A Study on the Regulation of MMP-9 and Other Inflammatory Factors in Rat Acute Lung Injury by Different Doses of Ulinastatin

Hao CHEN, Liwe ZHANG, Wenfang LI, Zhaofen LIN* & Tao WANG

Emergency Department of Shanghai Seventh People’s Hospital, Shanghai, 2000137, China

SUMMARY. The aim of this study is to research and compare levels of MMP-9, TNF-α, IL-6, IL-8 and IL-10 through different doses of ulinastatin (UTI). Furthermore, the mechanism of UTI against acute lung injury (ALI) induced by sepsis was explored using methods of cecal ligation and puncture. An animal model was established, in which 78 rats were randomly divided into three groups (n = 26): CLP group (ALI group), UTI 50,000 U/kg group (UTI1 group), and UTI 100,000 U/kg group (UTI2 group); six rats were treated as controls. A corresponding dose of UTI was given immediately after surgery in the UTI treatment groups. Blood samples and lung tissues were collected. Survival rate, lung wet weight index, pathology scores of the lung, as well as MMP-9, TNF-α, IL-6, IL-8 and IL-10 serum levels were detected at every time point. MMP-9, TNF-α, IL-6, IL-8 and IL-10 were determined by double antibody sandwich ELISA assay, and statistical analysis was performed. After using UTI intervention, lung injury was alleviated and no significant change in survival rate occurred. Furthermore, inflammatory mediators declined in each group, in which both the UTI1 and UTI2 groups had a descending trend compared with the ALI group; and this significantly decreased compared with the 48 h ALI group (P<0.01). TNF-α markedly increased in the ALI group, but this trend decreased in the UTI1 and UTI2 groups. IL-8 was elevated at every point in the ALI group, but this significantly decreased in the UTI1 and UTI2 groups, compared with the 48 h ALI group (P<0.01). The UTI1 and UTI2 groups revealed a descending trend compared with the ALI group, and the UTI2 group significantly decreased compared with the 24 h ALI group (P<0.05). Both the UTI1 and UTI2 groups deceased compared with the ALI group, although there was no significant difference. The most significant decrease was observed in MMP-9 and IL-8 (P<0.01). UTI plays a protective role against lung injury by inhibiting inflammatory factors such as MMP-9 and IL-8. This study may provide a new clue in the treatment of ALI.

KEY WORDS: inflammatory factors, lung injury, MMP-9, sepsis, UTI.

* Author to whom correspondence should be addressed. E-mail: lin_zhaofen123@126.com

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INTRODUCTION

Acute respiratory distress syndrome (ARDS) is the most serious condition of acute lung injury (ALI), which is a severe clinical syndrome with a high mortality of approximately 32-50% \(^1\,^2\). Uncontrolled waterfall inflammatory response for the lung is thought to be a main mechanism of ALI \(^3\). Some animal experiments and clinical studies overseas have revealed that matrix metalloproteinase-9 (MMP-9) is actively involved in lung injury with sepsis as an inflammatory factor \(^4\,^6\). Furthermore, other studies have suggested that ulinastatin (UTI) could inhibit the development of ALI \(^7\,^8\). However, the mechanism of this effect remains unclear. Through treatment with UTI in sepsis-induced acute lung injury in rats, this study investigated the effects of different doses of UTI on the level of inflammatory factors such as MMP-9. Moreover, lung pathological changes and its mechanism of action were also studied.

MATERIALS AND METHODS

Experimental animals

A total of 84 specific pathogen free (SPF) SD rats with a mean weight of 230-250 g were collected from the Medical Experimental Animal Center of The Second Military Medical University. All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Emergency Department of Shanghai Seventh People’s Hospital. The animal protocol was designed to minimize pain or discomfort to animals \(^9\).

Groupings and treatment on experimental animals

Among the 84 SD rats obtained for the study, 78 of the rats were randomly divided into three groups (26 rats per group): CLP operation group (ALI group), UTI of 50,000 U/kg treatment group (UTI1 group) and UTI of 100,000 U/kg treatment group (UTI2 group); while the remain six sham-operated rats were regarded as the control group. The 26 SD rats in each group were further divided into three groups: 12 h treatment, six rats; 24 h treatment, 10 rats; and 48 h treatment, 10 rats. After the experiments, all rats in each group were sacrificed at corresponding time points. After the operation, rats in the UTI treatment groups were immediately given corresponding doses of UTI.

