perfusion. Pulmonary pathological changes were observed by hematoxylin and eosin (HE) staining. The expression of TGF- β 1 and the mRNA were evaluated by immunohistochemical (IHC) scoring and real time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR), respectively. Results: SDS alleviated the symptoms of PQ induced ALI. Moreover, SDS reduced the expression of the inflammatory cytokine TGF- β 1 including TGF- β 1 IHC scores (at each time point from 6 to 72 hours after PQ perfusion) and mRNA level (at each time point from 1 to 72 hours after PQ perfusion) compared with PQ groups ($P < 0.05$). Conclusion: SDS alleviated the pulmonary symptoms of PQ-induced ALI, at least partially, by repressing inflammatory cell infiltration and the expression of TGF- β 1 resulting in delayed lung fibrosis.

Keywords: Salidroside, paraquat, acute lung injury, transforming growth factor- β 1

Introduction

Paraquat (PQ) is a highly effective and non-selective quaternary ammonium herbicide, which is widely used in the world, controlling weeds in a huge variety of crops [1]. However, it could cause acute lung injuries (ALI) and damages to other organ systems once being ingested by animals [2, 3]. The signs of ALI, usually observed in severe PQ-poisoned cases, were Clara and alveolar epithelial cell disruption, damage of surfactant generation, oedema, hypoxaemia, haemorrhage and infiltration of inflammatory cells into the interstitial and alveoli spaces [4-6]. Moreover, there have been frequent PQ poisoning incidents which have become a severe public health issue all over the world, especially in Asian region. The statistical data show that organophosphorus pesticide poisoning is the major toxic diseases, and among which PQ poisoning is the one with highest mortality (> 50%) [1, 7]. Currently, the

mechanism of PQ-induced lung toxicity has not been fully understood and there are no widely accepted guidelines on treatment of PQ poisoning. So developing an effective therapeutic method for PQ poisoning is badly needed.

Since no antidote is useful for PQ poisoning so far. Preventing the leukocyte infiltration and alveolar-capillary barrier dysfunction may be helpful to prevent PQ-induced ALI regardless of etiology. Qian *et al.* showed FTY720 (fingolimod) could alleviate PQ-induced lung injury [8]. Furthermore, methylprednisolone and cyclophosphamide have been reported to be valid in PQ poisoning [9]. Some studies also demonstrated that *Rhodiola rosea* exerts anti-inflammatory [10] and relieves pulmonary edema in rats [11].

Salidroside (SDS), a glucoside of tyrosol isolated from the plant *Rhodiola rosea*, is thought to be responsible for the antidepressant and anx-

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lyolytic actions [12]. SDS also has anti-inflammatory, anti-hypoxia, anti-tumor, anti-aging, and anti-liver fibrosis effects [13, 14]. Reports have shown that SDS has obvious therapeutic effects to rat's secondary lung injury and pulmonary dysfunction induced by burning [14-17]. SDS could not only alleviate bleomycin-induced rat pulmonary fibrosis by regulating the balance of MMP-2 (matrix metalloproteinase 2) and TIMP-1 (tissue inhibitor of metalloproteinases 1) to some extent [18], but also ease lipopolysaccharide and oleic acid induced ALI by repressing inflammatory mediators and anti-oxygenation [19]. Transforming growth factor- β 1 (TGF- β 1) is a polypeptide cytokine that promotes inflammatory responses by releasing related inflammatory cells [20]. TGF- β 1 plays a critical role in tissue injury development of multiple organs, including lung [21]. Expression level of TGF- β 1 is dramatically increased as early as 2 days after the lung injury, which is a critical mediator of ALI [20]. A previous study has demonstrated that TGF- β 1 not only participates in the early phase of ALI (exudative) contributing to pulmonary edema, but also is related with the late phase of ALI (fibrotic) leading to pulmonary fibrosis [4]. Additionally, TGF- β 1 has been reported to play an important role in ALI induced with PQ and hyperoxia [22]. Therefore, in this study, we investigated the protective role of SDS to PQ-induced ALI in rats, and explored the underlying mechanism by focusing on the change of TGF- β 1 level.

