The value of Heparin-binding protein in bronchoalveolar lavage fluid in ARDS—a case control study

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Research Article

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Abstract

Background

Heparin-binding protein (HBP) play an important role in ARDS. Plasma HBP is a good biomarker on predicting ARDS, but the value of bronchoalveolar lavage uid (BALF) HBP in ARDS has not been studied.

Methods

We use cecum ligation and puncture (CLP) to induce ARDS model in mice and study plasma HBP, BALF HBP and lung injury severity, lung wet/dry ratio and total protein levels in BALF. Also we included a total of 44 patients with ARDS and 38 patients with cardiogenic pulmonary edema (CPE). We compared BALF and plasma HBP levels between the two groups and studied their correlation.

Results

Animal study show, compare with sham group, CLP group mice show significant higher lung WD Ratio (P = 0.002), BALF protein (P < 0.001), BALF HBP (P = 0.013) and plasma HBP (P = 0.003) than sham group. Lung injury index WD Ratio and BALF protein have significant correlation with plasma HBP and BALF HBP in CLP group mice. Plasma and BALF HBP also have significant correlation (P = 0.026). Study in patients showed there are significant difference in BALF HBP (P < 0.001), BALF Protein (P < 0.001) and Plasma HBP (P < 0.001) between ARDS and CPE patients. There are significant correlation between P/F ratio and BALF HBP (P = 0.005) and plasma HBP (P = 0.021). We also found a strong correlation between BALF HBP and plasma HBP levels (P < 0.001).

Conclusions

Our study suggests that both in animal or human, Both BALF and Plasma HBP were significantly increased during lung injury, and were significantly correlated with the severity of lung injury, while BALF was better correlated with lung injury. BALF is associated with elevated plasma HBP levels. BALF HBP could be used as a biomarker to guide the diagnosis and treatment of ARDS.

1. Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening condition that causes acute respiratory failure in ICUs worldwide. The underlying pathological mechanism has yet to be fully elucidated, but it is widely believed that ARDS is a manifestation of systemic inflammatory response syndrome (SIRS) in the lung. The pathogenesis of ARDS is characterized by the injury of pulmonary vascular endothelial and alveolar epithelial cells, resulting in an excessive and uncontrolled inflammatory response in the lung. The increased permeability of pulmonary capillaries leads to the leakage of fluid and proteins into the
interstitial space and alveoli, impairing lung function. As such, the study of pulmonary vascular permeability is a crucial area of focus in ARDS research.

Neutrophils are considered to be among the most important effector cells involved in changes in capillary permeability. The accumulation of neutrophils and the release of cytokines can lead to alveolar capillary injury and increased permeability, which is a significant pathogenic factor in ARDS [1]. One of the most crucial inflammatory mediators released from neutrophils after being activated by signal chemokines and activators is Heparin-Binding Protein (HBP).

HBP, also known as azurocidin or cationic antimicrobial protein 37 (CAP37), is primarily found in the azurophilic granules of neutrophils, with a molecular weight of 37 kDa. As a multifunctional inflammation modulator, HBP derived from PMNs can promote the recruitment, adhesion, and extravasation of monocytes [2], and is considered the most crucial upstream signal that acts on vascular endothelial cells. HBP is involved in the pathological processes that lead to vascular leakage and lung edema [3], and thus plays a crucial role in ARDS.

Clinical studies have found that plasma HBP levels are significantly increased in patients with ARDS, which is related to its occurrence [4, 5]. HBP appears to be one of the primary effector molecules of transfusion-related acute lung injury and plays an important role in the pathogenesis of sepsis ARDS [6]. Previous studies by our team confirmed that ARDS patients have synchronous increases in neutrophils and HBP, and that HBP can be used as a good predictor of the occurrence of ARDS. Our studies also show that HBP release from PMNs is a β2 integrin-PI3K signaling pathway dependent process, revealing potential novel therapeutic targets for ARDS treatment [7].

We use CLP to induce ARDS model because it induces a lung injury similar to that observed in patients with intra-abdominal sepsis related ARDS.