Establishment and intervention of the animal model

All rats were allowed to acclimate for one week and fast for 12 h before the experiment. After being anesthetized by intraperitoneal injection of 40 mg/kg of sodium pentobarbital, rats were fixed in the supine position. The surgical area was routinely disinfected and hair-shaved. Under aseptic conditions, an incision of approximately 2 cm in length was made in the abdominal wall with a scalpel. The cecum was separated and ligated with 3-0 silk at the terminal ileocecal valve through the incision. Three punctures at the ligation were made by needle #18, and a small amount of feces was squeezed out. Then, both the peritoneum and skin were intermittently sutured with 4-0 silk, and 100 ml/kg of saline was injected for anti-shock. For the operation groups, rats were sacrificed after 12, 24 and 48 h, respectively; and samples were collected. For the sham groups, the abdomen was opened and the intestine was flipped without ligation. Then, venipuncture was conducted and physiological saline was used after the operation.

After CLP, intraperitoneal injection was immediately performed in the UTI1 and UTI2 groups using 50,000 of U/kg and 100,000 U/kg of UTI, respectively. The ALI group had no intervention treatment.

Determination of the evaluation index

Blood samples collection

MMP-9, TNF-\(\alpha\), IL-8, IL-6 and IL-10 were determined by double antibody sandwich ELISA.

Collection of lung tissues

After all rats were sacrificed at corresponding time points, the lung was taken through thoracotomy under sterile operations. Water and blood on the surface of the left lung was immediately cleaned with blotting paper, and its weight was measured by an electronic scale. Then, the lung was dried for 72 h at 90 °C to obtain the dry weight. W/D of the lung tissue was calculated. The lower lobe of right lung was fixed with 10% formalin for pathologic examination.

Determination of inflammatory factors in serum

MMP-9, TNF-\(\alpha\), IL-8, IL-6 and IL-10 were determined by double antibody sandwich ELISA.
The test kit was purchased from Shanghai Xitang Company. The protocol of the kit was strictly followed. The OD values were measured by a microplate reader (BioRad).

Pathologic examination

The lower lobe of the right lung was fixed with 10% formalin liquid, embedded in paraffin and stained with H&E; and lung tissue pathological changes were observed. According to Smith, morphological changes of the lungs such as pulmonary edema, inflammatory cellular infiltration and bleeding were observed by an inverted light microscope.

Statistical analysis

Data including lung injury scores were presented as $\bar{x} \pm s$, and analyzed with one-way analysis of variance using SPSS 17.0 statistical software. Survival rate was compared with Chi-square test. $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered very significant, as observed between two groups.

RESULTS

Survival rate

Survival rates are shown in Table 1, and there was no significant difference among the UTI1, UTI2 and ALI groups. Survival rate in the UTI2 group slightly increased compared with the ALI group, but there was no significant difference between these two groups by Chi-square test ($P > 0.05$).

W/D result

W/D level decreased 12 h after the CLP operation in the ALI group, and declined in the 24 h treatment group. As treatment duration was prolonged to 48 h, this level began to rise. However, W/D level in the UTI group increased for the 24 h group, while W/D in both the UTI1 and UTI2 groups were higher than the ALI group ($P < 0.01$). Moreover, for the 48 h group, W/D in the UTI2 group was higher than the ALI group ($P < 0.01$, Table 2). Changes in serum levels of TNF-$\alpha$, IL-8, IL-6, IL-10 and MMP-9 are listed in Table 2.

MMP-9

The UTI1 and UTI2 group had a descending trend compared with ALI the group, which significantly decreased compared with the 48-hour ALI group ($P < 0.01$).

