Expression of heat shock protein 70 in lung tissues of acute paraquat poisoned rats and intervention of ulinastatin

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BACKGROUND: Paraquat (PQ) is an effective herbicide and is widely used in agricultural production, but PQ poisoning is frequently seen in humans with the lung as the target organ. Clinically pulmonary pathological changes are often used to predict the severity and prognosis of the patients. In this study, we observed the expression of heat shock protein 70 (HSP70) in rat lung after PQ poisoning and to investigate the therapeutic effects of ulinastatin.

METHODS: Seventy-two adult healthy SD rats were randomly divided into a control group (group A, n=24), a poisoning group (group B, n=24), and an ulinastatin group (group C, n=24). The rat models of acute PQ poisoning were established by intra-gastric administration of 80 mg/kg PQ to rats of groups B and C, and the rats of group C were intra-peritoneally injected with 100 000 IU/kg ulinastatin 30 minutes after poisoning. The expression of HSP70 in lung tissue was observed, and W/D and histopathological changes in the lung tissue were compared 12, 24, 48 and 72 hours after poisoning. The expression of HSP70 in the lung tissue was assayed by using RT-PCR. All quantitative data were processed with one-way analysis of variance to compare multiple sample means.

RESULTS: Compared to group A, the expression of HSP70 in the lung of rats in groups B and C increased significantly at all intervals (P<0.05). The pathological changes in lung tissue of rats with PQ poisoning included congestion, leukocytes infiltration and local hemorrhage, whereas those of group C were significantly lessened.

CONCLUSION: Ulinastatin may ameliorate acute lung injury to some extent after PQ poisoning in rats by enhancing the expression of HSP70.

KEY WORDS: Paraquat; Poisoning; Ulinastatin; Heat shock protein; Acute lung injury

INTRODUCTION
Paraquat (PQ), an effective herbicide, is widely used in agricultural production, but PQ poisoning occurs frequently in humans. The function of organs such as the lung, liver, kidney and heart is impaired after poisoning, and the lung is the main target organ. At the early stage of poisoning, most patients die of acute lung injury, and at the late stage, some patients may develop pulmonary fibrosis with a mortality of over 80%. Clinically, pulmonary pathological changes are often used to predict the severity and prognosis of the patients, and it is the main task to alleviate the pulmonary damage. Ulinastatin, a kind of broad spectrum proteinase inhibitors, is effective to inhibit the activity of hydrolases, release inflammatory mediators, and produce oxygen radicals and hyperoxide. In this study, we produced the rat models of PQ poisoning to observe the changes of pulmonary heat shock protein 70 (HSP70) and the effect of ulinastatin.
METHODS
Main experimental reagents
20% PQ solution was produced by Shanghai Xianzhengda Company. Ulinastatin was purchased from Tianpu Biochemistry Medical Limited Company, and Coomassie brilliant blue kit from Nanjing Jiancheng Bioengineering Institute. HSP70 sheep polyclonal antibody was the product of Santa Cruz Company, USA, and cochlearia hydrogen peroxidase labelling rabbit-anti-sheep IgG was the product of Beijing Tiangen Biochemistry Technology Limited Company.

Grouping of animals and model establishment
A total of 72 clean Sprague-Dawley rats, body weight 220±40 g, were provided by the Department of Animal Science, Medical College of Nanchang University. The rats were randomly divided into a control group (group A), a poisoning group (group B) and an ulinastatin group (group C). Models of acute PQ poisoning were established according to Tong et al. The rats in groups B and C were intragastrically administered with PQ (80 mg/kg), while the rats in group A were intragastrically administered with the same volume of stroke-physiological saline. The rats in group C were peritoneally injected with 1000 000 U/kg per day ulinastatin at 30 minutes after poisoning, and the rats in groups A and B were peritoneally injected with the same volume of stroke-physiological saline. At 12, 24, 48, 72 hours after poisoning, the rats were anesthetized peritoneally with 3% pentobarbital (35 mg/kg) and executed through blood letting from the abdominal aorta.

Tissue specimens
After the rats were killed, the superior part of the left lung was taken for the measurement of the ratio of wet to dry weight. The central part of the left lung was washed completely, put in nitrogen canister and stored in a freezer at -80 °C for measurement of the expression of HSP70 by Western blotting. The inferior part of the left lung was fixed in 10% formaldehyde and used for pulmonary histopathological observation.

Measurement of arterial partial pressure of oxygen
The 0.5 ml blood was collected from the abdominal aorta 12, 24, 48, 72 hours after poisoning. The arterial partial pressure of oxygen was measured by a blood gas analyzer produced by Roche Company.