The role of HBP in acute respiratory distress syndrome is still under studied. Previous studies have focused on the plasma HBP level, but few studies have investigated BALF HBP and its relationship with ARDS. As fluid comes from the alveolar surface directly, BALF may have a higher cytokine concentration. Here we made a scientific hypothesis that the HBP concentration of BALF in patients with ARDS is also significantly increased. Herein, we try to figure out the level of HBP in BALF and its value in ALI lung injury animal model and ARDS patients. To evaluate the BALF HBP role in ARDS, we investigated the HBP level in BALF and blood in a CLP rat model of ALI. Wet/dry lung weight ratio and BALF protein was used as lung injury index. Furthermore, we collected and measured BALF and plasma HBP in patients with ARDS and patients with cardiogenic pulmonary edema as a control. Lung injury severity was determined by measuring lung wet/dry ratio and total protein levels in BALF.

2. Methods

Animals studies
Animals

6–8 weeks old male C57BL/6 mice weighing 22–25 g were obtained from the Experimental Animal Centre of Medical Collage of Tongji University (Shanghai, China). Animals were housed in pathogen-free mouse facility containing wood shaving and maintained in a room with a 12-hours light cycle with free access to food and water. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Groups and treatment

Twenty-four mice were randomly divided into two groups: Sham and CLP. Mice in the CLP group were subjected to cecal ligation and puncture (CLP) operation, while Sham mice were treated identically, except that the cecum was neither ligated nor punctured. Twenty-four hours after the operation, all mice were anesthetized and blood was drawn to measure plasma HBP. The right lungs were bronchoalveolar lavaged, and the left lungs were used to measure the wet/dry lung weight ratio.

Cecal ligation and puncture operation

The mice were anesthetized with 1.5% Pentobarbital Sodium via intraperitoneal injection, and a 1 cm incision was made at the ventral midline of the abdomen. The cecum was located and ligated with 4−0 silk just below the ileocecal junction, and punctured with an 18-gauge needle. Gentle pressure was applied to the cecum to express a small amount of fecal material, ensuring patency of the puncture holes. The cecum was then returned to the abdomen, and the incision was closed in layers.

Bronchoalveolar lavage

After the mouse was anesthetized, an 18-gauge catheter was inserted into the right bronchus. PBS (0.5 ml) was injected into the lung, and the effluent was collected. The procedure was repeated twice, and all collected material from one mouse was pooled for protein quantification. Half of the BALF was used to measure BALF protein using the BCA Protein Assay kit protocol, while the other half was immediately frozen at -80°C for HBP level measurement.

Blood and BALF HBP

“All mice were anesthetized, and blood was drawn from the arterial line into standard EDTA vacutainer tubes for measurement of plasma HBP. The blood samples were immediately placed on ice and centrifuged for 10 minutes at 2000 RCF. The resulting plasma was collected and frozen at -80°C.

Wet/dry lung weight ratio measurement

The left lungs were excised at the end of the experiment and weighed to determine the wet lung weight, then placed in an incubator at 55°C for 48 hours to obtain the final dry weight. The presence of pulmonary edema was examined by calculating the pulmonary wet/dry ratio (W/D).
Enzyme linked immunosorbent assay (ELISA)

Concentrations of HBP in both plasma and BALF were measured using the Azurocidin(HBP) ELISA kit (Boster, Wuhan, China), following the protocol provided. Briefly, 100 µL of sample was added to duplicate wells of microtiter plates coated with anti-human HBP antibody. After 90 minutes of incubation at 37°C, a biotin-conjugated antibody was added and incubated at 37°C for 60 minutes. After washing with Tris Buffered saline (TBS), avidin-biotin complex dilution was added and incubated at 37°C for 30 minutes. Then, tetramethylbenzidine (TMB) was added and allowed to react for 30 minutes before termination. The plate was read using a microtiter plate reader at 450 nm to obtain the results.