<table>
<thead>
<tr>
<th>Group</th>
<th>Survivors</th>
<th>Survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>6</td>
<td>100%</td>
</tr>
<tr>
<td>ALI</td>
<td>24 h 6</td>
<td>00%</td>
</tr>
<tr>
<td></td>
<td>48 h 3</td>
<td>00%</td>
</tr>
<tr>
<td>UTI1</td>
<td>12 h 6</td>
<td>00%</td>
</tr>
<tr>
<td></td>
<td>24 h 5</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>48 h 3</td>
<td>50%</td>
</tr>
<tr>
<td>UTI2</td>
<td>12 h 6</td>
<td>00%</td>
</tr>
<tr>
<td></td>
<td>24 h 6</td>
<td>00%</td>
</tr>
<tr>
<td></td>
<td>48 h 4</td>
<td>00%</td>
</tr>
</tbody>
</table>

Table 1. Survival rate of tested rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Lung Pathology score</th>
<th>W/D</th>
<th>TNF-$\alpha$ pg/ml</th>
<th>IL-6 pg/ml</th>
<th>IL-8 pg/ml</th>
<th>IL-10 pg/ml</th>
<th>MMP9 pg/ml</th>
<th>IL-10 pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0.67 ± 0.516</td>
<td>4.62 ± 0.06</td>
<td>8.59 ± 7.21</td>
<td>21.0 ± 5.11</td>
<td>150.15 ± 43.99</td>
<td>12.15 ± 5.66</td>
<td>13.08 ± 7.61</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>1.25 ± 0.50</td>
<td>4.63 ± 0.11</td>
<td>71.65 ± 37.79</td>
<td>47.67 ± 19.43</td>
<td>1605.12 ± 256.45</td>
<td>12.47 ± 2.50</td>
<td>53.09 ± 7.83</td>
<td></td>
</tr>
<tr>
<td>ALI</td>
<td>24 h 2.67 ± 0.52</td>
<td>4.37 ± 0.13</td>
<td>89.06 ± 33.85</td>
<td>91.34 ± 61.86</td>
<td>2201.55 ± 147.39</td>
<td>31.80 ± 3.62</td>
<td>61.00 ± 20.61</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>3.60 ± 0.55</td>
<td>4.47 ± 0.09</td>
<td>109.93 ± 10.99</td>
<td>76.97 ± 56.36</td>
<td>3582.41 ± 2304.93</td>
<td>45.27 ± 21.38</td>
<td>60.99 ± 40.09</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>1.30 ± 0.51</td>
<td>4.72 ± 0.22</td>
<td>45.95 ± 14.49</td>
<td>44.03 ± 6.51</td>
<td>1386.53 ± 86.85</td>
<td>22.80 ± 0.71</td>
<td>28.25 ± 19.20</td>
<td></td>
</tr>
<tr>
<td>UTI1</td>
<td>24 h 2.20 ± 0.59</td>
<td>4.72 ± 0.11**</td>
<td>73.59 ± 10.87</td>
<td>51.23 ± 32.75</td>
<td>1539.08 ± 109.51</td>
<td>24.72 ± 4.79</td>
<td>31.61 ± 15.74</td>
<td></td>
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<tr>
<td>48 h</td>
<td>3.33 ± 0.58</td>
<td>4.59 ± 0.15</td>
<td>148.62 ± 97.26</td>
<td>50.09 ± 12.04</td>
<td>1518.90 ± 96.04**</td>
<td>20.07 ± 3.97**</td>
<td>26.07 ± 9.39</td>
<td></td>
</tr>
<tr>
<td>12 h</td>
<td>1.27 ± 0.48</td>
<td>4.72 ± 0.04</td>
<td>116.25 ± 91.58</td>
<td>22.52 ± 18.16</td>
<td>1508.52 ± 685.58</td>
<td>22.71 ± 6.18</td>
<td>15.65 ± 8.49</td>
<td></td>
</tr>
<tr>
<td>UTI2</td>
<td>24 h 2.10 ± 0.53</td>
<td>4.85 ± 0.11**</td>
<td>77.12 ± 23.28</td>
<td>34.73 ± 13.70*</td>
<td>1959.02 ± 729.96</td>
<td>19.89 ± 8.51</td>
<td>30.38 ± 28.97</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>3.00 ± 0.82</td>
<td>4.74 ± 0.12**</td>
<td>57.00 ± 30.75</td>
<td>38.07 ± 15.12</td>
<td>1358.82 ± 260.78**</td>
<td>18.73 ± 3.39**</td>
<td>19.00 ± 4.63</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Comparison of inflammatory factors, lung injury scores and W/D results between the UTI and ALI groups ($P < 0.05$, $P < 0.01$).
TNF-α, IL-8, IL-6 and IL-10

TNF-α evidently increased in the ALI group, but decreased in the UTI1 and UTI2 groups.

