

# Antiinflammatory Effects of Heparin in Hemorrhage or LPS Induced Acute Lung Injury

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## 출혈성 및 내독소 투여로 유발된 급성폐손상에서 heparin의 항염증효과

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김재열, 최재철, 이영우, 정재우, 신중욱, 박인원, 최병휘

**배 경 :** 급성 폐손상은 폐내, 외의 원인질환들에 의해 폐포-모세혈관의 투과성이 증가하며, 폐부종에 의해 급성 저산소성 호흡곤란이 유발되는 증후군이다. 헤파린은 항응고작용 외에 자체적으로 항염증효과를 가지고 있으나, 염증성질환에 헤파린을 투여하면 출혈성 합병증이 발생하기 때문에 실제로 임상에서 이용하는데 제약이 있다. 하지만 헤파린에서 2-O와 3-O sulfate를 제거하면, 항응고 효과가 제거되고 항염증효과는 지니고 있는 비항응고성 헤파린 (nonanticoagulant heparin)으로 변화한다. 본 연구에서는 흰쥐에게 내독소 (LPS)를 투여하거나, 출혈성 쇼크를 일으켜서 유발된 급성폐손상에서 비항응고성 헤파린의 치료효과를 살펴보았다.

**방 법 :** 각 군당 5 마리 이상의 흰쥐 (Ballyc mouse)를 이용하였다. 미정맥 (tail vein)을 통해 생리식염수 또는 비항응고성 헤파린 (50 mg/kg)을 투여한 직후에 내독소를 복강으로 투여하거나 (1 mg/kg), 심장천자를 통해 총 혈액의 1/3 정도로 제거하여 출혈성 쇼크를 유도하여 급성폐손상을 유발하였다. 내독소 투여 또는 출혈성 쇼크 유발 1 시간 후에 흰쥐를 희생시키고 폐를 적출하였고, 폐의 염증성 변화는 사이토카인 (TNF- $\alpha$ , MIP-2, IL-1 $\beta$ )을 측정하여 살펴보고, 폐 손상의 정도는 myeloperoxidase (MPO) assay와 wet-to-dry weight ratio를 측정하여 알아보았다.

**결 과 :** 내독소를 투여한 흰쥐의 폐에서 대조군의 폐에 비해 사이토카인의 발현이 증가하고 (TNF- $\alpha$ ; 196.1 $\pm$ 10.8 vs 83.7 $\pm$ 18.4 pg/ml, MIP-2; 3,000 $\pm$ 725 vs 187 $\pm$ 26 pg/ml, IL-1 $\beta$ ; 6,500 $\pm$ 1167 vs 266 $\pm$ 25 pg/ml,  $p$ <0.05, respectively), 폐의 MPO 활성이 증가하였다 (27.9 $\pm$ 6.2 vs 10.5 $\pm$ 2.3 U/g of lung protein,  $p$ <0.05). 출혈성 쇼크를 일으킨 흰쥐의 폐에서 대조군의 폐에 비해 사이토카인의 발현은 증가되지 않았으나, MPO 발현은 증가되었다 (16.5 $\pm$ 3.2 vs 10.5 $\pm$ 2.3 U/g of lung protein,  $p$ <0.05). 내독소 투여 또는 출혈성 쇼크에 의해 급성폐손상이 유발된 흰쥐에서 생리적 식염수를 투여하거나 비항응고성 헤파린을 투여한 군 사이에 사이토카인의 발현이나 MPO 활성에 의미있는 차이는 관찰되지 않았다.

**결 론 :** 이상의 결과로 비항응고성 헤파린은 내독소를 투여하거나 출혈성 쇼크를 일으키고 한 시간 뒤에 측정된 흰쥐의 급성폐손상에서 의미있는 치료효과를 보이지 않았다. (*Tuberc Respir Dis* 2006; 60: 49-56)

**Key words :** 급성폐손상, 비항응고성 헤파린, TNF- $\alpha$ , MIP-2, IL-1 $\beta$ , MPO assay

## Introduction

Acute lung injury (ALI), which is called as acute respiratory distress syndrome (ARDS) in its most severe clinical manifestation, affects around 150,000 patients per year in the U.S, with recent mortality

rates being over 30 percent<sup>1,2</sup>. At present, there is no effective treatment for ALI except the low tidal volume ventilation, which is rather a way of reducing artificially induced ventilation-associated lung injury (VLI) than an active, specific way of treatment. ALI is characterized by neutrophil accumulation in the lungs, interstitial edema, disruption of epithelial integrity, and leakage of protein into the alveolar space<sup>3-6</sup>. Infection, associated with endotoxemia, and blood loss are frequent predisposing factors to the development of ALI. In experimental settings, endotoxemia or hemorrhage produces ALI<sup>7</sup>. Neutrophils, which play a central role in the ALI, produce proinflammatory mediators, including

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cytokines such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and macrophage inflammatory peptide-2 (MIP-2) and demonstrate increased activation of transcriptional regulatory factors including cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and nuclear factor kappa B (NF- $\kappa$ B)<sup>8-10</sup>.

