Effects of normal lymphatic fluid on rats with sepsis complicated with lung injury

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Abstract. – OBJECTIVE: Infection can be caused by severe burnt, trauma and hypoxia, further causing systemic inflammatory syndrome or sepsis. The sepsis occurs in about 2% of all hospitalizations and ranges from 6% to 30% in intensive care unit (ICU) in developed countries. This study aimed to investigate the effects of normal lymph fluid on sepsis complicated with pulmonary injury.

MATERIALS AND METHODS: Wistar rats were prepared for sepsis complicated with acute pulmonary model via cecal ligation and puncture, and received normal lymph fluid injection 60 min later. Artery blood-gas index, wet/dry weight (W/D) ratio of lung, Myeloperoxidase (MPO) and superoxide dismutase (SOD) activity of lung tissues were measured, along with protein content and cell count in bronchoalveolar lavage fluid (BALF). Real-time PCR (RT-PCR) and Western blot were employed to measure expression of nuclear factor κB (NF-κB) in lung tissues, whilst enzyme linked immunosorbent assay (ELISA) was used to analyze serum expression of tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2).

RESULTS: Model group had significantly depressed PaO2 and pH value, higher W/D ratio, and MPO activity, lower SOD activity, higher protein and cell count of BALF, and up-regulation of TNF-α, IL-2 and NF-κB expression (p < 0.05 compared to sham group). Infusion of lymph fluid effectively improved blood-gas function, decreased W/D ratio, MPO activity, elevated SOD activity, and lowered TNF-α, IL-2 and NF-κB expression (p < 0.05 compared to model group).

CONCLUSIONS: Normal lymph fluid can inhibit NF-κB expression, suppress inflammation, and improve blood-gas exchange in lung tissues. Therefore, the normal lymph fluid could effectively relieve the sepsis complicated with pulmonary injury.

Key Words: Sepsis, Pulmonary injury, Lymph fluid, Cytokines, NF-κB, Inflammation.

Introduction

Infection can be caused by server burnt, trauma, hypoxia and ischemia-reperfusion injury, as well as by systemic inflammatory syndrome called sepsis. As one common complication in major surgeries, sepsis is a predominant reason causing death in Intensive Care Unit (ICU). Generally, the sepsis occurs in about 2% of all hospitalizations and ranges from 6% to 30% in ICU in developed countries. Although progression in medical technology has brought measures for supporting organ function, and various new generation of antibiotics, thus impeding sepsis progression to certain extents. However, bacterial resistance caused by antibiotics abuse, aged population and traumatic treatment, the occurrence of sepsis and septic shock, are still a major challenge in clinical treatment. With the gradually increased incidence of sepsis, death case caused by severe sepsis and complicated multi-organ injury is increasing worldwide. Sepsis thus constitutes one major threat for patient life, and severely affects public health safety, thus causing heavy burdens for life quality and economy of patients. Severe sepsis leads to sequential injury of multiple organs, leading to multiple organs dysfunction syndrome (MODS), in which pulmonary tissue is the primary target, leading to acute pulmonary injury. Sepsis complicated with acute pulmonary injury occurs early and has higher incidence. As sepsis is a critical factor causing pulmonary damage, sepsis complicated with pulmonary injury is a common clinical symptom in ICU with high mortality. Currently no specific treatment method has been developed against sepsis complicated with pulmonary injury so far. Therefore, the establishment of effective treatment to alleviate such complication with high mortality is of critical implication. Previous studies showed that normal intestine lymph fluid can inhibit inflammatory response and decrease the expression of adhesion molecule in pulmonary vascular endothelial cells under endotoxin shock, indicating certain intervention effect on endotoxin by NML. Normal lymph fluid can
improve endotoxin shock caused by lipopolysaccharide, and improved dysfunction of micro-circulation and lymph circulation. The influence of normal lymph fluid in sepsis complicated with pulmonary injury rats, however, has not been reported.

**Materials and Methods**

**Experimental Animals**
A total of 60 healthy male Wistar rats (2 months old, specific-pathogen-free (SPF) grade, body weight 250 ± 20 g) were purchased from Laboratory Animal Center of Fujian Medical University (Fuzhou, China). Animals were kept in an SPF grade animal facility with fixed temperature (21 ± 1°C) and relative humidity (50-70%) in 12 h light/dark cycle. Animal experiments were performed by experienced persons strictly following experimental design to minimize animal pains. This study has been approved by the Ethical Committee of the first affiliated hospital of Fujian medical university.