IL-8 was elevated at every time point in the ALI group, but significantly decreased in the UTI1 and UTI2 groups compared with the 48-hour ALI group ($P < 0.01$).

The UTI1 and UTI2 groups exhibited a descending IL-6 trend compared with the ALI group, while the trend in the UTI2 group significantly decreased compared with the 24-hour ALI group ($P < 0.05$).

The IL-10 trends in the UTI1 and UTI2 groups decreased, compared with the ALI group; but there was no significant difference.

Pathological conditions

The lung tissue structure in the control group was very clear when observed by optical microscopy. There was no severe inflammatory reaction and edema variation. Hepatic injury became more serious along with the days after CLP operation. In the 12-hour group, little exudate occurred in the pulmonary alveoli, which revealed a slight interstitium thickness without obvious inflammatory cellular infiltration. However, in the 24 h group, the exudate in the pulmonary alveoli increased and a lot of inflammatory cellular infiltration was found; and this was accompanied by internal bleeding of the trachea. The alveolar septa was widened and the alveolar structure was damaged (Fig. 1A). As treatment time was prolonged to 48 h, interstitium thickness was more serious, the alveolar septa was further widened, plenty of proliferation of connective tissues occurred, and the alveolar structure was further damaged. The body exhibited that the alveolus cavity was collapsed, and focal atelectasis and emphysema was also observed. Infiltration in inflammatory cells was reduced, and more exudate continued to occur in the pulmonary alveoli (Fig. 1B). After the intervention of UTI, internal bleeding of the trachea and inflammatory cellular infiltration in the 24 h group was reduced (Fig. 1C), and destruction of the lung structure was lighter (Fig. 1D). Exudate in the pulmonary alveoli in UTI did not have marked changes, which was also the same for pathology treated by different doses of UTI. Lung injury scores are shown in Table 2.

DISCUSSION

UTI has been widely used in China. It has been reported that the use of UTI in high doses is more effective in the treatment of sepsis in secondary organ injury. UTI and glucocorticoid play important roles in the inhibition of the excess expression of inflammation factors. However, the side effects of glucocorticoids limits its clinical use; while UTI has been widely used without obvious side effects.

This study found that survival rate did not significantly improve after treatment with UTI. Although survival rate in the 48 h UTI group slightly increased, these changes did not have any significant differences. Severe CLP was considered to be related to model choices. Severe CLP could lead to widespread necrosis and exudation in the abdomen, which presented a severe inflammation response. The use of monotherapies might not have good curative effects. Moreover, we found that IL-10 level, as an anti-inflammatory cytokine of sepsis, was also reduced after treatment with UTI. IL-10, also known as cytokines synthesis inhibitor factor, is an important anti-inflammatory cytokine of sepsis. IL-10 can effectively inhibit the lung tissue invasion of inflammatory cells such as lymphocytes and neutrophils, and thus reduce lung injury. The decline in IL-10 levels against improving sepsis may induce ARDS. This might be the main reason that survival rate did not significantly improve after treatment with UTI. In addition, sample number was also a reason for the absence of significant difference.

Lung pathology injury after the operation confirms the presence of acute lung injury. Ac-
According to pathologic results, significant lung injury of tested rats occurred after 24 h of treatment, which peaked after 48 h; and rats in the 48 h group all appeared to have significant lung injury. In the 24 h group, inflammatory cell soak was obvious and exudation was minimal. However, lung injury was further aggravated, alveolar tissue septum hyperplasia was severely damaged, inflammatory cell soak was significantly reduced, and exudation increased in the 48 h group. After treatment with UTI, lung injury was relieved, the pathological score of lung injury decreased, and lung injury was relieved. Therefore, treatment with UTI was considered to reduce lung injury, but there was no significant difference. Meanwhile, the pathological score declined in the UTI2 group compared with the UTI1 group, and the difference was also not statistically significant.

Uncontrolled inflammatory response for lung injury mediated by many inflammatory factors is the major pathophysiologic bases of ALI, and the key factor in treatment is to effectively control and alleviate the excessive inflammation response of the lung. Many cytokines are involved in the pathogenesis of ALI such as TNF-α, IL-6, IL-8 and IL-10, which participate in the process of lung injury by a series of inflammatory cascade reactions. As proven by the experiment, all serum levels of inflammatory factors significantly decreased after treatment with UTI. The serum level of UTI2 inflammatory factors was lower than UTI1, but the difference was not statistically significant.