Measurement of the expression of pulmonary HSP70 protein
Lung tissues of rats in each group were put into cell lysate and homogenated on ice at corresponding time points. Bradford method was adopted to quantitate protein, and tissue sample was added into 2×SDS loading buffer, and then processed with SDS-PAGE gel separation, and the protein strap was electrotransferred onto the PVDF membrane. TTBS was used to block for 1 hour, then HSP70, HSP70 one antibody and IgG-HRP two antibody were used to brood in succession, finally enhanced chemiluminescence method was applied to detect positive signal. Images were gathered and analyzed semiquantitatively, the level of HSP70 protein was indicated with the ratio of HSP70/β-actin. The process was repeated three times in each group.

Statistical analysis
Experimental data were analyzed with SPSS 12.0 software. Quantitative data were demonstrated in the form of mean±SD, and group comparison was made with one-way analysis of variance and two samples t test. The difference was considered statistically significant when P<0.05.

RESULTS
General conditions of rats
Compared with group A, rats in group B had the manifestations of poisoning at 24-72 hours. The main manifestations included poor spirit, slow reaction, cyanosis in oral lips and four limbs at varying degrees, increased respiratory frequency, less activity, and easy capture. Blood-like substance outflowed from the nasal cavity in some rats. In group C, the above manifestations alleviated, activity increased, dyspnea reduced, but compared with group A, there were significant differences.

Histopathological observation
In group A, the lung tissue was pink and retracted well under naked eyes. Under a light microscope, the alveolar structure was distinct, the alveolar wall was thick, inflammatory cell infiltration was not found in the alveolar space (Figure 1).

In group B, the lung tissue didn’t retract well under naked eyes. The size of the lung increased significantly, the color of the lung was not asymmetrical, a flake of ecchymosis and hemorrhagic spots were observed. Under a light microscope, edematous fluid in the alveolar space and a small quantity of cellulose were observed at 12 hours after poisoning; at 3 days after poisoning, the most obvious clinical manifestations included interstitial pulmonary edema, and alveolar edema. The alveolar space was filled with dissociative neutrophils,
macrophages, and homogeneous edema fluid associated with diffuse pulmonary hemorrhage. The alveolar space collapsed, and hyaline membrane formed partially. For a long time, no changes took place in the clinical manifestations but there was an absorption trend (Figure 2).

In group C at 12 hours after poisoning, focus or foliated inflammatory cell infiltration was observed, the degree was lower than that in group B. The clinical manifestations were obvious at 3 days after poisoning in group C, but less obvious in group B (Figure 3).

**Pulmonary W/D ratio**

Compared to group A, the W/D ratio of groups B and C increased significantly at corresponding time points, especially group B, and there was statistical significance among the three groups \( P<0.05 \) (Table 1).

**Arterial partial pressure of oxygen**

Compared to group A, the arterial partial pressure of oxygen in groups B and C in corresponding time points decreased significantly, especially in group B. There was statistical significance among the three groups \( P<0.05 \) (Table 2).

**Expression of pulmonary HSP70 and effect of ulinastatin**

The expression of pulmonary HSP70 in group A at corresponding time points was low, and there was no statistical significance among subgroups \( P>0.05 \). Compared to group A, the expression of pulmonary HSP70 in group B increased gradually with time, and peaked at 24 hours. Compared to group B, the expression of pulmonary HSP70 in group C increased, and peaked at 24 hours (Table 3, Figure 4).

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**Table 1. Comparison of lung W/D in different time point in rats (mean ±SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( (n=6) )</td>
<td>4.78±0.057</td>
<td>4.79±0.049</td>
<td>4.78±0.067</td>
<td>4.77±0.051</td>
</tr>
<tr>
<td>B ( (n=6) )</td>
<td>5.05±0.060*</td>
<td>5.89±0.018*</td>
<td>6.65±0.030*</td>
<td>7.50±0.025*</td>
</tr>
<tr>
<td>C ( (n=6) )</td>
<td>4.95±0.033*</td>
<td>5.29±0.020*</td>
<td>6.02±0.030*</td>
<td>6.80±0.045*</td>
</tr>
</tbody>
</table>

Compared with group A, \*\( P<0.05 \), \#\( P<0.01 \); compared with group B, \#\( P<0.05 \).

**Table 2. Blood gas analysis changes in each group (mean ±SD, mmHg)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( (n=6) )</td>
<td>93.76±1.06</td>
<td>95.02±1.24</td>
<td>94.52±2.12</td>
<td>93.85±1.98</td>
</tr>
<tr>
<td>B ( (n=6) )</td>
<td>75.96±6.62*</td>
<td>64.13±5.23*</td>
<td>59.55±5.15*</td>
<td>52.23±5.06*</td>
</tr>
<tr>
<td>C ( (n=6) )</td>
<td>88.23±5.03*</td>
<td>82.85±6.01*</td>
<td>76.49±7.32*</td>
<td>63.03±7.51*</td>
</tr>
</tbody>
</table>

Compared with group A, \*\( P<0.05 \), \#\( P<0.01 \); compared with group B, \#\( P<0.05 \), \#\( P<0.01 \).