2.2 Human studies

Study population

All patients admitted to the intensive care units at Tongji University Shanghai East Hospital between May 2018 and October 2020 were screened for the onset of ARDS. Inclusion criteria were: (a) aged between 18–89 years who met the criteria for ARDS; (b) patients who underwent fiberoptic bronchoscopy in ICU; (c) signed informed consent. Exclusion criteria were primary pregnancy, a history of malignant tumors, after cardiopulmonary resuscitation, chronic interstitial lung disease or COPD, pulmonary embolism, immunosuppression due to medication or disease, and patients on hemodialysis. At the same time, patients who were intubated for cardiogenic pulmonary edema and hypoxemia were enrolled in the CPE group.

Patients in the ARDS groups met the ARDS Berlin Definition as defined using the following criteria: (1) Within 1 week of a known clinical insult or new/worsening respiratory symptoms. (2) Chest radiograph or computed tomography scan show bilateral opacities not fully explained by effusions, lobar/lung collapse, or nodules. (3) Mild: 200mmHg < PaO2/FIO2 ≤ 300mmHg with PEEP or CPAP ≥ 5cmH2O; Moderate: 100mmHg < PaO2/FIO2 ≤ 200mmHg with PEEP or CPAP ≥ 5 cm H20; Severe: PaO2/FIO2 ≤ 100mmHg. (4) Respiratory failure not fully explained by cardiac failure or fluid overload, or objective assessment excludes hydrostatic edema if no risk factor is present.

Measure BALF and blood HBP

All patients were examined by fiberoptic bronchoscopy within 24 hours after enrollment. They were intubated and ventilated at the time of BAL. Preoxygenation was performed for 15 minutes. The bronchoscope was inserted through the tracheal tube and wedged into a bronchus of the middle or lingual lobe. Three 20-ml aliquots of sterile 0.9% NaCl at room temperature were instilled, with a total volume of 60 ml, and recovered by gentle hand suction. The alveolar lavage fluid was retained and centrifuged in a 3500rpm centrifuge at room temperature for 10 minutes within 2 hours. Half of the supernatant was used to measure BALF protein following the protocol of the BCA Protein Assay kit, and the other half was added to buffer and immediately frozen in aliquots at -80°C for HBP level measurements.
Blood samples from patients were collected from the arterial line using standard EDTA vacutainer tubes. The blood samples were immediately put on ice and centrifuged for 10 minutes at 3500rpm. The plasma was then recovered and immediately frozen at -80°C.

The HBP concentration in BALF and plasma was measured using enzyme-linked immunosorbent assay (ELISA).

Calculate P/F ratio

All patients underwent Arterial blood gas Analysis before fiberoptic bronchoscopy. The P/F ratio was calculated as the ratio of the partial pressure of oxygen PaO2 (mmHg) to the fraction of inspired oxygen FiO2 (%).

2.3 statistical methods

SPSS 26.0 and graphpad prism 9 software were used for statistical analysis. The significance of differences between categorical variables was evaluated using Fisher’s exact test. Quantitative data use mean ± standard deviation (̅ ± s) indicates that independent sample t-test is used for comparison between groups. The person method was used for correlation analysis. The correlation between plasma and BALF HBP was analyzed by logistic regression. Probabilities of less than 0.05 were accepted as significant.

3 Results

Animal studies

Compare with sham group, CLP group mice show significant higher lung WD Ratio(4.72 ± 0.26 VS 6.81 ± 0.52, P = 0.002) and BALF protein 0.32 ± 0.05 VS 0.74 ± 0.08 , P < 0.001 and BALF HBP 50.75 ± 5.17 VS 545.81 ± 182.92, P = 0.013 and plasma HBP 33.75 ± 4.53 VS 93.00 ± 17.34, P = 0.003 from are also higher than sham group. (See Fig. 1)

Further analysis show lung injury index WD Ratio (r = 0.644, P = 0.007) and BALF protein(r = 0.644, P = 0.024) have significant correlation with plasma HBP in CLP group mice. There is also significant correlation between WD Ratio(r = 0.746, P = 0.005), BALF protein(r = 0.845, P = 0.001) and BALF HBP in CLP group mice. Plasma and BALF HBP also have significant correlation (r = 0.635 P = 0.026). (See Fig. 2)

