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Unfractionated heparin attenuated histone-induced pulmonary endothelial glycocalyx injury through Ang/Tie2 pathway

Jia Yin[1], Yawen Chi[1], Danyan Liu[1], Xinghua Li[1] and Xu Li[1*]

Abstract

Purpose This study aimed to investigate the involvement of angiopoietin (Ang)/Tie2 pathway in mediating pulmonary endothelial glycocalyx injury in histone-induced acute lung injury in mice, and the protective mechanism of unfractionated heparin (UFH).

Methods Twenty-four male C57BL/6 mice (20–25 g), 8–12 weeks old, were randomly divided into control, histone, and histone + UFH groups. The histone (50 mg/kg) was administered via tail vein. UFH (400 U/kg) was administered 1 h after histone injection. The control group was administered by an equal amount of sterile saline solution. The lungs of all groups were harvested 4 h after the injection of histones or sterile saline.

Results UFH attenuated histone-induced lung histopathological changes and edema. UFH alleviated pulmonary endothelial injury and glycocalyx shedding by reducing histone-induced low expression of thrombomodulin (TM) and decreased lung syndecan-1 levels. UFH improved histone-induced low mRNA expression of TM, syndecan-1, Ang-1, Tie2 and high expression of heparinase (HPA), Ang-2.

Conclusion UFH may attenuate histone-induced lung injury and pulmonary endothelial glycocalyx degradation via the Ang/Tie2 pathway.

Keywords Histone, Glycocalyx, Unfractionated heparin, Angiopoietin

Introduction

Sepsis is a severe clinical syndrome that can lead to multiple organ dysfunction syndrome (MODS). There are 50 million cases of sepsis worldwide each year, with a mortality rate of up to 23%, placing a significant economic burden on society [1]. Acute respiratory distress syndrome (ARDS) represented 10.4% of intensive care unit (ICU) admissions, and the in-hospital mortality rate is approximately 40% [2]. Therefore, it is vital to explore the pathogenesis and potential therapeutic targets and strategies.

Histones are positively charged nucleoproteins composed of nucleosomes and DNA [3]. In sepsis, histones are released passively from dead cells or released actively as components of neutrophil extracellular traps (NETs) [4–6]. As a member of damage-associated molecular patterns (DAMPs), extracellular histones can mediate inflammatory reaction, coagulation activation and organ dysfunction in sepsis and ARDS [7, 8]. Increased levels of extracellular histones are associated with higher mortality in sepsis patients [9, 10]. Therefore, extracellular

*Correspondence:
Xu Li
vincentzh002@outlook.com
[1]Department of Critical Care Medicine, The First Affiliated Hospital, China Medical University, North Nanjing Street 155, Shenyang, Liaoning 110001, China

histones are regarded as significant mediators and therapeutic targets in sepsis [11].

The endothelial cells and the glycocalyx on their surface are the first barrier that separate blood and organs in sepsis [12]. The angiopoietin (Ang)/Tie2 pathway participates in the maintenance of endothelial homeostasis [13]. Ang-1 is an agonist of Tie2, which phosphorylates Tie2 and inhibits FOXO1 by activating Akt, thereby promoting endothelial cell stability [14]. Ang-2 acts as a Tie2 antagonist to counter the stabilizing effects of Ang-1. Recent studies have demonstrated that Ang-2 is a central regulator of coagulation, inflammatory responses, and endothelial dysfunction in sepsis [15]. Ang-2 levels were positively correlated with sepsis severity, especially within 24 h after sepsis [16]. Ang-2 levels can predict the prognosis of septic ARDS patients [17]. Therefore, it is possible to consider the Ang/Tie2 pathway as a valuable therapeutic target in sepsis.

Unfractionated heparin (UFH), a commonly used anticoagulant, also possesses various non-anticoagulant properties such as suppressing inflammation, regulating the immune response, and protecting the endothelial glycocalyx [18]. We have investigated the toxicity of histones and the protective effect of UFH from different aspects: Li et al. [19] explored the effect of histones on coagulation activation. Fu et al. [20] investigated the influence of histones on glycocalyx and the involvement of heparinase (HPA). However, the exact mechanism remains unclear. This study aimed to explore histone-mediated glycocalyx destruction and endothelial cell damage, focusing on the involvement of Ang/Tie2 signaling pathway.

Materials and methods

Animals

Male C57BL/6 mice ($n = 24$), 8-12weeks old, weighing 20–25 g from Changsheng Biotechnology Company (Liaoning, China). The mice were housed in a room with a standard 12-h light/dark cycle for at least 1 week, with free access to food and water during the experiment.

This study was performed in strict accordance with the Guide for Care and Use of Laboratory Animals to ensure animal welfare. All experimental procedures were performed under approval from the Laboratory Animal Ethical Review Committee of China Medical University (approval no.: CMU20231000).

Experimental protocol

Mice were randomized to the control, histone, and histone + UFH groups ($n = 8$ in each group). All the drugs were injected via the tail vein. All mice, except those in the control group, were injected with histones (Sigma H9250) at a dose of 50 mg/kg. Mice in the histone + UFH group were injected with UFH (Shanghai No. 1 Biochemistry & Pharmaceutical Co., China) at a dose of 400 U/kg 1 h after the histone administration. The mice in the control group were administered an equivalent amount of sterile saline. The dosage and injection forms of histones and UFH were based on preliminary experiments and previous studies [19].