**Reagent and Equipment**

Pentobarbital sodium and lidocaine were purchased from Beijing Chemical (Beijing, China). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Covina, CA, USA). Ethylenediaminetetraacetic acid tetrasodium salt (EDTA) was purchased from HyClone (South Logan, UT, USA). Myeloperoxidase (MPO) and superoxide dismutase (SOD) test kits were purchased from Jiancheng (Nanjing, China). Western blotting reagents were purchased from Beyotime Biotech. (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, England). Rabbit anti-mouse nuclear factor κB (NF-κB) monoclonal antibody and goat anti-rabbit horseradish peroxidase (HRP)-labeled IgG secondary antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). ABI 7700 Fast fluorescent quantitative PCR cycler was purchased from ABI (Foster City, CA, USA). Tumor necrosis factor α (TNF-α) and interleukin 2 (IL-2) enzyme linked immunosorbent assay (ELISA) kits were purchased from R&D systems Inc. (Minneapolis, MN, USA). Other common reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Labsystem Version 1.3.1 microplate reader was purchased from Bio-Rad Laborato-

**Animal Model Preparation and Grouping**
A total of 60 healthy male Wistar rats were normally kept for 2 weeks and were then randomly divided into three groups (n=20 each) including sham group, model group, which was prepared from sepsis complicated with pulmonary injury by cecal ligation and puncture, and lymph fluid treatment group, which received injection of normal lymph fluid via femoral artery 60 min after generating the model.

**Acquisition of Normal Lymph Fluid**
Rats were anesthetized by intraperitoneal injection of 50 mg/kg 1% pentobarbital, and were made for an incision right of the abdominal middle line to open the cavity for exposing mesenterium. An intubation was made on intestinal lymph tube for drainage of lymph fluid in 60 min to collect 50 μl fluid, which was then centrifuged at 1500 r/min for 5 min to remove cell components. Supernatant was saved at -80°C for further use.

**Generation of Rat Sepsis With Pulmonary Injury Model and Animal Treatment**
Rats were anesthetized by intraperitoneal injection of 50 mg/kg 1% pentobarbital, and were prepared for septic model using cecal ligation and puncture (CLP) according to previous literature. Middle abdominal skin was shaved and made for a vertical incision along the middle line to expose cecal tissues with integrity. A ligation was performed at 1.5 cm from the blind end using 4.0 silk suture. Two times of punctures were performed at distal site of cecal ligation site using 18 G needle to squeeze little feces. Cecal tissues were then re-located into peritoneal cavity, which was then closed by layers. Body fluid loss was replenished using 24 ml/kg saline. Sham group received identical treatment as those in model group but without ligation or puncture. 60 min after preparing the model, 150 μl normal lymph fluid was replenished into femoral artery of treatment group.

**Sample and Tissue Collection**
Six hours after treatment, rats were anesthetized by 10% hydrate chloral. Blood samples were collected from abdominal artery for 10 min
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centrifugation at 2000 r/min to remove the supernatant serum, which was kept at -20°C. Rats were then sacrificed to collect right upper lung tissues for measuring W/D. Other lung tissues were frozen at -80°C.

**Blood-Gas Analysis and W/D Test**

Automatic blood-gas analyzer was used to measure artery blood oxygen pressure (PaO₂) and pH values. Surface water on right upper pulmonary lobes was removed by filter paper to weigh the wet mass. After drying in incubator for 24 h, dry mass was measured to calculate W/D ratio for evaluating the condition of pulmonary edema.

**Myeloid Peroxidase (MPO) Activity in Lung Tissues**

A total of 100 mg frozen pulmonary tissues were homogenized on ice by adding phosphate potassium buffer following the manual instruction. The homogenate was centrifuged at 4°C under 30 000×g for 30 min. Precipitation was performed by adding 50 mmol/l phosphate potassium buffer containing 0.5% hexadecyl trimethyl ammonium bromide (HTAB). After 60°C incubation for 2 h, the mixture was centrifuged at 4°C under 40 000×g for 130 min. The supernatant was added with reaction buffer for measuring absorbent value at 460 nm wavelength. MPO activity (U/g) was measured as absorbance × 13.5/wet weight of lungs (g).