Methods

Laboratory animals

Ninety healthy male Sprague-Dawley rats (SPF grade, *Rattus rattus*, 190-210 g) were provided by The Laboratory Animal Center, Zhejiang Chinese Medicine University [Animal production license No.: SCXK (z) 2008-0022; animal use permit No.: SYXK (z) 2008-0115]. The rats were kept under a 12-h light/12-h dark cycle with free access to food and water. The animal experiments were approved by local ethics committee.

Animal grouping and model establishment

PQ and SDS were purchased from Shandong Sanyuan Industry and Trading, Co., Ltd. and National Institutes for Food and Drug Control, respectively. PQ and SDS were dissolved in 0.9% saline solution. The molecule structure of

SDS was shown in **Figure 2**. A total of 90 male rats were randomly and evenly divided into 9 groups: control group, PQ groups (4 groups) and PQ + SDS groups (4 groups). The rats in control group were treated with equal volume of physiological saline intraperitoneally. The rats in PQ groups were exposed to PQ solution (20 mg/kg) by gastric gavage at 1, 6, 24, and 72 hours, respectively. While the rats in PQ + SDS groups were intraperitoneally injected once with 1% SDS (10 mg/kg) every 12 hours after PQ perfusion. The injection style of SDS was done as previously reported [19].

Pathological observation of lung tissue

Five mice were randomly chosen from each group and sacrificed for further study at 1, 6, 24, 72 hours after PQ perfusion respectively. The animals were anesthetized with intraperitoneal injections of 10% chloral hydrate and rapidly perfused fixed with saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Lung tissue was obtained and stored at -70°C until use. The upper right lung tissue was used to prepare paraffin sections as follows: fixation in neutral formaldehyde overnight at 4°C; decalcification by immersion in 0.12 M EDTA, pH 7.0 for 1 week at room temperature (RT); dehydration by gradient concentration of ethanol; vitrification by dimethylbenzene; embedding in 52-54°C paraffin. Finally, sections were cut in a microtome. Then, all sections were collected for hematoxylin and eosin (HE) and immunohistological (IHC) staining. The pathological changes were observed under optical microscope.

HE staining

To observe general histological pathology of lung tissue, HE staining was used. Digital images were collected by a conventional light microscope in five visual fields/per section with 100 \times magnification under bright-field viewing.

IHC observation and scoring

The TGF- β 1 kit (Invitrogen, San Diego, CA, USA) and IHC kit (Boster Company, Wuhan, China) were used in this experiment. The expression of TGF- β 1 was detected using an IHC staining kit. The lung slides were dewaxed and hydrated. Endogenous peroxidase was inactivated by H₂O₂. The antigens were repaired in sodium citrate at pH 6.0 by microwave. Goat serum was

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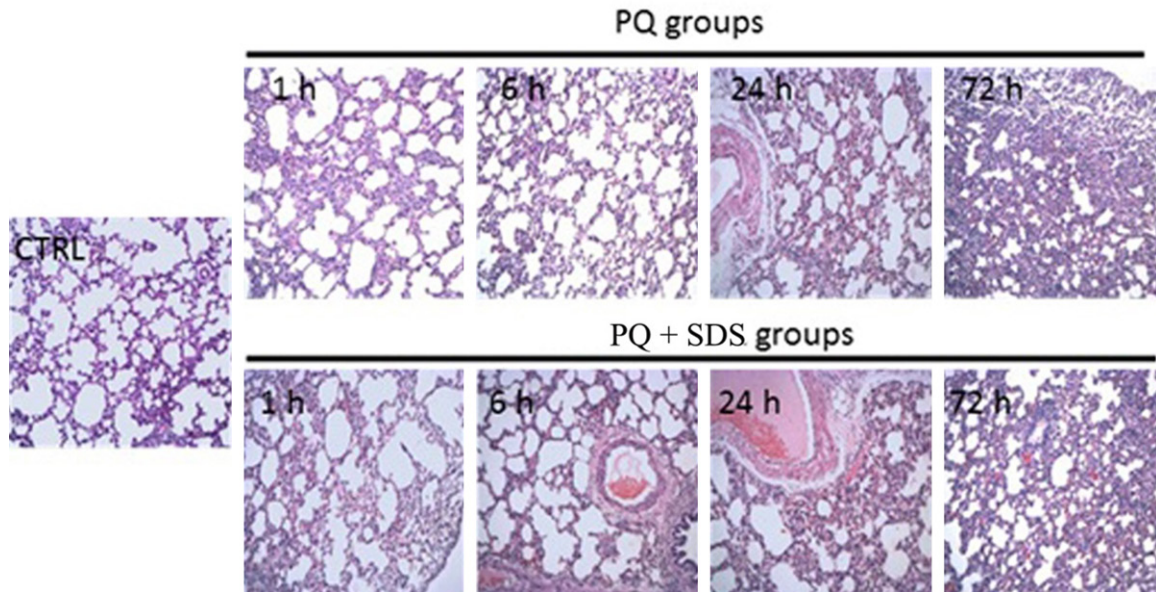