All mice were anesthetized and sacrificed for lung tissues 4 h after injection with histone or sterile saline. The left lung lobes were paraffin-embedded for hematoxylin and eosin (HE) staining and immunohistochemistry (IHC). The tongue lobes were measured for wet weight/dry weight and lung water content. The right upper lobes were collected to detect the immunofluorescence intensity of CD31, syndecan-1, and thrombomodulin (TM). The remaining lobes were harvested to measure the mRNA expression of TM, syndecan-1, HPA, Tie2, Ang-1, and Ang-2.

Pulmonary histological evaluation

The lungs were immersed in 4% paraformaldehyde for at least 72 h, then embedded in paraffin after dehydration with gradient alcohol. Lung slides (3 μm) were stained with HE staining. Two pathologists assessed tissue injury using a blinded method based on a previous report [21].

The extent of pulmonary leakage

To assess the extent of pulmonary edema, we determined the wet/dry lung weight ratios [(wet weight– dry weight) / wet weight (%)] and water content of the lung tissues. After measuring the wet weight, we dried lung lobes in an oven at 70 °C for 72 h, and their dry weight was subsequently measured.

Immunohistochemistry staining

Paraffin-embedded lung slides were dewaxed and rehydrated and microwaves were used for antigen retrieval. The experimental protocol was based on the instructions provided in the IHC Kit (MXB Biotechnologies). Sections were incubated with rabbit anti-mouse-von Willebrand factor (vWF) (Servicebio, China), then stained with a DAB Kit (Servicebio, China). Two pathologists assessed staining results in a blinded manner.

Immunofluorescence

Immunofluorescence was used to determine the expression of syndecan-1 and TM in the endothelium. CD31 is an endothelial biomarker expressed on the luminal surface of the blood vessels. CD31 was used to label the endothelial cells. Fresh frozen lung sections (6 μm) were embedded in optimal cutting temperature compound and observed after double immunofluorescence staining. The sections were dried at room temperature for 90 min and washed with phosphate buffered saline (PBS) (Solarbio, China) three times. After blocking with serum for 1 h at 37 ℃, the sections were incubated with mouse

anti-mouse-CD31 (Servicebio, China) and rabbit anti-mouse-syndecan-1 (Servicebio, China) or mouse anti-mouse-TM (Servicebio, China) simultaneously. The slides were then stained with secondary antibody for 1 h at 37 ℃. The secondary antibodies were goat anti-mouse IgG Cy3 (Servicebio, China) and goat anti-rabbit IgG Alexa-488 (Servicebio, China). After washing with PBS, the sections were stained with DAPI (Servicebio, China) prior to measurement. Images were taken by Olympus BX53.

Quantitative realtime PCR

A universal RNA extraction kit (Accurate Biology, China) and reverse transcription kit (Accurate Biology, China) were used to extract total RNA from the lung tissues and transcribe the total RNA to cDNA, respectively. The levels of mRNA were detected by QuantStudio™ Real-Time PCR system (Thermo Fisher Scientifc, USA), the operation protocol was based on the instructions of the SYBR Green Premix Pro Taq™ HS qPCR Kit (Accurate Biology, China). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a reference gene. All primers were synthesized by Biomed Bioengineering Company (Beijing, China) (Table 1). Each sample was assessed at least three times. The $2^{-\Delta\Delta}$Ct value was used to analyze the results.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v8. All statistics are shown as the mean ± SD. Statistical significance between multiple group comparisons was evaluated using one-way analysis of variance (ANOVA) and Tukey's post hoc test. Differences were considered statistically significant at $P < 0.05$.

Table 1 Sequences of the primers

Name	Primer sequence
GAPDH	Forward:5`-GGTTGTCTCCTGCGACTTCA-3` Reverse:5`-TGGTCCAGGGTTTCTTACTCC-3`
HPA	Forward:5`-AACCTCCATAATGTCACCAA-3` Reverse:5`-GTCTGCTCATCCACCATC-3`
Tie2	Forward:5`-AATGGCTCCTTCATCCACTCAG-3` Reverse:5`-GCTTCACATCTCCGAACAATCA-3`
TM	Forward:5`-GGTCAGTGGTGCGGACTTTCA-3` Reverse:5`-GGTTCATTCGTGCTCCTATTGC-3`
Ang-1	Forward:5`-GAAGATGGAAGCCTGGATT-3` Reverse:5`-TACTGCCTCTGACTGGTTA-3`
Ang-2	Forward:5`-CAACAACTCGCTCCTTCA-3` Reverse:5`-CCACACTGCCATCTTCTC-3`
Syndecan-1	Forward:5`-CGGCACTTCTGTCATCAA-3` Reverse:5`-CCTTCGTCCTTCTTCTTCAT-3`

Ang: angiopoietin; GAPDH: glyceraldehyde-3-phosphate phosphate dehydrogenase; HPA: heparinase; TM: thrombomodulin

Results

UFH alleviates histone-induced pulmonary histological damage

The alveoli in the control group were intact without infiltration of erythrocytes or inflammatory cells (Fig. 1A). In the histone group, the alveolar walls were thickened and the structure was unclear, accompanied by alveolar hyperemia and edema and large amounts of inflammatory cells infiltration in the interstitial tissue, suggesting acute lung injury caused by histones ((Fig. 1B). In the histone + UFH group, the alveolar structure was intact, the alveolar wall was locally thickened, and the infiltration of inflammatory cells in the alveolar interstitium decreased, suggesting that UFH alleviated histone-induced acute lung injury (Fig. 1C). The lung injury score demonstrated that histone induced severe lung damage (Fig. 1D) and UFH ameliorated the histone-induced histopathological changes.