3.2.1 Characteristics of the Study Population

At last, 44 ARDS and 38 cardiogenic pulmonary edema patients were enrolled in the study. The patients from two groups had similar age(mean age 60.5 versus 55.6 year) and gender(male 54.5% versus 52.4%) distributions.
The ARDS group exhibited 10 cases (22.7%) of abdominal infection, 6 cases (13.6%) of urinary infection, 24 cases (54.5%) of pulmonary infection, 4 cases (9.1%) of soft tissue infection, and 2 cases of blood transfusion. The CPE group was patients with cardiogenic pulmonary edema and hypoxic endotracheal intubation. (See Table 1)

### Table 1

<table>
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<th>CPE group</th>
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<tr>
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### 3.2.2 Comparison of two groups

We compared the levels of BALF HBP, plasma HBP, CRP, and lactate between the ARDS and CPE groups. Results show that there were significant differences in BALF HBP (t = 6.298, P < 0.001), BALF Protein (t = 5.846, P < 0.001) and Plasma HBP (t = 7.034, P < 0.001) between the two groups. (See Fig. 3.)

### 3.2.3 BALF HBP is related to the degree of lung injury in patients

P/F ratio was used as a severity index of lung injury. The results of this study indicate a significant correlation between BALF HBP (r = 0.415, P = 0.005) as well as plasma HBP (r = 0.346, P = 0.021) with the P/F ratio. BALF HBP also shows significant correlation with Plasma HBP (r = 0.563, P < 0.001) .(See Fig. 4)
4 Discussion

Although the mechanism of acute lung injury/acute respiratory distress syndrome has not been fully elucidated, most scholars currently believe that the syndrome is primarily caused by the systemic inflammatory response syndrome resulting from infection. When the body is stimulated by infection, the immune system responds by releasing a large number of inflammatory mediators that trigger neutrophil degranulation, release various proteolytic enzymes, and cause capillary endothelial cells to become more permeable to fluids and proteins. This damage to the pulmonary vascular endothelial cells and alveolar epithelial cells leads to the leakage of fluid and protein into the interstitial spaces and alveoli from the vascular lumen, resulting in ARDS. Neutrophil-dependent damage to the lung endothelial cells and epithelial barriers is considered the primary cause of ARDS.

Heparin-Binding Protein (HBP) is a cytokine that is released from activated immune cells and is known to cause inflammation and oxidative stress in the lungs. As the main ingredients of granules protein in PMNs, HBP plays an important role in the early stage of cascade inflammation pathologic process and vascular permeability [8]. Secreted from emigrating PMNs, HBP binds to endothelial glycocalyx and is presented to leukocytes in the blood stream [9]. HBP can activate monocytes rolling along the endothelium and ultimately induces stable monocyte arrest. Adhesion of monocytes is followed by transendothelial extravasation and directed migration to the site of injury. HBP could provoke a rapid rise in cytosolic free Ca2+ in adjacent endothelial cells, formation of actin stress fibers, and increased paracellular permeability [10, 11]. Immunoneutralization of HBP in neutrophil-derived secretion completely inhibits the activity, substantiating the critical role of this protein in neutrophil-evoked alterations in vascular permeability.

Vascular hypermeability is one the most important pathophysiological processes of ARDS, so there were lots of clinical studies focus on HBP and ARDS. HBP was believed be a potential mediator of sepsis-induced acute lung injury through enhanced endothelial permeability [12]. In a study about ARDS, compared with cardiogenic pulmonary edema patients, ARDS patients had significant higher plasma HBP levels. HBP was a strong prognostic marker for short-term mortality and was associated with severity of hypoxemia in ALI/ARDS patients [13]. In a transfusion-related acute lung injury study, after stimulated by human antibodies, PMNs in whole-blood cell released substantial amounts of HBP without the release of interleukin-6, tumor necrosis factor-alpha. So HBP was believed as one of the primary effector molecules of transfusion-related acute lung injury [14]. Our previous study showed early stage HBP was a good biomarker to predict the occurrence of sepsis related ARDS. Also in lung injury model animals, HBP level dramatically ascended and showed significant correlation with lung wet/dry ratio and BALF total proteins. As lung wet/dry ratio and BALF total proteins reflected the impaired of alveolar-capillary barrier that leads to leukocyte migration and influx of protein. Results also suggested that plasma HBP was positive correlated with lung injury severity.