Figure 1. Pathological changes of lung tissue from different groups of rats determined by HE staining ($\times 100$) after PQ poisoning. PQ groups: rats were treated by paraquat solution; PQ + SDS groups: rats were treated by PQ plus SDS at each time point from the 1 hour after PQ poisoning; CTRL (control group): rats were only treated by saline intraperitoneally.

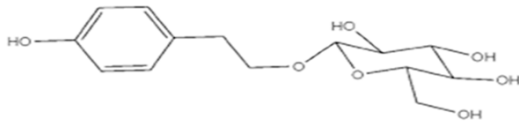


Figure 2. Molecular structure of SDS.

used to block non-specific antigen sites. Then, the slides were incubated successively with 50 μ l diluted primary antibody (TGF- β 1) overnight at 4°C. The sections were then washed with PBS and incubated with biotin-conjugated secondary antibody for 1 hour at RT, then, streptavidin/HRP was added and 50 μ l diaminobenzidine was added until reaction terminated by the tap water, with dyeing time controlled under microscope. Finally, the slides were re-dyed with hematoxylin, dehydrated, vitrified and mounted before observed under light microscope. Two slides were chosen randomly for negative control, which were incubated with antibody dilution instead of primary antibody. Under microscope observation, the positive cells for TGF- β 1 were counted in 5 randomly selected fields with Image-Pro Plus 6.0 software.

Description of the level of TGF- β 1 is on the basis of the ratio of positive cells and intensity of the reaction. The parameter is classified and

a combined score is used to decide positive or negative result according to criteria defined previously [23-25]. For the score of TGF- β 1 positive cell ratio, 0-1%, 1-10%, 10-50%, 50-80% and 80-100% were scored as 0, 1, 2, 3 and 4, respectively. For intensity score, negative, weakly positive, positive and strongly positive were scored as 0, 1, 2, and 3, respectively. IHC score value = positive cell ration score \times intensity score.

Quantitative polymerase chain reaction (QPCR)

The total RNA of lung tissue was extracted with Trizol method (Invitrogen, San Diego, CA, USA). The reverse transcription (RT) reaction was performed in 20 μ l system using RT kit from TaKaRa. TGF- β 1 and GAPDH mRNA sequences were obtained from PUBMED database. All the primers were designed with Primer Premier 5.0. qPCR assay was used to detect the mRNA expression level of TGF- β 1. qPCR reaction was performed in 20 μ l system containing 10 μ l SYBR® Premix Ex Taq™, 0.4 μ l forward primer (10 μ M), 0.4 μ l reverse primer (10 μ M), 2 μ l cDNA template, and 7.8 μ l distilled H₂O (dH₂O). PCR program: 95°C pre-denaturation 2 min, 95°C denaturation 15 s, 60°C annealing 30 s, 40 cycles. The Primers used were listed in **Table 1**.

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Table 1. Real-time PCR reactions for each gene-specific primer

Gene	Primer	Sequence	Product Length (bp)
TGF-β1	TGF-F-uc	5'-GAGATGCAATGCTATTCCCT-3'	101
	TGF41TC	5'-CGACGTCACAGCGCACTT-3'	
GAPDH	GAPDH-F	5'-CCTCAAGATTGTCAGCAAT-3'	141
	GAPDH-R	5'-CCATCCACAGTCTTCTGAGT-3'	

Statistical analysis

All the data were presented as \bar{X} (mean) \pm s (standard deviation). SPSS 17.0 was used for statistical analysis. Multiple comparisons among groups were performed by one-way ANOVA. Paired comparisons were carried out using least significant difference method. $P < 0.05$ was designated as significant difference.