UFH reduced histone-induced lung leakage

As shown in the results (Fig. 2A and B), the degree of pulmonary edema increased obviously after histone administration ($P < 0.01$), and UFH treatment significantly ameliorated histone-induced increase in lung leakage ($P < 0.01$).

UFH alleviates histone-induced pulmonary endothelial injury

The expression of vWF was increased when the endothelial cells were damaged. IHC staining showed that the expression of vWF was increased in the histone group (Fig. 3B), and significantly decreased in the histone + UFH group (Fig. 3C).

TM inhibits thrombosis and is also a biomarker of endothelial cell injury. The expression of TM in endothelial cells decreased compared with the control group, which was reversed in the histone + UFH group (Fig. 4). CD31 is enriched at the endothelial intercellular junctions and is a specific biomarker of the endothelial cells. Double immunofluorescence staining for CD31 and TM confirmed the differences in the expression levels of endothelial cell biomarker between groups.

These results show that UFH effectively protected pulmonary endothelial cells from histone-induced injury in mice.

UFH alleviates histone-induced glycocalyx shedding

Immunofluorescence results showed that the fluorescence intensity of syndecan-1 in endothelial cells decreased remarkably in the histone group compared to that in normal cells, whereas UFH restored the exfoliated glycocalyx (Fig. 5). We also performed double immunofluorescence for CD31 and syndecan-1 to further confirm

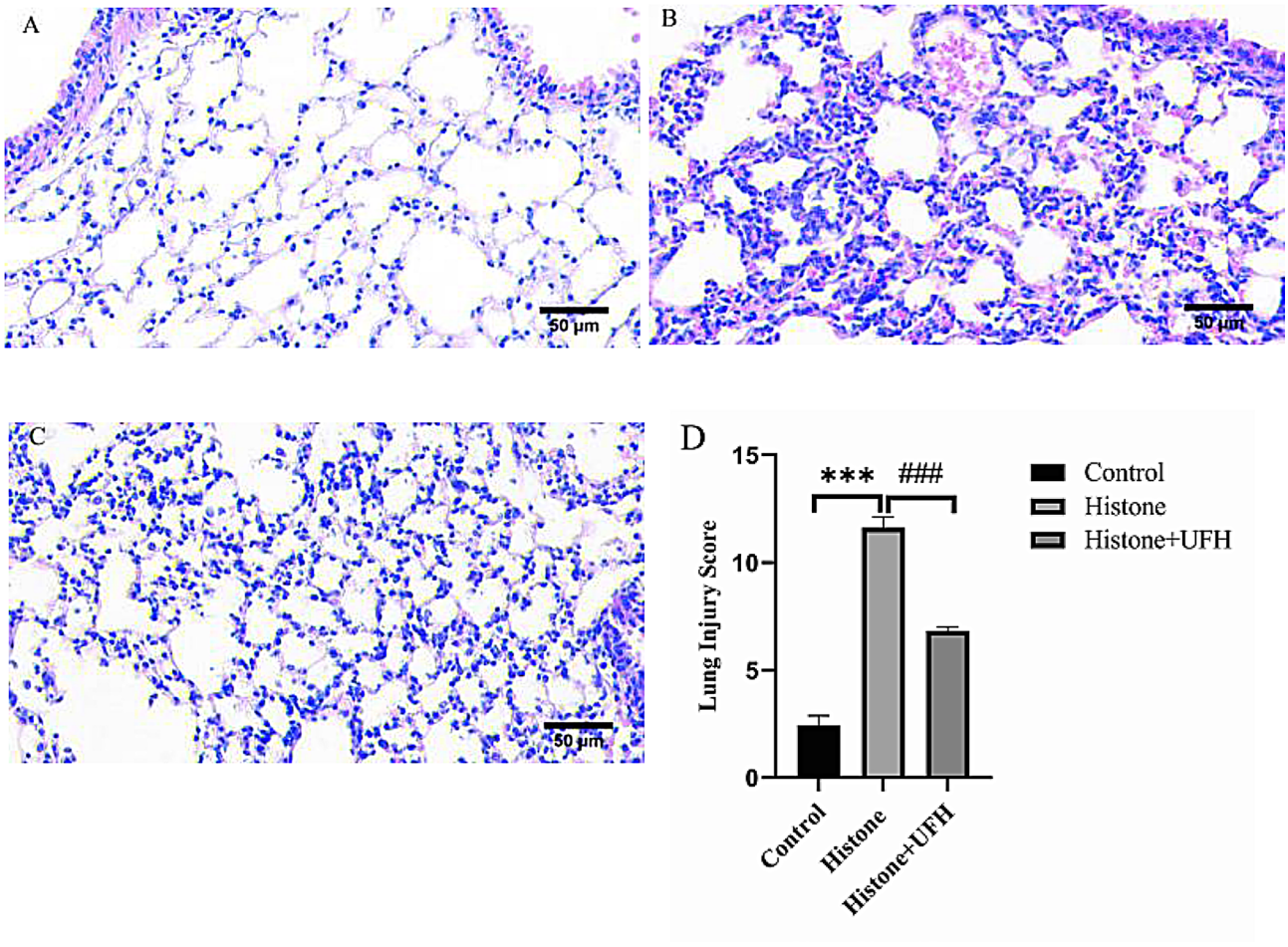

Fig. 1 HE staining of lung tissues in each group (200×, scale bar 50 μm). **A**: control group; **B**: histone group; **C**: histone + UFH group. **D**: Lung injury score in each group. Mean ± SD. $n = 8$. $^{***}P < 0.001$, compared with control group; $^{\#\#\#}P < 0.001$, compared with histone + UFH group

