The Protection Effect of Ulinastatin on Freshwater Instillation-Induced Acute Lung Injury in Rabbits

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Research

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Abstract

Background: Drowning is an important cause of accidental death in humans. The main cause of death following drowning is pulmonary oedema or lung injury, eventually leading to acute respiratory distress syndrome. The present study aimed to determine the protective effects of Ulinastatin on freshwater-induced acute drowning lung injury.

Methods: Rabbits were randomly divided into a control, freshwater, freshwater + small-dose Ulinastatin, freshwater + medium-dose Ulinastatin, freshwater + large-dose Ulinastatin group. The arterial blood gas analysis was performed before modelling (baseline) and at various time points after freshwater instillation. And then, the wet-to-dry weight ratio lung permeability index were measured to detect the effect of Ulinastatin on lung endothelial permeability. Furthermore, histopathological staining and ELISAs were used to analyse the histological changes and inflammatory cytokines expression resulted from lung injury, respectively. Western blotting and Quantitative real-time polymerase chain reaction were used to measure the protein and mRNA levels of Hypoxia inducible factor-lα (HIF-1α)/ Vascular endothelial growth factor (VEGF) in the lung tissues.

Results: By inhibiting the HIF-1α/VEGF pathway, treatment with Ulinastatin at a large dose could markedly attenuate changes in the PaO2/FiO2 (P/F), lung endothelial permeability, histopathology, and the expression of inflammatory cytokines induced by freshwater instillation.

Conclusion: Ulinastatin is a potential candidate treatment for freshwater drowning-induced acute lung injury that targets the HIF-1α/VEGF pathway.

Introduction

According to the World Health Organization, approximately 400,000 people die from drowning every year, making drowning an important cause of accidental death in humans and the second most common cause of unintentional injury among children [1]. When drowning occurs, acute asphyxia may be caused by reflex spasm of the larynx and trachea in the early stage, and in the late stage, the main cause of death is pulmonary oedema or lung injury following drowning, eventually leading to acute respiratory distress syndrome (ARDS). Approximately 99.87% of drowning victims die of pulmonary oedema after drowning [2]. Freshwater drowning often occurs in bathtubs, swimming pools, rivers and lakes [3]. Implementation of effective therapies may be limited by lack of recognition of instillation-induced acute drowning lung injury (ADLI) by clinicians, and there is no effective drug for it. Previous study in our group has shown that hydrogen gas inhalation attenuates seawater instillation-induced ADLI in rabbits and that the protective effects observed may be related to the activation of the Nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [4]. Additionally, the release of reactive oxygen species and increased cell apoptosis stimulated by seawater instillation were possible mechanisms of lung injury [5, 6].

Vascular endothelial growth factor (VEGF), a heparin-binding glycoprotein involved in the physiological process of vascular endothelial cells, can promote pulmonary development and the synthesis and

secretion of pulmonary surfactant, increase vascular permeability and potentiate angiogenesis [7]. Hypoxia is the main factor that increases the expression of VEGF [8, 9]. Hypoxia inducible factor-lα (HIF-1α) is an upstream regulatory factor of VEGF and can induce VEGF expression, which plays an important role in hypoxia [8]. The human urinary trypsin inhibitor Ulinastatin, a protease inhibitor extracted from human urine, is an endogenous anti-inflammatory substance with anti-inflammatory function that reduces oxidative stress injury. Previous studies have shown the efficacy of Ulinastatin in the treatment of acute pancreatitis and acute circulatory failure [10, 11]. Ulinastatin administration has also exhibited the effective in inhibiting excessive expression of proinflammatory cytokines in septic mice [12].

Experimental studies have shown that Ulinastatin can alleviate acute lung injury (ALI) induced by sepsis and oleic acid [8, 13]. In addition, Ulinastatin can relieve the inflammatory response after drowning lung injury, but the specific mechanism remains unclear [14]. In the present study, the protection effect and mechanism of Ulinastatin were investigated in freshwater-induced ADLI and found to be mediated by the HIF-1α/VEGF signalling pathway.

Materials And Methods

Animal modelling and grouping

Healthy New Zealand white rabbits weighing 2.18 ± 0.20 kg were purchased from the Animal Center of Second Military Medical University (SCXK 2012-0007, Shanghai, China). Rabbits were group-housed at 20–22 °C on a 12 h light–dark cycle with free access to a standard diet and water. Animals remained in the housing facilities for at least one week to acclimatize prior to initiation of the experimental protocols. Freshwater was collected from Dianshan Lake, Shanghai (the water quality essentially met the third-level national surface water quality standard, pH 6.5–8.5, chloride content lower than 0.01 mg/l).