Heparin presents a dazzling array of properties. In lung disease, it is used as an anticoagulant for thromboembolism, but its polyanionic nature confers a wide variety of other actions not related to anticoagulation<sup>11</sup>. Heparin is a potent antiinflammatory agent that inhibits neutrophil-derived elastase<sup>12</sup>, complement activation<sup>13</sup>, tumor necrosis factor-induced lung edema<sup>14</sup>, L- and P-selectins<sup>15</sup>, leukocyte rolling<sup>16</sup> and neutrophil-induced injury of pulmonary alveolar epithelium<sup>17</sup>. However, therapeutic potential of heparin as an antiinflammatory treatment in lung injury is limited by its inherent anticoagulant activity. Attachment of heparin to antithrombin III and some other proteins is critically dependent on binding energies conferred by specific saccharide sequences or charged side groups, and anticoagulant activity can be removed from heparin by partial chemical desulfation<sup>18</sup>. However, removal of sulfates may have variable effects on other heparin-related activities, which appear to be related to simple charge neutralization of cationic proteins by the anionic polysaccharide<sup>11,14</sup>. This change often results in the loss of important pharmacological activities. Lyophilization of porcine mucosal heparin under extreme alkaline conditions (pH>13) produces a nonanticoagulant heparin (NCH) remarkable for the selective loss of only 2-O and 3-O sulfates, leaving 6-O and N-sulfates intact. Selectively O-desulfated heparin retains potent activity as an inhibitor of neutrophil protease, elastase and cathepsin G<sup>19</sup>. It also inhibited translocation of the NF- $\kappa$ B from the cytoplasm

to the nucleus in human endothelial cells and attenuated myocardial reperfusion injury<sup>20</sup>. It is plausible that NCH could show therapeutic effects on inflammatory diseases of lung. However, as far as authors have searched, there has been no report which evaluated the therapeutic effects of NCH on animal models of the acute lung injury.

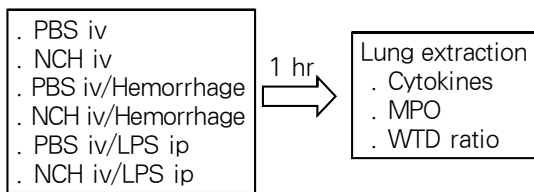
In the present study, we evaluated the therapeutic effects of selectively O-desulfated heparin on the mouse model of acute lung injury developed by endotoxemia or hemorrhage.

## Materials and Methods

*Mice.* Male BALB/c mice, 8-12 week of age, were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were kept on a 12:12-hour, light-dark cycle with free access to food and water. At least five mice were used for each experimental group. All experiments were conducted in accordance with institutional review board-approved protocols.

*Materials.* Nonanticoagulant heparin (2-O,3-O desulfated porcine intestinal heparin, NCH) was kindly donated by Dr. Thomas Kennedy (University of North Carolina, Chapel Hill, NC, USA). *Escherichia coli* 0111:B4 endotoxin (LPS) was purchased from Sigma (St. Louis, MO). Isoflurane was obtained from Abbott Laboratories (Chicago, IL, USA). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL). All other reagents were purchased from Sigma unless otherwise noted in the text.