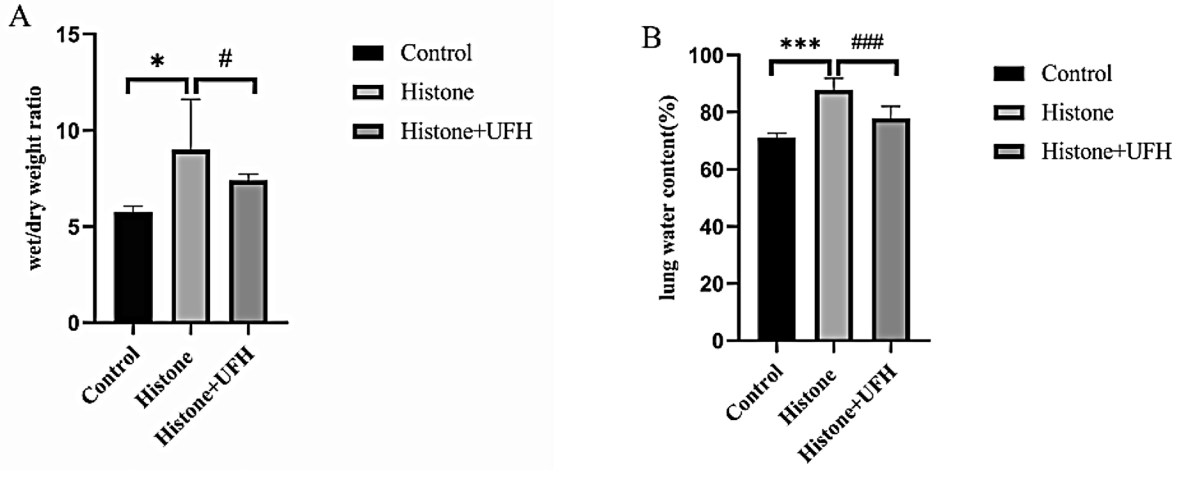

Fig. 2 The lung W/D ratios in each group (**A**). The pulmonary water contents in each group (**B**). Mean ± SD. $n = 8$. $^{*}P < 0.05$, $^{***}P < 0.001$, compared with control group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, compared with histone + UFH group

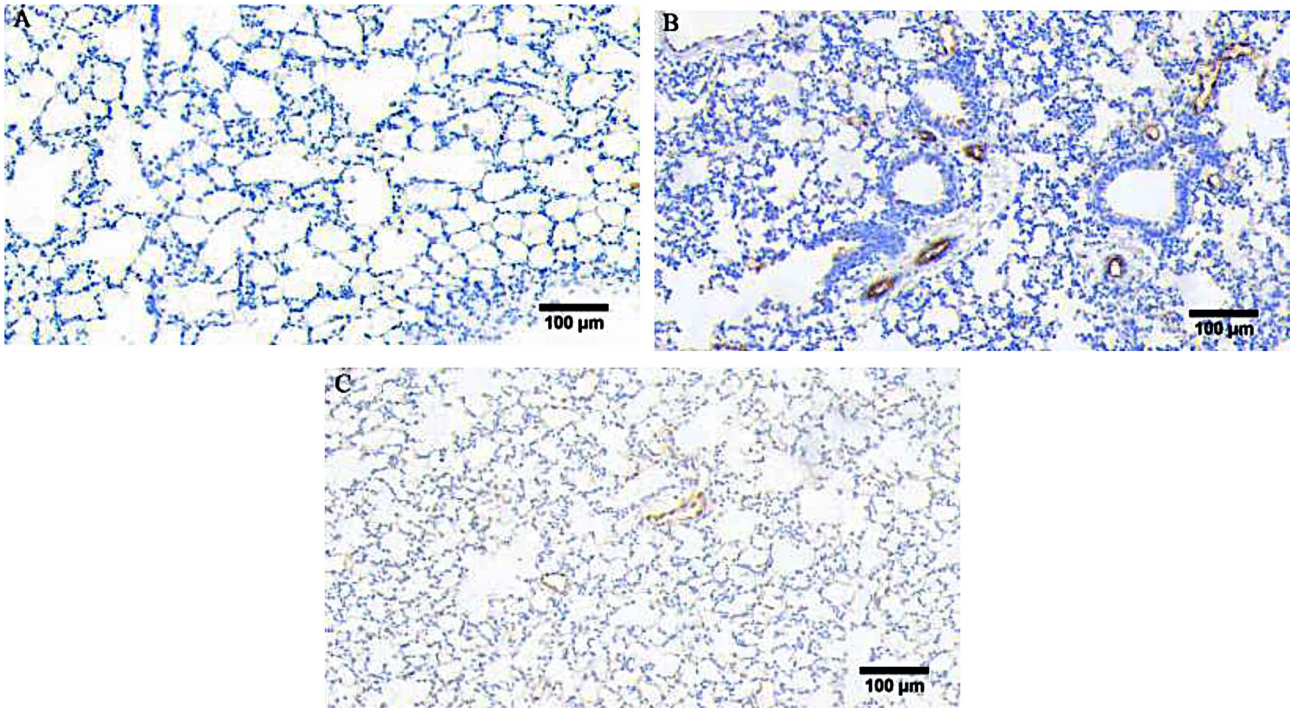

Fig. 3 UFH ameliorates histone-induced overexpression of vWF (IHC staining) (200×). **A**: control group; **B**: histone group; **C**: histone + UFH group. Scale bar = 100 μm

the occurrence and repair of endothelial glycocalyx injury.