**SOD Activity in Rat Pulmonary Tissues**

Superoxide dismutase (SOD) activity was measured in pulmonary tissues from all groups following instruction of test kit. Lung tissues were extracted for proteins using 95°C water bath for 40 min. After cooling down, the mixture was centrifuged at 4°C under 40 000×g for 130 min. The supernatant was added with reaction buffer for measuring absorbent value at 460 nm wavelength. MPO activity (U/g) was measured as absorbance × 13.5/wet weight of lungs (g).

**Protein Quantification of Bronchoalveolar Lavage Fluid (BALF) and Cell Count**

Following the manual instruction, bicinchoninic acid (BCA) method was used to quantify protein contents in BALF. Standard curve was plotted based on concentration of standard samples. Absorbance values at 565 nm were measured. Protein content of samples was deduced based on the standard curve. BALF protein content was presented as mg/ml. An elevated number indicated higher permeability of pulmonary micro-vessels. BALF was centrifuged, and cell precipitation was re-suspended in 0.1 ml saline for preparing smear slides. After staining, differential counting of neutrophils and lymphocytes was performed.

**Real-Time PCR for NF-κB mRNA Expression in Rat Pulmonary Tissues**

Under sterile condition, mRNA of pulmonary tissues was extracted by TRizol reagent. Complementary DNA (cDNA) was synthesized using specific primers (Table I). Real-time PCR was then performed to detect target gene expression under the following conditions: HMGB1: 52°C 1 min, followed by 35 cycles each containing 90°C 30 s, 58°C 50 s and 72°C 35 s; RAGE and NF-κB: 55°C 1 min, followed by 35 cycles each containing 90°C 30 s, 62°C 50 s and 72°C 35 s. ABI 7700 Fast fluorescent quantitative PCR cycler was used to collect related data. Cycle threshold (CT) value was measured for standard samples based on internal reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene for plotting standard curve. Quantitative analysis was performed by 2-ΔΔCt approach.

**ELISA for Rat Serum TNF-α and IL-2**

Rat serum was stored at -80°C for further use. ELISA kit was used to measure TNF-α and IL-2 levels in the supernatant. Following manual instruction of ELISA kit, a linear regression function was calculated based on concentration of standard samples and absorbance values. Sample concentration was calculated from the regression function based on absorbance value of samples.

**Western Blot for Measuring NF-κB Protein Expression**

Proteins were extracted from pulmonary tissues. In brief, tissues were mixed with lysis

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Table I. Primer synthesis sequence.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer 5'-3'</th>
<th>Reverse primer 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>GADPH</td>
<td>AGTGCCAGCCTCGTCATAG</td>
<td>CGTTGAACCTGCGTGGTAG</td>
</tr>
<tr>
<td>NF-κB</td>
<td>GATATCCAGGGAGTTGGA</td>
<td>ATGTCAGGGGTTATTGCTGT</td>
</tr>
</tbody>
</table>
buffer for 15-30 min iced incubation. Using ultrasonic rupture (5 s, 4 times) and centrifugation (10,000 ×g, 15 min), proteins were quantified from the supernatant and were kept at -20°C for Western blotting. Proteins were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and were transferred to polyvinylidene difluoride (PVDF) membrane by semi-dry method. Non-specific binding sites were blocked by 5% defatted milk powders for 2 hours. Anti-NF-κB monoclonal antibody (1:500) was applied for 4°C overnight incubation. Goat anti-rabbit IgG (1:2,000) was then added for 30 min incubation. After phosphate-buffered saline and Tween-20 (PBST) washing and ECL development for 1 min, the membrane was exposed under X-ray. An imaging analyzing system and Quantity one software were then used to scan X-ray films and to detect the density of bands with repeated measures (n=4).

**Statistical Analysis**

SPSS16.0 software (SPSS, Inc., Chicago, IL, USA) was used for analyze all collected data, of which measurement data were presented as mean ± standard deviation (SD). Tukey’s post-hoc test was used to validate the ANOVA for comparing measurement data among the groups. A statistical significance was defined when \( p < 0.05 \).