*Model for Hemorrhage.* Either PBS (200  $\mu$ l) or NCH (6.25 mg/ml, 200  $\mu$ l, 50 mg/kg) was injected into the tail vein. Just after the injection of either solution, hemorrhage model was induced into the mouse. In brief, mice were anesthetized with inhaled isoflurane. Cardiac puncture was used to



**Figure 1.** Design of the experiment. Mice were divided into 6 groups and each group was composed of 5 mice. Nonanticoagulant heparin (NCH, 6.25 mg/ml, 200  $\mu$ l, 50 mg/kg) or PBS (200  $\mu$ l) was injected into the tail vein. Immediately after the injection of NCH or PBS, hemorrhage or LPS-injection was followed. One hour after the induction of hemorrhage or LPS-injection, mice were anesthetized and lungs were removed for the evaluation of cytokines, MPO assay and wet-to-dry ratio.

remove 30% of the calculated total blood volume (0.27 ml/10g body wt) over 60 seconds into a heparinized syringe. One hour after the induction of hemorrhage, mice were again anesthetized with isoflurane, and the previously removed blood was infused into the retro-orbital venous plexus. The sham procedure involved cardiac puncture under isoflurane anesthesia, without blood removal, followed by the second episode of anesthesia and retroorbital puncture 1 hour later. One hour after the induction of hemorrhage, Mice were anesthetized with inhaled isoflurane and chest was opened and flushed by infusing 10 ml of PBS into beating right ventricle. After then, lungs were removed and stored at  $-70^{\circ}\text{C}$  before being used for cytokines and MPO assay (Figure 1).

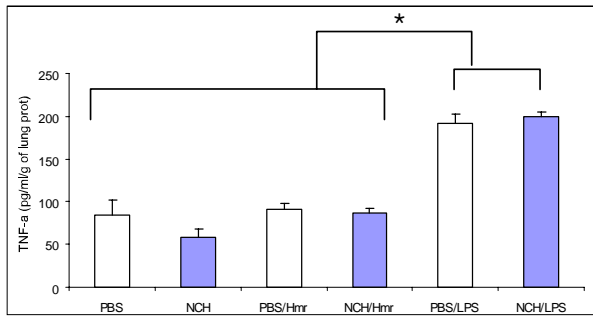
**Model of endotoxemia.** Either PBS (200  $\mu$ l) or NCH (6.25 mg/ml, 200  $\mu$ l, 50 mg/kg) was injected into the tail vein. Just after the injection of either solution, endotoxemia-induced lung injury was made by injecting LPS (125  $\mu$ g/ml, 200  $\mu$ l, 1 mg/kg) into the peritoneum. One hour after the injection of LPS, mice were anesthetized with inhaled isoflurane and chest was opened and flushed by infusing 10 ml of PBS into beating right ventricle. After then, lungs were removed and stored at  $-70^{\circ}\text{C}$  before being used for cytokines and MPO assay

(Figure 1).

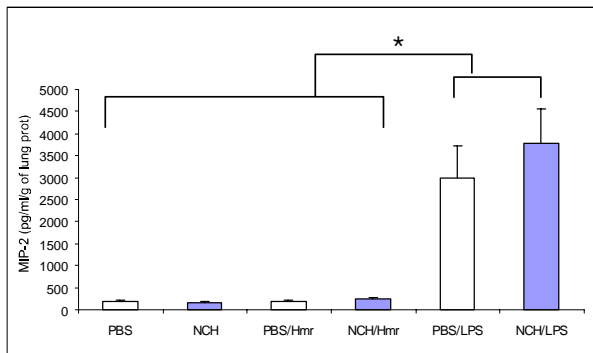
**Preparation of lung homogenate for ELISA.** Lung tissues were homogenized in ice cold lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300  $\mu$ M p-nitrophenyl phosphate, 1 mM PMSF, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, pH 7.3) containing 1 mM of protease inhibitor cocktail (Sigma, St Louis, MO). Homogenates were centrifuged at 14,000 g for 15 minutes and supernatants were collected. The protein concentration of each sample was assayed using the micro BCA protein assay kit standardized to BSA, according to manufacturer's protocol (Pierce, Rockford, IL).

**Cytokine ELISA.** Immunoreactive TNF- $\alpha$ , MIP-2 and IL-1 $\beta$  were quantitated using commercially available ELISA kits (R&D Systems, Minneapolis, MN), according to manufacturer's instructions.

**Myeloperoxidase assay:** MPO activity was assayed as described as follows. Lung tissue was homogenized in 1.0 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 10 mM N-ethylmaleimide for 30 seconds on ice. The homogenate was centrifuged at 12,000 g for 30 minutes at  $4^{\circ}\text{C}$ . The proteinous pellet was homogenized once more in ice-cold buffer, and the homogenate was centrifuged once more at 12,000 g for 30 minutes at  $4^{\circ}\text{C}$ . The pellet was resuspended and sonicated on ice for 90 seconds in 10 times vol of hexadecyltrimethylammonium bromide buffer (0.5% hexadecyltrimethylammonium in 50 mM potassium phosphate, pH 6.0). Samples were incubated in a water bath ( $56^{\circ}\text{C}$ ) for 2 hours and then centrifuged at 12,000 g for 10 minutes. The supernatant was collected for assay of MPO activity as determined by measuring the  $\text{H}_2\text{O}_2$ -dependent oxidation of o-DA (3,3'-dimethoxybenzidine dihydrochloride) at 460 nm.



