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Low molecular weight heparin may prevent acute lung injury induced by sepsis in rats

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ABSTRACT

The purpose of this study was to assess the protective effect of low molecular weight heparin (LMWH) on acute lung injury (ALI) in rats induced by sepsis. Rat ALI model was reproduced by cecal ligation and puncture (CLP). All rats were randomly divided into three groups (n = 50): control group (A), ALI group (B), and LMWH-treated group (C). Group A received a sham operation and the other groups underwent CLP operation. Groups A and B accepted intraperitoneal injection (i.p.) of normal saline (NS) at a dose of 2.0 ml kg^{-1} and ceftriaxone (30 mg kg^{-1}), group C was intraperitoneally injected with additional LMWH (150 U kg^{-1}) except saline and ceftriaxone. Blood was collected and lung tissue was harvested at the designated time points for analysis. The lung specimens were harvested for morphological studies, immunohistochemistry examination. Lung tissue edema was evaluated by tissue water content. The levels of lung tissue myeloperoxidase (MPO) were determined. Meanwhile, the nuclear factor-kappa B (NF-κB) activation, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) levels, high mobility group box 1 (HMGB1) and intercellular adhesion molecule-1 (ICAM-1) protein levels in the lung were studied. There was a significant difference in each index between groups A and B ($P < 0.05$). Treatment with LMWH significantly decreased the expression of TNF-α, IL-1β, HMGB1 and ICAM-1 in the lungs of ALI rats. Similarly, treatment with LMWH dramatically diminished sepsis-induced neutrophil sequestration and markedly reduced the enhanced lung permeability. In the present study, LMWH administration inhibited the nuclear translocation of NF-κB in the lungs. These data suggest that LMWH attenuates inflammation and ameliorates lung pathology in CLP-induced sepsis in a rat model.

1. Introduction

Sepsis is a kind of serious medical condition that is characterized by dysregulated systemic inflammatory responses (von Dadelszen et al., 2000; Lever and Mackenzie, 2007; de Jong et al., 2010). Sepsis is one of the most major reasons in intensive care unit (ICU), and many patients may occur dysfunction of organs (Brun-Buisson, 2000; Horeczko et al., 2014). Dysfunctional microcirculation has an important role in organ function. When sepsis occurs, coagulation will be activated in almost all patients with serious infection, and blood coagulation changes can be detected in patients with septic shock (Corrigan et al., 1968; Marti-Carvajal et al., 2012). Researchers have shown that thrombin can be generated, and fibrin deposition can be generated in microcirculation after several minutes of pathogen invasion in the body. If damage is not further aggravated, formed blood clots will be rapidly dissolved; while if infection is continually stimulated, local low perfusion will occur, and neutrophil granulocyte and blood platelet are deposited that form sludged blood and necrosis. These results will lead to irreversible tissue damage. If the process is occurred in the whole microcirculatory system, these will lead to multiple organ dysfunction (MOD) and increase the risk of death (Li et al., 2013). Therefore, some factors, including the activation of blood coagulation, anti-freezing and fibrinolytic activity decline, are major features of sepsis, and are also important factors that affect the prognosis. Anti-freezing is quite crucial in the treatment of sepsis. More experimental data suggest that an anti-inflammatory coagulation drug has a potential preventive and therapeutic effect for syndrome of sepsis cross-activated by inflammation and cruor (Bernard et al., 2001; Abraham et al., 2003; Liu et al., 2014).

Heparin has a protective effect in shock led by lipopolysaccharides (LPSs). Mortality rate can be reduced in rats if heparin is used in rats injected with *Salmonella* and *Escherichia coli* LPS. Heparin has an important role in generation and release of proinflammatory factors, and can inhibit nuclear transcription factor kB(NF-kB), which leads to activation of proinflammatory factor in mononuclear cells and endothelial cells,

Abbreviations: LMWH, low molecular weight heparin; CLP, becal ligation and puncture; NS, normal saline; MPO, myeloperoxidase; NF-κB, nuclear factor-kappa B; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; HMGB1, high mobility group box 1; ICAM-1, intercellular adhesion molecule-1; ICU, intensive care unit; MOD, multiple organ dysfunction; LPS, lipopolysaccharide; DAB, diaminobenzidine; OD, optical density.

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and inhibit proinflammatory response of ischemia/reperfusion injury and reduce endothelium dysfunction by increasing generation of nitric oxide (NO) and the top ring (Page, 2013). In vitro, heparin can inhibit the expression, generation and release of proinflammatory factors in mononuclear cell activated by LPS and other cells by acting on thrombin and NF-KB. In high performance sepsis, heparin can improve endothelial cell function and vascular reactivity, and also adjust adhesion, migration and excitation of leukocyte. Heparin blocks the process of leukocyte adhesion to endothelial cells, and prevents the infiltration to the organization by connection with P-selectin and L-selectin (Nelson et al., 1993; Nasiripour et al., 2014). Polymicrobial sepsis induced by cecal ligation and puncture (CLP) is the most frequently used model, because it closely resembles the progression and characteristics of human sepsis (Ritter et al., 2003; Sener et al., 2005).