Results

PQ poisoning symptoms

In the PQ groups, the rats displayed series of symptoms including listlessness, decreased appetite, squinting, back arching, sluggish, and an increased respiratory rate half an hour after PQ perfusion. Six hours after PQ perfusion, rats showed lip cyanosis, buccal respiration or respiratory distress. The poisoning symptoms became progressively severe from 24 to 72 hours after PQ perfusion, the damage of respiratory system is the most obvious along with weight loss, diarrhoea, and hematuria compared to the control group. Compared with PQ groups, rats in PQ + SDS groups displayed milder symptoms. Twelve rats died in PQ groups (2 at 1 hour, 2 at 6 hours, 4 at 24 hours, 4 at 72 hours) and 5 rats died in PQ + SDS groups (1 at 1 hour, 1 at 6 hours, 2 at 24 hours, 1 at 72 hours). The mortality between PQ and PQ + SDS groups were significantly different ($P < 0.05$).

Pathological changes of lung tissue

The HE staining results of control group showed clear alveolar structure, thin alveolar wall, no alveolar edema, and no inflammatory cell infiltration in pulmonary interstitial. While in PQ groups, the rats showed alveolar inflammation features 1 hour after PQ perfusion, such as obvious telangiectasia and hyperemia. The rats of 6 hours after PQ perfusion displayed widened alveolar septum, edema fluid in alveolar cavity, and partial hyaline membrane forma-

tion. More serious symptoms, such as obvious lung tissue inflammation and exudation, hemorrhage, necrotic and inflammatory cell infiltration, partial bronchiolar epithelium exuviation, alveolar structure collapse, atelectasis, and pneumonectasis, were observed in rats at 24-72

hours post PQ perfusion. Compared with PQ groups, the rats in PQ + SDS groups showed similar but slighter symptoms at each time point from 1 to 72 hours after PQ perfusion (**Figure 1**).

IHC detection of TGF-β1 in lung tissue

Compared with control group, TGF-β1 positive cells and TGF-β1 IHC scores in PQ groups were significantly increased at each time point ($P < 0.01$) from 6 hours after PQ perfusion and peaked at the 72 hours after PQ perfusion. While in PQ + SDS groups, TGF-β1 positive cells and TGF-β1 IHC scores ($P < 0.05$) were decreased significantly at each time point from 6 hours after PQ perfusion compared with PQ groups (**Table 2**).

Detection of TGF-β1 mRNA level via qPCR

Gene expression level for TGF-β1 was examined by qPCR in the lung tissue of rats in 9 groups. The gene expression of TGF-β1 in PQ groups was significantly increased at each time point from 1 to 72 hours after PQ perfusion compared with that of control group (**Table 3**, $P < 0.01$), while TGF-β1 mRNA level in PQ + SDS groups was significantly decreased at each time point from 1 to 72 hours after PQ perfusion relative to PQ groups ($P < 0.05$) (**Table 3**).

Discussion

In the present study, we demonstrated that SDS ameliorated the symptoms of PQ-induced ALI as measured by HE staining, IHC and qPCR for inflammatory cytokine level of TGF-β1. Our observations implied that SDS exhibited protective effects though repressing inflammatory cell infiltration and the expression of TGF-β1 at least partially in ALI induced by PQ as potentially pathophysiological mechanisms explaining the functional impairment.

Due to the presence of polyamines uptake system in lung tissue, PQ could accumulate in lung

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Table 2. TGF-β1-positive cell number ($\bar{X} \pm s$) and TGF-β1 IHC scoring for all the groups

Group	TGF-β1 positive cell number				TGF-β1 IHC scores			
	1 h	6 h	24 h	72 h	1 h	6 h	24 h	72 h
Control	16±1.31				2.11±0.20			
PQ groups	17±1.34	21.33±2.59 ¹⁾	26.67±3.12 ¹⁾	32.39±4.36 ¹⁾	2.35±0.19	3.54±0.54 ¹⁾	5.63±0.98 ¹⁾	6.46±1.41 ¹⁾
PQ + SDS groups	16±1.54	18.73±2.71 ^{1),2)}	22.67±3.03 ^{1),2)}	28.31±4.41 ^{1),2)}	2.22±0.18	2.85±0.46 ^{1),2)}	4.15±1.09 ^{1),2)}	5.15±1.32 ^{1),2)}

Note: ¹⁾P < 0.01 vs. control group; ²⁾P < 0.05 vs. PQ groups.