**Blood-Gas Analysis and W/D Comparison**

Artery blood oxygen pressure (PaO \(_2\)) and pH blood-gas index were tested, along with the comparison of pulmonary tissue W/D to evaluate condition of pulmonary edema. Results showed significantly lower PaO \(_2\) and pH values after sepsis complicated with acute pulmonary injury \( (p < 0.05 \) compared to sham group). After infusion of normal lymph fluid, PaO \(_2\) and pH values of model rats were significantly elevated \( (p < 0.05 \) compared to model group). Model rats had higher W/D of pulmonary tissues \( (p < 0.05 \) compared to sham group). After infusion of normal lymph fluid, W/D ratio of lung tissues was decreased \( (p < 0.05 \) compared to model group, Table II). These results showed that normal lymph fluid could improve respiration function of rats having sepsis complicated with pulmonary injury and pulmonary edema status.

**Differential Counting of BALF Cells**

We performed differential counting of cells in BALF. The results showed significantly elevated counts of neutrophil and macrophage in BALF of model group \( (p < 0.05 \) compared to sham group). With infusion of normal lymph fluid, both neutrophil and lymphocyte numbers were remarkably decreased in rat BALF \( (p < 0.05 \) compared to model group, Figure 1).

**Oxidative Stress Index in Rats**

MPO and SOD contents were measured from all groups of rats. Results showed significantly elevated MPO activity in lung tissues after pul-

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<table>
<thead>
<tr>
<th>Group</th>
<th>PaO (_2) (mmHg) ± SD</th>
<th>pH    ± SD</th>
<th>W/D ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>122.37 ± 11.15</td>
<td>7.36 ± 0.06</td>
<td>4.21 ± 0.46</td>
</tr>
<tr>
<td>Model</td>
<td>78.21 ± 109.73*</td>
<td>7.10 ± 0.03*</td>
<td>6.87 ± 0.71*</td>
</tr>
<tr>
<td>Lymph fluid</td>
<td>98.93 ± 11.28**</td>
<td>7.31 ± 0.05*</td>
<td>5.16 ± 0.51*</td>
</tr>
</tbody>
</table>

*Note: *\( p < 0.05 \) compared to sham group, \#\( p < 0.05 \) compared to model group.

**Figure 1.** Cell counting in rat BALF. *\( p < 0.05 \) compared to sham group, \#\( p < 0.05 \) compared to model group.
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Pulmonary injury, plus decrease of SOD content $p < 0.05$ compared to sham group). The infusion of lymph fluid significantly inhibited MPO activity and enhanced SOD content in model rats ($p < 0.05$ compared to model group, Table III). These data showed that normal lymph fluid could exert protective effects against sepsis complicated with pulmonary injury via modulating oxidative stress response.

Serum TNF-α and IL-2 Expression Level in Rat

ELISA was used to test serum levels of TNF-α and IL-2. Results showed that in rats with sepsis complicated with pulmonary injury, serum TNF-α and IL-2 levels were increased ($p < 0.05$ compared to sham group). After infusion of lymph fluid, rat serum TNF-α and IL-2 were significantly inhibited ($p < 0.05$ compared to model group, Figure 2). These findings showed that normal lymph fluid could exert protective effects against sepsis complicated with pulmonary injury via inhibiting secretion of inflammatory factors.

NF-κB mRNA Expression Level of Rat Pulmonary Tissues

Real-time PCR was used to test NF-κB mRNA expression in pulmonary tissues of rats having sepsis complicated with pulmonary injury. Results showed significantly elevated NF-κB mRNA expression in rat pulmonary tissues after sepsis and associated pulmonary injury ($p < 0.05$ compared to sham group). The infusion of lymph fluid significantly lung tissue injury-induced up-regulation of NF-κB ($p < 0.05$ compared to model group, Figure 3).

NF-κB Protein Expression Level of Rat Pulmonary Tissues

Western blot was further used to test NF-κB protein expression in pulmonary tissues of rats having sepsis complicated with pulmonary injury. Consistent with mRNA levels, results showed significantly elevated NF-κB protein expression in rat pulmonary tissues after sepsis and associated pulmonary injury ($p < 0.05$ compared to sham group). The infusion of lymph fluid significantly lung tissue injury-induced up-regulation of NF-κB ($p < 0.05$ compared to

Table III. Oxidative stress index of all rats.