Many studies have confirmed that HBP is associated with infection. Plasma levels of HBP appeared to be the most robust parameter for differentiating patients with severe sepsis and circulatory failure from
those with less severe infections[15]. There are few studies about HBP in body fluids other than plasma. A multicenter study in Sweden observed the diagnostic and predictive value of HBP in urinary tract infection. The results suggest HBP was a good diagnostic marker for UTI, with an area-under-curve value of 0.94. An elevated level of HBP in the urine is associated with UTI and may be a useful diagnostic marker in adult patients with a suspected UTI.[16]. Bronchoalveolar lavage fluid (BALF) is a fluid that is obtained from the lungs through a procedure called bronchoalveolar lavage. In theory, as a marker of pulmonary exudation due to vascular leakage, HBP level in BALF is strong link to the severity of lung injury. Previous studies on ARDS mainly focused on plasma HBP, which represent the systemic inflammatory mediators. There is no report on the level of HBP in alveolar lavage fluid directly from the lung. In this study, we investigated the BALF levels of HBP and analyzed its value in animal and patients with ALI/ARDS.

In animal study, we chose wet/dry ratio of lung and total protein levels in BALF as markers of the severity of lung injury. Both plasma HBP and BALF HBP levels are higher in animal models of lung injury than control. Result also show degree of injury was positively correlated with HALF HBP level. Clinical study shows there were higher BALF HBP and plasma HBP level in patients in ARDS group compared with cardiogenic pulmonary edema group. There is significant correlation between BALF HBP, plasma HBP and the severity of ARDS.

The results in ARDS patients suggest that balfhbp in ARDS patients is significantly higher than that in cardiogenic pulmonary edema caused by elevated hydrostatic pressure. The elevation of HBP in alveolar lavage fluid is consistent with that in plasma. This result is consistent with the pathological mechanism of pulmonary neutrophil aggregation and release of HBP leading to vascular leakage and the decline of pulmonary oxygen exchange capacity in the pathological process of ARDS. The level of HBP derived from the lungs is more closely related to lung injury.

Conclusions

In conclusion, our study suggests that both in animal or human, Both BALF HBP were significantly increased during lung injury, and were significantly correlated with the severity of lung injury. Compare with plasma HBP, BALF HBP was better correlated with lung injury. This study provide more evidence for the pathological value of HBP in ARDS and a theoretical basis for intervening HBP in the local of the lungs to reduce or prevent ARDS in the future.

Declarations

Ethics approval and consent to participate: The study protocol was approved by the ethics committee of East Hospital, Tongji University School of Medicine, in accordance with the Declaration of Helsinki.

Consent for publication: Written informed consent was obtained from all participating individuals. The informed consent of patients or their families was obtained before they were included in the study.
Availability of data and materials:

Animal data:  http://dx.doi.org/10.6084/m9.figshare.22663795

Patient data:  http://dx.doi.org/10.6084/m9.figshare.22663807

Competing interests: All authors declare no conflicts of interest.

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Authors' contributions: FC analyzed and interpreted the patient data regarding the hematological disease and the transplant. RH performed the histological examination of the kidney, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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Authors' information (optional): Not applicable

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Figures
Figure 1

Compare lung WD Ratio, BALF protein, BALF HBP and plasma HBP between sham and CLP group mice.

Figure 2

The correlation between BALF HBP, plasma HBP and P/F in CLP group mice.
Figure 3

Compared with the CPE group, there were significant differences in BALF HBP (P = 0.002), BALF Protein (P=0.01) and plasma HBP (P = 0.02) in ARDS group.

Figure 4

The correlation between BALF HBP, plasma HBP and P/F in ARDS patients.