UFH alleviates histone-induced low mRNA expression of syndecan-1, TM, Ang-1, Tie2 and high expression of HPA, Ang-2

To assess the extent of endothelial damage caused by histones, mRNA expression of syndecan-1, TM, and HPA was detected (Fig. 6A–C). To detect the involvement of the Ang/Tie2 pathway in histone-induced endothelial injury, the mRNA expression of Tie2, Ang-1, and Ang-2 was evaluated (Fig. 6D–F). The amplification efficiency of all primers reached 90-100%, the melting curve was unimodal and Tm > 80℃, so the primers were considered to be effective. The results revealed that histones significantly decreased the mRNA expression of syndecan-1 and TM (Fig. 6A and B) and enhanced the mRNA expression of HPA (Fig. 6C). Compared with the control group, the mRNA expressions of Tie2 and Ang-1 were decreased in the histone group (Fig. 6D and F), and Ang-2 mRNA expression was increased (Fig. 6E). The administration of UFH reversed the histone-induced changes. These data suggest that UFH ameliorated histone-induced endothelial glycocalyx injury and that the Ang/Tie2 pathway may participate in this process.

Discussion

In this study, the experimental results indicated that histones caused acute lung injury in mice, with endothelial glycocalyx disruption and increased endothelial permeability. Histones exerted toxic effects via the Ang/Tie2 pathway. UFH attenuated the effect of histones on the Ang/Tie2 pathway and ameliorated histone-induced glycocalyx shedding. To the best of our knowledge, this study is the first to demonstrate that the Ang/Tie2 pathway is associated with histone-induced acute lung injury and pulmonary endothelial glycocalyx degradation.

As a common clinical syndrome with high morbidity and mortality, sepsis is difficult to treat, and effective treatments are urgently needed. Histones are important mediators in the development of sepsis, and the levels of extracellular histones are positively correlated with the severity of sepsis [16]. Extracellular histones, as a type of DAMPs, exert toxic effects in the following ways: (1) binding to endothelial cells, causing increased cell permeability, calcium inflow, and endothelial cell damage [22]; (2) promoting inflammatory response through toll-like receptors (TLRs) signaling [23, 24]; (3) inducing oxidative stress and pyroptosis of endothelial cells [24]; (4) inducing the expression of adhesion molecules and the breakdown of intercellular adhesion junctions [25, 26]; (5) damaging endothelial glycocalyx [20]. In sepsis, histone-mediated inflammatory response, endothelial cell injury, glycocalyx disruption, and thrombosis collectively contribute to MODS and even death. Therefore, it may