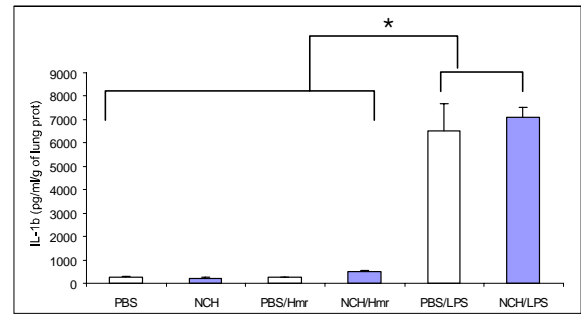
**Figure 2.** NCH treatment did not reduce TNF- $\alpha$  expressions in the lung after LPS injection. Mice were injected with either PBS (200  $\mu$ l) or NCH (50 mg/kg). Hemorrhage or LPS-injection (1 mg/kg, ip) was followed immediately. One hour after the induction of hemorrhage of LPS-injection, mice were anesthetized and the expression of TNF- $\alpha$  was evaluated by ELISA method. \*:  $<0.05$ . PBS; phosphate buffered saline, NCH; nonanticoagulant heparin, PBS/ Hmr; PBS injection followed by hemorrhage, NCH/ Hmr; NCH injection followed by hemorrhage, PBS/ LPS; PBS injection followed by LPS injection, NCH/ LPS; NCH injection followed by LPS injection



**Figure 3.** NCH treatment did not reduce MIP-2 expressions in the lung after LPS injection. Mice were injected with either PBS (200  $\mu$ l) or NCH (50 mg/kg). Hemorrhage or LPS-injection (1 mg/kg, ip) was followed immediately. One hour after the induction of hemorrhage of LPS-injection, mice were anesthetized and the expression of MIP-2 was evaluated by ELISA method. \*:  $<0.05$

*Wet-to-Dry Lung Weight Ratios.* Lungs were excised, rinsed briefly in PBS, blotted, then weighed to obtain the "wet" weight. Lungs were dried in an oven at 80°C for 7 days to obtain the "dry" weight.

*Statistical Analysis.* Data are expressed as means  $\pm$  SEM. ANOVA was performed with SPSS Win-



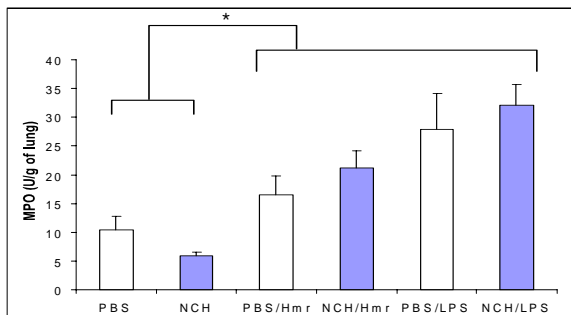
**Figure 4.** NCH treatment did not reduce IL-1 $\beta$  expressions in the lung after LPS injection. Mice were injected with either PBS (200  $\mu$ l) or NCH (50 mg/kg). Hemorrhage or LPS-injection (1 mg/kg, ip) was followed immediately. One hour after the induction of hemorrhage of LPS-injection, mice were anesthetized and the expression of IL-1 $\beta$  was evaluated by ELISA method. \*:  $<0.05$

downs 9.0 statistical analysis software, and a difference was accepted as significant if the p value was less than 0.05, as verified by Duncan and Tukey post hoc test.