**Table 3. Comparison of the expression of HSP70 in different groups (mean ±SD)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>A ( (n=6) )</td>
<td>0.281±0.037</td>
<td>0.282±0.040</td>
<td>0.279±0.038</td>
<td>0.286±0.039</td>
</tr>
<tr>
<td>B ( (n=6) )</td>
<td>0.402±0.018*</td>
<td>0.515±0.045*</td>
<td>0.424±0.034*</td>
<td>0.394±0.040*</td>
</tr>
<tr>
<td>C ( (n=6) )</td>
<td>0.534±0.039*</td>
<td>0.844±0.052*</td>
<td>0.765±0.053*</td>
<td>0.513±0.042*</td>
</tr>
</tbody>
</table>

Compared with group A, \*\( P<0.05 \), \#\( P<0.01 \); compared with group B, \#\( P<0.05 \), \#\( P<0.01 \).
DISCUSSION

The lung is the major target organ of PQ poisoning. Acute lung injury, multiple organ dysfunctions, and pulmonary interstitial fibrosis are the main causes of death for PQ poisoning.\[^{8,9}\] PQ can cause histocytic pathophysiological damage by producing oxygen radicals, releasing inflammatory mediators, and inducing lipid peroxidation, especially pulmonary oxidative damage.\[^{10,11}\] Some studies show that antioxidant treatment can significantly reduce the degree of lung injury for the PQ poisoning.\[^{12-15}\] In this study, the rats had the following clinical manifestations after poisoning, including poor spirit, slow reaction, cyanosis in oral lips and limbs at different degrees, increased respiratory frequency, inactivity, easy capture, and blood-like substance in the nasal cavity. Diffuse hemorrhage, alveolar space collapse, hyaline membrane formation, increased pulmonary water content, inflammatory cells aggregation in alveoli were observed. These indicated the occurrence of lung injury.

Heat shock protein is a kind of protein, which is conservative in organic evolution. Many physiological, pathological and stress factors can induce the production of heat shock protein, and this is important in protecting the body from excessive stress damage. The HSP70 family is closely associated with pulmonary biology, and has protective effects on lung injury induced by anti-inflammation, anti-oxidation, anti-apoptosis or molecular chaperone roles.\[^{16-19}\] Weiss et al\[^{20}\] administered a vector containing the porcine HSP-70 cDNA driven by a CMV promoter (AdHSP) into the lungs of rats subjected to 2CLP or sham operation. The administration of AdHSP after either sham operation or 2CLP increased HSP-70 protein expression in lung tissue, as determined by immunohistochemistry and Western blot hybridization. The administration of AdHSP significantly attenuated interstitial and alveolar edema and protein exudation and dramatically decreased neutrophil accumulation, relative to the control of adenovirus. Hiratsuka et al\[^{21}\] transduced HSP70 DNA into the lungs of rats using adenovirus vector, and found that donor adenovirus-mediated gene transfer of HSP70 decreases subsequent ischemia-reperfusion injury in rat lung isografts.

Other studies found that glutamate and rhubarb can both increase the expression of HSP70 after lung injury and exert protective effects.\[^{12-15}\] Ulinastatin is a broad spectrum protease inhibitor, and can protect the lung from injury through antioxidation, inhibiting the release of inflammatory mediators, increasing stability of pulmonary cell membrane and mitochondria, endoplasmic reticulum, lysosome membrane, and improving cell energy metabolism.\[^{25-29}\] Another study discovered that ulinastatin can inhibit the release of TNF-α, IL-1β, IL-2, SOD, MDA, MMP-9 and neutrophil elastase in serum of rats after acute PQ poisoning, exerting protective effect against lung injury. Chen et al\[^{20}\] found that ulinastatin can protect the kidney in the early phase of trauma by upregulating the renal expression of HSP70. In this study, we found that compared with the control group, the pulmonary expression of HSP70 in poisoning group increased significantly at 12 hours, and peaked at 24 hours. The results indicated that PQ poisoning may enhance the expression of HSP70 while inducing acute lung injury, but serious pulmonary damage indicated the production of HSP70 is unable to induce injury after poisoning. In the ulinastatin group, the pulmonary expression of HSP70 in the corresponding time points was obviously higher than that in the poisoning group. At the same time, pulmonary pathological changes minimized and pulmonary water content decreased, indicating that ulinastatin may alleviate PQ-induced lung injury by enhancing the pulmonary expression of HSP70. Hence our study provided theoretical evidence for the application of ulinastatin in treatment of PQ poisoning.

**REFERENCES**


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