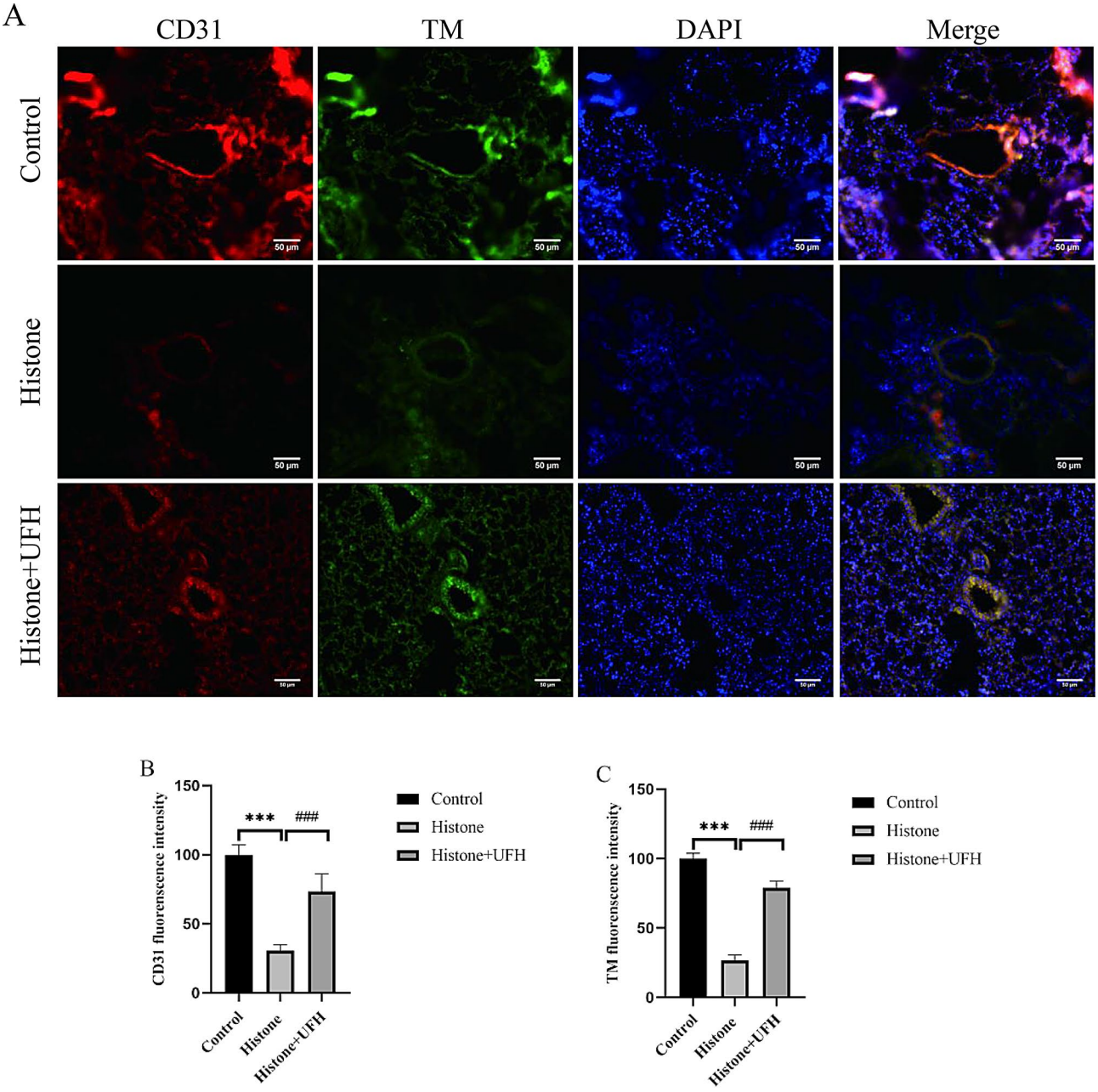

Fig. 4 The fluorescence intensity of CD31 and TM in each group (200×). Scale bar = 50 μm. Mean ± SD. $n = 8$. $^{***}P < 0.001$, compared with control group; $^{###}P < 0.001$, compared with histone + UFH group

be helpful to target and antagonize the toxic effects of histones. In our study, UFH attenuated histone-induced inflammation and endothelial glycocalyx shedding, which was consistent with the previous studies [19, 20]. This study provides a theoretical basis for the application of UFH against toxic effects of histones in sepsis.

Endothelial cells and the glycocalyx are the first barrier in sepsis. TM is an important component of the protein C system and is a biomarker of endothelial injury [27]. In sepsis, endothelial injury results in reduced TM expression and thrombosis, ultimately leading to organ dysfunction. The immunofluorescence intensity of TM decreased after histone administration, indicating that histones could lead to lung endothelial cell injury. Disruption of the glycocalyx leads to vascular barrier impairment and vascular leakage [28]. During glycocalyx shedding, endothelial cell adhesion molecules are exposed to plasma, triggering further inflammatory response, leukocyte rolling and adhesion, and platelets activation [29]. When the glycocalyx is disrupted, components such as heparan sulfate (HS) and syndecan-1 are released into the blood, and syndecan-1 is widely