<table>
<thead>
<tr>
<th>Index</th>
<th>Sham</th>
<th>Model</th>
<th>Lymph fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO (U/g)</td>
<td>1.62 ± 0.56</td>
<td>6.71 ± 0.85*</td>
<td>4.31 ± 0.27*#</td>
</tr>
<tr>
<td>SOD (U/mg)</td>
<td>34.22 ± 5.77</td>
<td>17.21 ± 3.65*</td>
<td>22.31 ± 6.18*#</td>
</tr>
</tbody>
</table>

Note: *$p < 0.05$ compared to sham group, # $p < 0.05$ compared to model group.

Figure 2. Serum TNF-α and IL-2 expression level in rat. *$p < 0.05$ compared to sham group, # $p < 0.05$ compared to model group.

Figure 3. NF-κB expression in rat pulmonary tissues. *$p < 0.05$ compared to sham group, # $p < 0.05$ compared to model group.
Discussion

Sepsis firstly caused acute pulmonary injury, which mainly consists of diffused lung injury, causing structural change of lung structure, elevated permeability of alveoli micro-vessels, and aggregation of edema fluids which cannot be effectively removed. Pulmonary tissue inflammation and edema are major pathological changes of acute pulmonary injury, and can cause collapse of alveoli, leading to dysfunction of lung respiration function. We showed the occurrence of blood-gas dysfunction in rat model with sepsis complicated with pulmonary damage, which also had elevated W/D ratio, suggesting that CLP generated sepsis complicated with pulmonary damage could aggravate pulmonary edema and respiration dysfunction. Sepsis complicated with pulmonary injury can cause activation of neutrophil and macrophage, followed by the abundant release of inflammatory factors including TNF-α and IL-2 by activated cells. Nuclear factor NF-κB plays an important role in gene transcription of inflammatory factors. During the occurrence and progression of pulmonary damage, NF-κB could modulate various factors closely related with pulmonary damage including inflammatory cytokine and adhesion molecules. Inflammatory mediator functions at endothelial or epithelial cells of pulmonary alveoli vessels, leading to cell injury and disruption of lung-blood barrier. The exfiltration of alveoli fluids with abundant proteins causes the existence of abundant proteins and macrophage or neutrophil in BALF, accompanied with elevated lymphocytes, further decreasing lung compliance, lower gas exchange or pulmonary volume, lower lung volume, imbalance of ventilation/blood flow, eventually leading to respiratory failure. This study showed that rat model with sepsis plus pulmonary damage could potentiate NF-κB mRNA and protein expression, and further accelerated the release of inflammatory factors including TNF-α and NF-κB, aggravating pulmonary damage. In previous reports, lymph fluid was believed to be correlated with bone marrow failure, red blood cell dysfunction and cardiac constriction failure, all of which elevated permeability of pulmonary vessels, plus aggravation of neutrophils and apoptosis of alveoli tissues. No study, however, has been performed regarding the effect of normal lymph fluid on sepsis complicated with pulmonary injury. Our findings demonstrated that the infusion of normal lymph fluids significantly improved blood-gas dysfunction caused by sepsis and other complications, producing lower WD and MPO activity, elevating SOD activity, and suppressing expression of TNF-α, IL-2 and NF-κB. SOD is one of the most important anti-oxidation enzyme for clearing free oxygen radicals, which plays a critical role for body oxidation-anti oxidation balance. The activity of SOD indirectly reflects body’s ability for clearing free oxygen radicals. MPO is a neutrophil lysosome enzyme, and can reflect the aggregation of pulmonary neutrophils with features. This study showed that the enhancement of SOD activity and decrease of MPO activity could modulate aggregation of neutrophils.

Conclusions

Normal lymph fluid can relieve inflammation, improve blood-gas exchange in lung tissues, and effectively relieved sepsis complicated with pulmonary damage via inhibiting NF-κB expression. This study provided evidences for using normal lymph fluids to treat sepsis complicated with pulmonary damage, and provided novel insights for disease prevention and diagnosis.

Acknowledgements
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Conflict of Interest
The authors declare that they have no conflict of interests.

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