## Results

### The expressions of lung cytokines were increased in endotoxemia model

The expression of TNF- $\alpha$  in PBS injected mice (control group) was  $83.7 \pm 18.4$  pg/ml/g of lung protein and that of LPS injected mice was  $191.6 \pm 10.8$  pg/ml/g of lung protein ( $p < 0.05$  compared to control group or hemorrhage group). In the while, lung TNF- $\alpha$  level in mice with hemorrhage was  $91.1 \pm 6.3$  pg/ml/g of lung protein ( $p > 0.05$  compared to control group) (Figure 2). The lung MIP-2 expressions in the control, hemorrhage, and endotoxemic mice were  $187 \pm 26$ ,  $200 \pm 22$ , and  $3000 \pm 725$  pg/ml/g of lung protein, respectively. The level of lung MIP-2 in the endotoxemic mice was significantly higher than those of control group or hemorrhage group ( $p < 0.05$ ) (Figure 3). The level of IL-1 $\beta$  in endotoxemic mice ( $6,500 \pm 1167$  pg/ml/g of lung protein) was significantly elevated compared to those of control

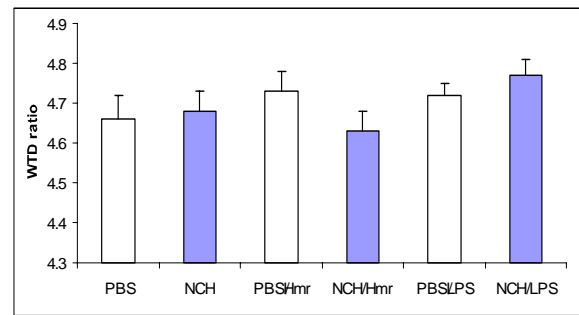


**Figure 5.** NCH treatment did not reduce neutrophil recruitment in the lung after LPS injection. Mice were injected with either PBS (200  $\mu$ l) or NCH (50 mg/kg). Hemorrhage or LPS-injection (1 mg/kg, ip) was followed immediately. One hour after the induction of hemorrhage or LPS-injection, mice were anesthetized and lung neutrophil recruitment was evaluated by MPO assay. \*:  $<0.05$

group ( $266 \pm 25$  pg/ml/g of lung protein) or hemorrhage group ( $224 \pm 24$  pg/ml/g of lung protein) ( $p < 0.05$ ) (Figure 4).

#### NCH treatment had no effects on the expressions of lung cytokines

There was no difference in the expressions of lung cytokines between mice injected by PBS or NCH in control states. In detail, lung TNF- $\alpha$ , MIP-2, and IL-1 $\beta$  levels of PBS- vs NCH-injected mice were  $83.7 \pm 18.4$  vs  $57.8 \pm 10.4$ ,  $187 \pm 26$  vs  $168 \pm 33$ , and  $266 \pm 25$  vs  $204 \pm 28$  pg/ml/g of lung protein, respectively (Figure 2, 3, and 4). Both in hemorrhage and endotoxemic models, NCH injection didn't affect the expressions of three cytokines. In hemorrhage model, lung TNF- $\alpha$  levels in PBS-injected or NCH-injected mice were  $91.1 \pm 6.3$  and  $87.5 \pm 5.5$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 2). Those of MIP-2 levels were  $200 \pm 22$  and  $231 \pm 41$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 3). Lung IL-1 $\beta$  levels were  $224 \pm 24$  and  $492 \pm 44$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 4). In endotoxemic model, lung TNF- $\alpha$  levels in PBS-injected or NCH-injected mice were  $192 \pm 11$  and  $199 \pm 6$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 2). Those of MIP-2 levels



**Figure 6.** Either hemorrhage nor LPS-injection did not increase lung leakage at one hour after insults. Mice were injected with either PBS (200  $\mu$ l) or NCH (50 mg/kg). Hemorrhage or LPS-injection (1 mg/kg, ip) was followed immediately. One hour after the induction of hemorrhage or LPS-injection, mice were anesthetized and lung leakage was evaluated by wet-to-dry ratio.

were  $3000 \pm 725$  and  $3791 \pm 779$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 3). Lung IL-1 $\beta$  levels were  $6500 \pm 1167$  and  $7080 \pm 447$  pg/ml/g of lung protein ( $p > 0.05$ ) (Figure 4).

#### NCH treatment did not reduce the recruitment of neutrophils in the lung

The MPO activities in PBS- or NCH-injected control mice were  $10.5 \pm 2.3$  and  $5.9 \pm 0.7$  U/g of lung ( $p > 0.05$ ). Those of PBS- or NCH-injected mice in hemorrhage model were  $16.5 \pm 3.2$  and  $21.2 \pm 3.0$  U/g of lung, which were higher than those of control group but no significant difference between them ( $p > 0.05$ ). In endotoxemic model, MPO activities were increased both in PBS-injected ( $27.9 \pm 6.2$  U/g of lung,  $p < 0.05$  compared to control group) and NCH-injected mice ( $32.1 \pm 3.6$  U/g of lung,  $p < 0.05$  compared to control group). However, there was no significant difference in MPO activity between PBS-injected and NCH-injected mice in endotoxemic model ( $p > 0.05$ ) (Figure 5).