TNF-α is the main proinflammatory factor in inflammation response on burns and wounds. The aggregation of polymorphonuclear neutrophils (PMN) is a characteristic of ALI/ARDS lung inflammation, which is mediated by TNF-α in alveolar macrophages. Plasma IL-6 is an important sign that cytokines of sepsis and other inflammatory diseases have become activated, which is related to multisystem organ failure and prognosis. Results have shown that TNF-α and IL-6 levels significantly declined after treatment with UTI. (TNF-α in the 48-hour UTI1 and UTI2 groups had no significant decrease due to large intra-group mutation in the UTI2 group.) Therefore, the inhibition of TNF-α and IL-6 was also considered to be one of the mechanisms to lung protection.

IL-8 and MMP-9 levels in the 48-hour UTI1 group and UTI2 group declined compared with the ALI group, and the difference was statistically significant. The inhibition of MMP-9 and IL-8 by UTI treatment was the most severe among all inflammatory factors. It is more important to inhibit MMP-9 and IL-8 by UTI treatment to protect lung injury, than to inhibit TNF-α and IL-6.

MMP-9, also known as Gelatinase B, is an enzyme with the largest molecular weight in the metalloproteinase (MMPs) family, which exists in invertebrates as a 92k zymogen. In the MMPs family, MMP-9 is most important. MMP-9 plays an important role in lung injury by degrading and destroying the key component of the extracellular matrix such as IV and V collagen, gelatin, fibronectin, laminin and plasminogen. MMP-9 can improve the effects of inflammatory factors, as well as neutrophil migration, into lung tissues for local invasion; and it can stimulate parenchymal cells in local lung tissues such as lung fibroblasts, alveolar epithelial cells type II and endothelium to release MMPs. Lastly, lung injuries were aggravated. The main biological effect of IL-8 was to attract and activate neutrophils. In the pathogenesis of acute lung injury, IL-8 is a kind of late inflammatory factor of sepsis, which can help in adhering neutrophils and basophils to release histamine and leukotrienes. It has been reported that MMP-9 can also crack and activate pro-inflammatory factor, IL-8. In addition, IL-8 is also a significant chemotactic agent for neutrophils. After activation, neutrophils can release MMP-9. Research has shown that IL-8 and MMP-9 form a positive-feedback effect in inflammatory response. MMP-9 can enhance the chemotaxis of IL-8 to 10 times.

UTI has shown a wider inhibition for the level of inflammatory factors, and its lung protective effect on ALI was most likely predominantly performed by inhibiting inflammatory factors. In this study, the level of MMP-9 and IL-8 was significantly reduced more than TNF-α and IL-6 after treatment with UTI. Acute lung injury caused by sepsis was relieved after treatment with UTI by lowering the level of inflammatory media, especially for MMP-9 and IL-8.

In the present study, the level of W/D did not present a similar trend with lung injury, which was not consistent with a literature report that the level of W/D declined after treatment with UTI. Under the influence of severe sepsis, both injury of vascular endothelial cell integrity and effect of DIC could lead to plasma extravasation and a lack of circulatory blood volume. In experiments, it was observed that experimen-
tal rats stopped eating and drinking after CLP operation, and had significant weight loss. An average of 35 g was reduced in rats in the 48 h ALI group. We considered that severe CLP might cause severe shock to rats, and this shock did not increase lung water, which was also confirmed by pathologic examination. The level of W/D decreased after 24 h of treatment, and the shock in rats was compensated due to eating and drinking; thus, W/D level began to rise after 48 h of treatment. Compared with the CLP group, rats in the UTI treatment group were considered in a better position, with more eating and drinking accompanied with increased W/D levels.

In conclusion, UTI has protective effects on lung injury by the inhibition of the levels of inflammatory factors, and can relieve damage to lung injury. MMP-9 and IL-8 may be more effective for the protection of septic lung injury than early inflammatory factors such as TNF-α and IL-6. Ultra-high doses of UTI can further reduce levels of inflammatory factors. However, the use of UTI could not ameliorate the survival rate of the tested rats.

REFERENCES