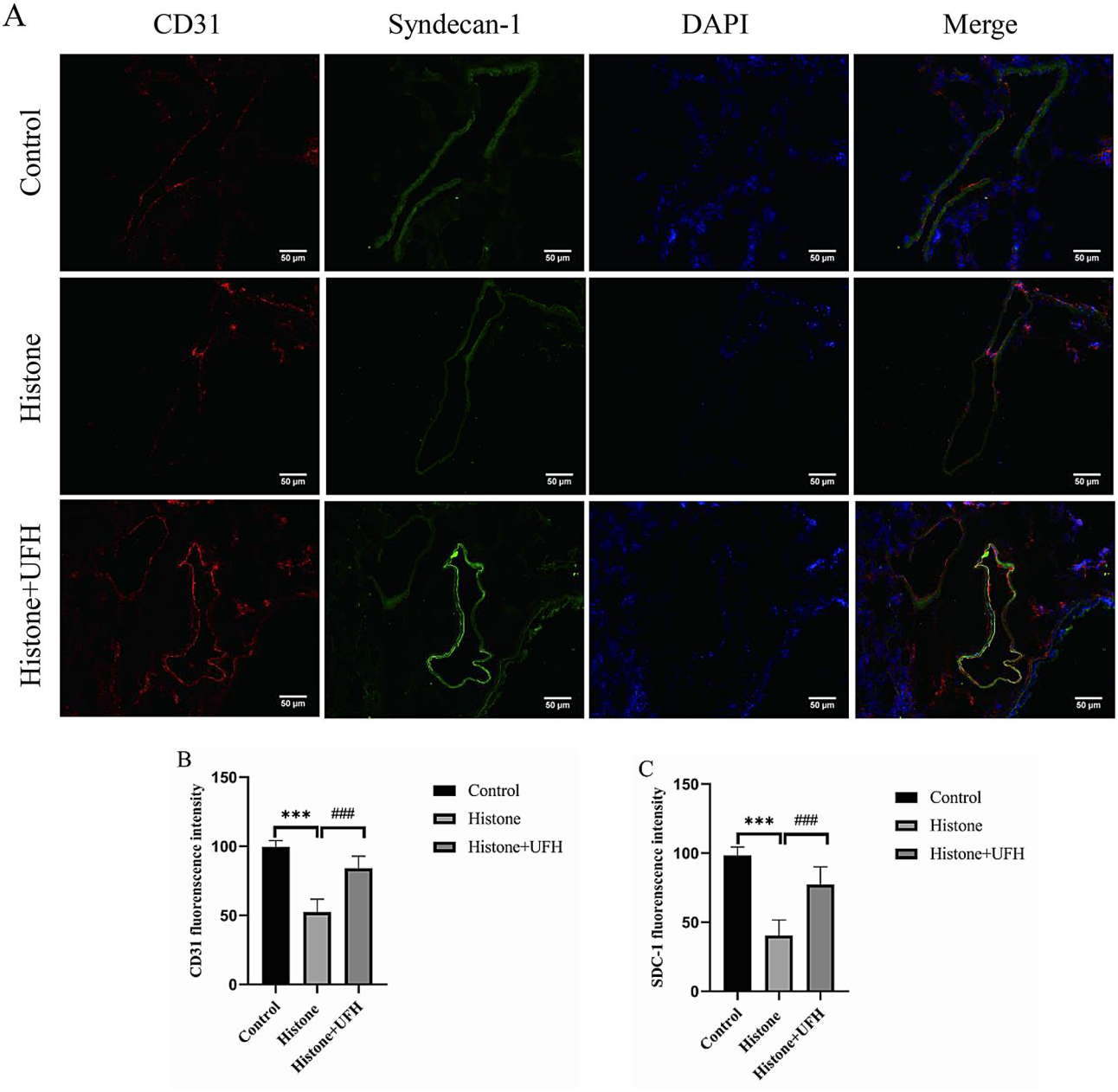

Fig. 5 The fluorescence intensity of CD31 and syndecan-1 in each group (200×). Scale bar = 50 μm. Mean ± SD. $n = 8$. $^{***}P < 0.001$, compared with control group; $^{###}P < 0.001$, compared with histone + UFH group

recognized as a marker of glycocalyx shedding [30]. Therefore, we measured the level of syndecan-1 in endothelial cells to assess the severity of glycocalyx disruption. Our results showed that histones induced a decrease in syndecan-1 levels in the lung tissues, suggesting the disruption of glycocalyx. Double immunofluorescence for CD31—a protein specifically expressed in endothelial cells, and syndecan-1 revealed that histones damaged the glycocalyx of pulmonary endothelial cells. The glycocalyx can be degraded by various proteases, such as HPA. Increased expression of HPA also promotes the expression of matrix metalloproteinases, which in turn directly cleave proteoglycans. Cross-talk exists between these degrading enzymes [31]. However, the exact signaling pathways involved in the glycocalyx degradation remains unclear.

Increasing attention has been devoted to the Ang/Tie2 pathway, because it has the potential to become a target for therapeutic intervention in sepsis. Our studies have shown that the Ang/Tie2 pathway plays an important role in the pathogenesis of sepsis and mediates the interaction of inflammation, coagulation and complement