#### Either hemorrhage or endotoxemic model didn't increase in the leakage of lung

Lung leakage was evaluated by wet-to-dry ratio.

The wet-to-dry ratios of PBS- or NCH-injected mice in control mice were  $4.66 \pm 0.06$  and  $4.68 \pm 0.05$  ( $p > 0.05$ ). Those of PBS- or NCH-injected mice in hemorrhage mice were  $4.73 \pm 0.05$  and  $4.63 \pm 0.05$  ( $p > 0.05$ ). In endotoxemic mice, they were  $4.72 \pm 0.03$  and  $4.77 \pm 0.04$  ( $p > 0.05$ ) (Figure 6).

## Discussion

Sepsis, which is one of the most important causes of acute lung injury, is the leading cause of death in critically ill patients world-wide. In the United States, sepsis develops in 750,000 people annually, and more than 210,000 of them die<sup>21</sup>. Despite more than 20 years of extensive research, sepsis and systemic inflammatory response syndrome (SIRS) remain the chief causes of death in intensive care units, with mortality rates between 30 to 70%. Further while, according to a recent report, the incidence is rising at rates between 1.5% and 8% annually<sup>22</sup>. In the while, hemorrhage is often associated with severe acute lung injury<sup>23</sup>. The ischemia and reperfusion that occur with hemorrhage result in production of reactive oxygen species (ROS) that are thought to cause some of the lung injury that follows hemorrhage<sup>24</sup>. A common consequence of sepsis or hemorrhage associated with systemic inflammation is multiple organ dysfunction syndrome (MODS)<sup>25</sup>. Whereas the clinical manifestations of this syndrome are inconsistent, alterations in pulmonary functions are almost always observed<sup>26,27</sup>. For example, systemic administration of LPS and/or hemorrhagic shock induced an increase in the expression of inflammatory cytokines (TNF- $\alpha$ , IL-6) in serum and in the lung as early as 1 hour after insults<sup>7,28,29</sup>. Components of the pulmonary dysfunction associated with MODS include widening of the alveolar-arterial PO<sub>2</sub> gradient, decreased lung compliance and pulmonary edema.

Most clinical trials targeting blockade of specific inflammatory mediators have not been successful except activated protein C (APC), which has recently been approved for treatment of severe sepsis. In the same sense, transfusion is the sole therapeutic option for the victims of major trauma which doesn't prevent the development of acute lung injury after severe bleeding. In the context of these dire situations in the management of sepsis- and hemorrhage-associated acute lung injury, we tried to evaluate the therapeutic potential of NCH on acute lung injury. Previously in *in vitro* study, NCH not only decreased the neutrophil-induced injury of pulmonary alveolar epithelium<sup>17</sup>, but also prevented ischemia-reperfusion injury of the lung<sup>30,31</sup>. With these actions, heparin might pose an ideal possible treatment for acute lung injury. With these in mind, we evaluated the therapeutic effects of NCH on the two kinds of acute lung injury model (hemorrhage and LPS injection). Both model induced the increase of MPO activity in the lung and in the LPS injection model, the expression of lung cytokines (TNF- $\alpha$ , MIP-2, IL-1 $\beta$ ) increased. These results imply that hemorrhage and endotoxemia could result in the development of acute lung injury. Unfortunately, however, NCH failed to reduce the expression of lung cytokines (Figure 2, 3, 4) and neutrophil recruitment (Figure 5). By the way, both models for acute lung injury, endotoxemia and hemorrhage, didn't even increase the leakage of the lung (Figure 6), which was evaluated with wet-to-dry ratio. The validity of this method for the evaluation of lung leakage needs to be tested in the future.

The failure of NCH to reduce the inflammation of lung might be related it's inefficacy in the *in vivo* model of acute lung injury. However, because we tried a fixed dose of NCH (50 mg/kg) at fixed time point (one hour after the induction of acute lung injury), it is still a too hasty conclusion to give off

the hope for NCH as a potential agent in the treatment of acute lung injury.

In conclusion, NCH failed to reduce acute lung injury associated with endotoxemia and hemorrhage. However, considering the fact that the history of therapeutic intervention for sepsis has been referred to as 'the graveyard for pharmaceutical companies', this result is not quite surprising. Still the research to find the exact time point of injection and effective dose of NCH needs to be continued.

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