Table 3. TGF-β1 mRNA levels detection via qPCR for all the groups

Group	Fold changes			
	1 h	6 h	24 h	72 h
Control	1			
PQ groups	2.15±0.24 ¹⁾	3.31±0.76 ¹⁾	4.47±1.02 ¹⁾	5.11±1.12 ¹⁾
PQ + SDS groups	1.55±0.22 ¹⁾	2.45±0.66 ^{1),2)}	3.52±1.03 ^{1),2)}	4.12±1.05 ^{1),2)}

Note: ¹⁾P < 0.01 vs. control group; ²⁾P < 0.05 vs. PQ groups.

against a concentration gradient, reaching to 6-10 times of PQ concentration in serum [13, 14]. Compared with other tissues, the PQ clearance rate in lung tissue is much slower. After PQ poisoning, the early pulmonary symptoms are alveolar epithelial injury, hemorrhagic edema, and inflammatory cell infiltration, while the latter symptom is the alveolar and interstitial fibrosis [3]. There is considerable evidence to suggest that the inflammatory features, histological pulmonary impairment and metabolic processes are similar in human and rats in acute PQ intoxication [26]. In our study the rats in PQ groups showed the similar pulmonary symptoms at both early and latter stages post PQ perfusion, which indicates our rat model is very resemble to the symptoms of human PQ poisoning. The rats in PQ + SDS groups showed much slighter symptoms, indicating SDS has therapeutic effects to PQ poisoning.

Growing evidences showed that several cytokines are involved in the initiation and development of lung injuries and fibrosis [27, 28]. TGF-β1 is a polypeptide cytokine that not only controls proliferation, differentiation, and apoptosis [29], but also promotes inflammatory responses by releasing related inflammatory cells during the development of ALI and lung fibrosis [20]. The down-regulation of the inflammatory processes is an important mechanism for decreasing PQ-induced ALI. TGF-β1 could also be involved in ALI and lung fibrosis by enhancing the pulmonary extracellular matrix deposition and inducing the synthesis of collagen and fibronectin [30]. TGF-β1 is now an

acknowledged starting hub in the development of lung injury and fibrosis. Previous researches had exhibited that inhibition of TGF-β1 expression or its signaling pathways can abrogate pulmonary fibrosis induced by bleomycin [31]. In the current study, the number and

scores of TGF-β1 positive cells measured by IHC was up-regulated in lung tissue following PQ administration, this result is in line with previous research results [32]. While that of TGF-β1 positive cells decreased after SDS intervention. Additionally, quantitative analysis of qPCR demonstrated that level of TGF-β1 was accordance with the outcomes of the IHC. Obviously, our results showed that SDS treatment repressed the expression of TGF-β1 along with inflammatory indicating that the anti-inflammatory effect of SDS may be partly contributed to the inhibition of TGF-β1, which further improved the symptom of PQ induced ALI.

Compared to western medicine, there is more interest in natural plant products which may be used in the development of new drugs and applications in lung diseases induced by toxic agents [33, 34]. SDS is herbal extract from *Rhodiola rosea L.* Compared with synthetic drugs, natural compounds has less side effects to human body. Guan et al. also exhibited that SDS treatment caused the reduction of inflammatory cells in the bronchoalveolar lavage fluid in LPS-induced ALI in mice [19]. But necessary evaluations are needed for the usage of SDS in PQ poisoning treatment.

Although we observed that SDS plays an important role of alleviating the symptoms of PQ induced ALI, there remained some shortcomings in our study. We examine the pathological changes only under optical microscope, but transmission electron microscopy (TEM) was not used which might have shown the structure changes of lung tissue more clearly. Additionally,

the level of TGF-β1 of lung tissue was only observed under microscope, but electrophoregram was not observed which might be more convincing. Moreover, the mechanisms of PQ poisoning are so complicated and unclear. Hence, further experiments are necessarily to solve these problems above in our future study.

To conclude, our study suggested that SDS alleviated the symptoms of PQ induced ALI in rats via down-regulation of the expression of TGF-β1. To a certain degree, our results could provide informative clues for pathogenesis of PQ induced ALI and shed lights for developing clinical treatment for PQ poisoning.

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Disclosure of conflict of interest

None.

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