Herein, this study is to observe the influence of low molecular weight heparin on acute lung injury induced by cecal ligation and puncture (CLP), and the morphological studies, immunohistochemistry examination and the MPO were performed. Meanwhile, the expression of inflammation factors including TNF-α, IL-1β, HMGB1 and ICAM-1 were determined.

2. Material and methods

2.1. Animals

All animal experiments in this investigation were conducted in adherence to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and approved by the Shandong University, Shandong province, China. Male Wistar rat weighing 200–300 g (Experiment Animal Center of Shandong University, China) were used. An esthesis was induced by 4% sevoflurane. All rats were randomly divided into three groups ($n = 50$): sham group (A), CLP group (B), and LMWH-treated group (C). Group A received a sham operation and the other groups underwent CLP operation. Groups A and B accepted intraperitoneal injection (i.p.) of normal saline (NS) at a dose of 2.0 ml kg^{-1} and ceftriaxone (30 mg kg^{-1}), and group C were intraperitoneally injected with additional low molecular weight heparin (LMWH) (Fragmin, Pfizer) (150 U kg^{-1}) except saline and ceftriaxone.

2.2. Morphometric analysis & immunohistochemistry

The rats were sacrificed and the left lung parenchyma was isolated, fixed in 4% paraformaldehyde solution for 24 h in situ, processed for paraffin embedding, and cut into 4 μm transverse sections. The tissue sections were either subjected to routine H&E staining for the detection of collagenous fibers and muscle fibers or incubated with indicated primary antibodies at 4 °C overnight for the later immunostaining by using diaminobenzidine (DAB). Non-immune IgG was used as a negative control. Six cross-sectional areas of various blood vessel layers (the lumen, intima and media) were randomly selected and measured with a computer-based Image Pro Plus Analyzer 4.5 (Media Cybernetics) system.

A pathologist blind to group assignment analyzed the samples and determined levels of lung injury according to Murakami's technique [16]. Briefly, 24 areas in the lung parenchyma were graded on a scale of 0 to 4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, edema, infiltration of inflammatory cells, and hemorrhaging.

2.3. Detection of inflammatory factors

Contents of TNF-α, IL-1β, and IL-6 were detected based on balance method using radioimmunoassay. Some round bottom polystyrene tubes were numbered (according to protocol of kit), mixed, and put for 15 min in room temperature. Then, they were centrifuged for 15 min at 3500 r/min. The supernatant was deleted, and radioactivity of each tube was detected.

2.4. Levels of ICAM-1 using double antibody sandwich enzyme immunoassay

Solid-phase antibody was made using purified microwell plate containing antibody, ICAM-1 antigen, biotinylated anti-human ICAM-1 antibody, and avidin marked by HRP were added in order in microwell. TMB substrate was used to color after completely washing, and the depth of the color was positively associated with the level of ICAM-1 in samples. Optical density (OD) values were detected in 450 nm using microplate reader, which was used to estimate concentration in samples.

2.5. Western blot

Lung parenchymas were homogenated and lysed in RIPA buffer containing 50 mmol/L pH 8.0 Tris–HCl, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecylsulfate, 0.1 mmol/L dithiothreitol, 0.05 mmol/L phenylmethyl–sufonylfluoride, 0.002 mg/mL aprotinin, 0.002 mg/mL leupeptin, and 1 mmol/L $NaVO_3$. DC protein assay reagent (Bio-Rad, Hercules, CA) was used to quantify the protein concentration. Proteins were subjected to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated with appropriate primary antibodies overnight. HRP-conjugated secondary antibodies (Santa Cruz) were applied to visualize and were bound to primary antibodies. For the antibody information, the antibodies against NF-κB, TNF-α, IL-1β, and HMGB1 were purchased from Cell Signaling Technology, the antibodies against ICAM-1 and GAPDH were purchased from Santa Cruz. We used the NIH Image J software (National Institutes of Health, Bethesda, MD, USA) to quantitate protein band concentrations.

2.6. Determination of the wet/dry ratio

The lung tissue was cleaned of blood stains with absorbent paper, weighed wet, torrefied in a 75 °C thermostatic baking oven for 72 h, and weighed again dry to calculate the lung D/W weight ratio.

2.7. Statistical analysis

Groups of data are presented as mean ± standard error. Data were analyzed using one-way ANOVA followed by Fisher's LSD post-hoc test. Calculations were performed using SPSS for Windows version 12.5S statistical package (SPSS, Chicago, USA). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Histological change

Sham-operated group: pulmonary lobule structure was clear, the alveolar cavity was clean, alveolar walls did not thicken; inflammatory cell infiltration was not detected in alveolar interstitial, hyperplasia was not detected in fibrous connective tissue, and bronchial mucosal epithelium was complete (Fig. 1A). CLP model group: pulmonary lobule structure was unclear, the alveolar cavities were detected with bleeding and exudation, alveolar walls thickened, inflammatory cell infiltration was detected in alveolar interstitial, hyperplasia was detected in fibrous connective tissue, and bronchial mucosal epithelium was not complete (Fig. 1B). CLP + LMWH group: part of the pulmonary lobule structure was clear, the alveolar cavity was clean, alveolar walls mildly thickened, inflammatory infiltration in alveolar interstitial and bleeding and exudation in the alveolar cavities were significantly reduced, fibrous connective tissues were detected with a