Freshwater drowning animal modelling: The rabbits were anaesthetized by left ear vein injection of sodium pentobarbital (30 mg/kg, Sigma-Aldrich, St. Louis, MO, USA). A 14-F endotracheal tube (Smiths Medical International Ltd. Kent, UK) was inserted into the trachea, and the groin was cleanly shaven. A 23-gauge PE-50 polyethylene catheter (Becton, Dickinson and Company, NJ, USA) was inserted into the right femoral artery to measure arterial blood pressure and obtain blood samples. During the operation, an electric blanket was used to maintain the animal's anal temperature at 38 ± 0.1 °C. Following 15 min of stabilization, freshwater (18 ml/kg) was instilled into both lungs over 5 min through the endotracheal tube. After instillation, the rabbits were maintained in a supine position with the head elevated 30°. Arterial blood gas was measured after 15 min of freshwater instillation was complete. When the PaO2/FiO2 (P/F), which was calculated by the ratio of arterial partial pressure of oxygen (PaO2) to the fraction of inhaled oxygen (FiO2) (21%), had decreased to less than 300 and remained at this level for over 1 h, the freshwater instillation-induced ADLI model was successfully established.

Rabbits (n = 30) were randomly divided into five groups: i) control group (C, n = 6), no instillation; ii) freshwater group (F, n = 6), freshwater-induced ADLI; iii) small-dose (2.5×10^4 U/kg) Ulinastatin treatment

group (SU, n = 6), Ulinastatin (2.5×10^4 U/kg) administered intravenously immediately following successful establishment of freshwater-induced ADLI; iv) medium-dose (5×10^4 U/kg) Ulinastatin treatment group (MU, n = 6); v) large-dose (10×10^4 U/kg) Ulinastatin treatment group (LU, n = 6). The dose of Ulinastatin used was determined on the basis of a previous study [15].

Blood Gas Analysis

In each group, arterial blood gas analysis was performed before modelling (baseline) and at 0 h (T0), 0.5 h (T0.5), 1 h (T1), 3 h (T3), and 6 h (T6) after freshwater instillation. Then, PaO2 was measured with a blood gas analyser (GEM Premier 3000, Instrumentation Laboratory Company, USA).

Wet-to-dry Weight (w/d) Ratio

The W/D ratio was used to represent the severity of pulmonary oedema. At the end of the experiment, the left lower lungs were weighed and then dried to a constant weight at 80 °C for 72 h. The W/D ratio was finally calculated by dividing the wet weight by the dry weight.

Assessment Of The Lung Permeability Index (lpi)

At the end of the experiment, the rabbits were anaesthetized, and the left upper lungs of rabbits in all groups were lavaged twice with 10 ml of normal saline. The recovery rate of the lavage fluid was approximately 90%. The bronchoalveolar lavage fluid (BALF) was centrifuged at 3000 rpm for 10 min at 4 °C, after which the supernatant was collected. The total protein (TP) content in the lavage fluid and serum was detected by using an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Gen. Med. Corporation, Shanghai, China). The LPI was then calculate as follows [16]:

$LPI = TP_{\text{lavage fluid}}/TP_{\text{serum}}$

Histological And Pathological Injury Score

The Smith score was used to evaluate lung injury, including alveolar, bronchial, and vascular injury, characterized by inflammatory infiltration, alveolar wall rupture and fusion [17]. The total lung injury score (LIS) was the sum of the scores for the above items assigned as followed: score of 0, no injury; score of 1, injured area of < 25%; score of 2, injured area of 25%-50%; score of 3, injured area of 50%-75%; score of 4, injury observed in the full vision field. At the end of the experiments, the right lower lung tissues were extracted, fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. After deparaffinization and dehydration, the lungs were cut into 5 μm sections and stained with haematoxylin and eosin (HE). The sections were scored by observing 10 visual fields for each section from each animal at high magnification (200×), and the average of the total scores was recorded.

ELISA

The concentrations of tumour necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) in the BALF were detected by using ELISA kits (R&D Systems; Minneapolis, MN, USA). After freshwater instillation, the rabbits were anaesthetized, and the left upper lungs of rabbits in all groups were lavaged twice with 10 ml of normal saline. The recovery rate of the lavage fluid was approximately 90%. The lavage fluid was centrifuged at 3000 rpm for 10 min at 4 °C, after which the supernatant was collected and processed for ELISA according to the manufacturer's instructions.