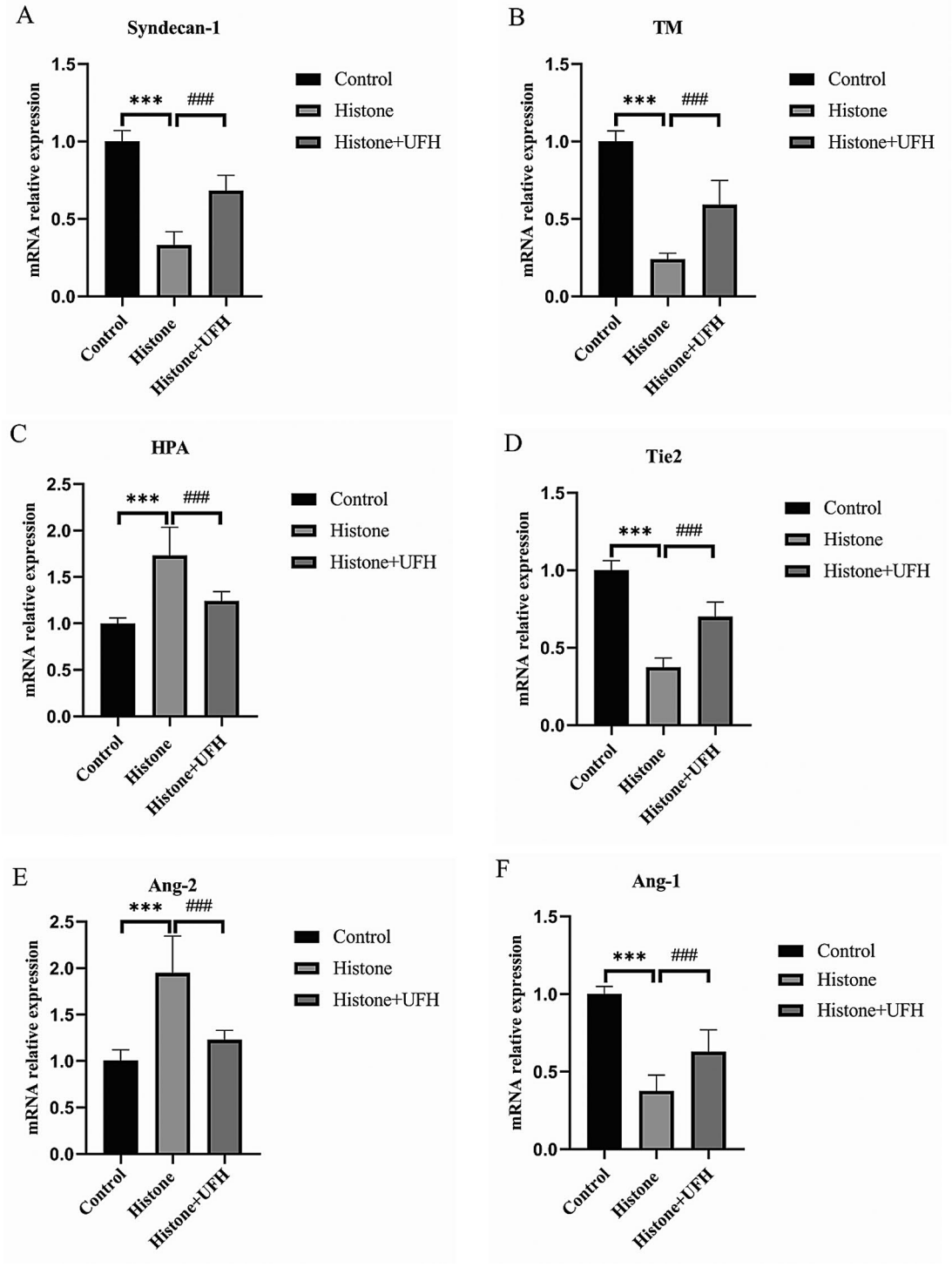

Fig. 6 The mRNA levels of syndecan-1, TM and HPA in each group (**A**, **B**, and **C**). The mRNA levels of Tie2, Ang-2 and Ang-1 in each group (**D**, **E**, and **F**). Mean ± SD. $n = 8$. $^{***}P < 0.001$, compared with control group; $^{###}P < 0.001$, compared with histone + UFH group

pathways in sepsis [15]. Ang-1 and Ang-2 regulate the quiescence and activation of endothelial cells through the same receptor, Tie2. Ang-1 is highly expressed, and Ang-2 is expressed at low levels in the physiological state to maintain the normal function of endothelial cells [32]. Under pathological conditions, the expression of Ang-2 increases, which antagonizes the action of Ang-1 through Tie2 receptors, leading to endothelial instability [33]. Clinical studies have suggested that circulating Tie2 levels are particularly important in predicting the prognosis

of septic ARDS patients, which may be related to high expression of Tie2 in the pulmonary vascular endothelium [34]. An increased Ang-2/Ang-1 ratio is correlated with a higher survival rate in septic ARDS patients [35]. In a cecal ligation and puncture (CLP)-induced acute lung injury mouse model, Ang-2 levels in the plasma increased, which was consistent with clinical condition [36]. Ang-2 promotes the release of HPA [37] and therefore plays a role in glycocalyx injury. In our study, we demonstrated that histones damaged endothelial cells and disrupted the glycocalyx, and that histones caused a decrease in Tie2 and Ang-1 mRNA expression and an increase in Ang-2 mRNA expression, suggesting that histones may cause acute lung injury and pulmonary endothelial glycocalyx shedding in mice through Ang/Tie2 pathway, thus verifying the previous hypothesis.

The Ang/Tie2 axis is closely related to endothelial cell injury and glycocalyx disruption. Regulation of the Ang/Tie2 signaling pathway, especially reducing Ang-1/Tie2 downregulation and endothelial glycocalyx injury, is helpful for early intervention to delay the progression of sepsis. Previous animal studies have confirmed that application of the Ang-2 monoclonal antibody can reduce the severity of lung injury and vascular leakage in mice with sepsis [36]. Intravenous application of Ang-1 analogs in CLP mice attenuated the renal inflammatory response and the expression of adhesion molecules [38]. In our experiments, histones caused changes in the expression of Ang/Tie2 pathway, which were relieved by UFH administration. Therefore, UFH may protect endothelial cells and the glycocalyx via the Ang/Tie2 pathway. Sepsis is a complex clinical syndrome involving inflammation, immunity, and coagulation. UFH has a variety of biological activities and has been demonstrated to exert protective effects in basic research, so it may become a new therapeutic strategy for sepsis. This study provides a new theoretical foundation for the use of UFH in the treatment of sepsis.

In our experiments, UFH was administered 1 h after histone administration, simulating clinical practice. UFH exerted a protective effect on maintaining the stability of the endothelial glycocalyx and endothelial function. There are some limitations to our study. First, our study was not designed to use a Tie2 agonist or Ang-2 antibody to determine the involvement of Ang/Tie2 pathway. This mechanism requires further investigation. Second, our experiments were designed to be conducted at only one time point (4 h) after histone injection, and multiple time points should be observed to explore the dynamic effects of histones and UFH on the endothelial glycocalyx in the future. Third, we only studied lung tissue. However, further research of other tissues and blood samples is warranted.

Conclusion

In summary, our study showed that UFH ameliorated histone-induced pulmonary injury and endothelial glycocalyx shedding through the Ang/Tie2 pathway, and that UFH might exert protective effects.

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Author contributions
J Yin: conceptualization, methodology, writing — original draft preparation. D Liu: methodology. Y Chi: formal analysis and investigation. X Li: formal analysis and investigation. Xu Li: conceptualization, writing — review and editing, funding acquisition. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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