Western Blotting

Western blotting
The lung tissues were lysed in RIPA lysis buffer (containing 1/100 PMSF, Solarbio, China). The lysates were centrifuged at 12,000 rpm for 30 min at 4 °C, after which the supernatants were collected. The protein concentration was determined using the bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific Inc., USA). TP extracts were separated on 8% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gels before being electro-transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, USA). After they had been blocked for 1 h with 5% milk in Tris-buffered saline with Tween-20 (TBST), the membranes were incubated with various primary antibodies overnight at 4 °C. The following day, the membranes were washed three times with TBST and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Cell Signalling Technology) for 1 h at room temperature. Reactive bands were visualized using an enhanced chemiluminescence kit (Millipore Corporation, USA), and image acquisition was performed using an Amersham Imager 600 (GE, USA). To ensure the loading of equal samples in each lane, membranes were stripped and re-probed with anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) antibody. Signal intensity was semi-quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA). The following primary antibodies were used according to the manufacturers' instructions: anti-HIF-1α (1:1000; Millipore, USA), anti-VEGF (1:1000; LSBio, USA), and anti-GAPDH (1:1000; Cell Signalling Technology, USA).

Quantitative Real-time Polymerase Chain Reaction (q-pcr)

Total RNA was extracted from 100 mg of right upper lung tissue with a TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was converted to cDNA using a cDNA synthesis kit (Bio-Rad). Reverse transcriptase-generated cDNAs encoding *VEGF*, *HIF-1a*, and *GAPDH* (used as a control for RNA integrity and internal standard) were amplified by Q-PCR. The sequences of the primers used for amplification were as follows: *VEGF*, (forward) 5'-CGGTTCCAGAAGGGAGAGGA-3' and (reverse) 5'-CTGGGACCACTTGGCATGG-3'; *HIF-1a*, (forward) 5'-AGAGTCAAGCCCAGAGTCAC-3' and (reverse) 5'-TGGGACTGTTAGGCTCAGGT-3'; and *GADPH*, (forward) 5'-GGGCTCTCTGCTCCTCCCTGT-3' and (reverse) 5'-ACGGCCAAATCCGTTCACACC-3'. Forty amplification

cycles consisting of 15 s of denaturation at 95℃, 30 s of annealing at 60℃, and 30 s of extension at 72℃ were performed. The relative mRNA expression of each target gene was determined by the 2 -ΔΔCT method.

Statistical analysis

All data are expressed as the mean ± SD or mean ± SEM. The statistical significance of differences was determined by one-way analysis of variance using GraphPad Prism (v.5.0.) (GraphPad Software, USA). A P value of less than 0.05 indicated significance.

Results

Effect of Ulinastatin on the PaO2/FiO2 (P/F) in rabbits with freshwater instillation-induced ADLI

The P/F at various time points after freshwater instillation was measured. As shown in Table 1, the P/F in F group decreased significantly over the first 3 h and was significantly lower than that of the C group ($P < 0.05$). However, there were no significant differences in P/F between the groups treated with Ulinastatin at various doses (SU, MU, LU) and the F group ($P > 0.05$).

Table 1
Effect of Ulinastatin on PaO2/FiO2 (P/F) in freshwater-instilled rabbits.

Groups (n = 6)	BL	T 0 h	T 0.5 h	T 1 h	T 3 h	T 6 h
C	484.92 ± 67.30	448.41 ± 65.60	506.35 ± 47.87	519.84 ± 68.37	542.86 ± 102.49	534.13 ± 133.18
F	389.68 ± 30.03	185.71 ± 50.12**	247.62 ± 61.28**	292.06 ± 62.50**	392.06 ± 17.73*	437.30 ± 78.27
SU	470.63 ± 57.57	192.06 ± 35.46	242.86 ± 46.27	296.03 ± 41.78	437.30 ± 72.12	492.06 ± 137.54
MU	411.11 ± 70.54	174.60 ± 42.77	264.29 ± 61.92	311.11 ± 72.82	411.90 ± 53.09	438.89 ± 32.63
LU	424.60 ± 45.91	193.65 ± 18.24	261.11 ± 28.95	315.08 ± 60.79	438.89 ± 58.51	440.48 ± 65.21

All data are representative of the mean ± standard deviation (SD) (n = 6). *$P < 0.05$, and **$P < 0.01$ versus control group (C). ADLI: acute drowning lung injury; C: Control group; F: Freshwater instillation group; SU: Freshwater instillation + Small doses (2.5×10^4 u/kg) Ulinastatin treatment group; MU: Freshwater instillation + Medium dose (5×10^4 u/kg) Ulinastatin treatment group; LU: Freshwater instillation + Large doses (10×10^4 u/kg) Ulinastatin treatment group; BL: Baseline.

Effect of Ulinastatin on lung endothelial permeability in rabbits with freshwater instillation-induced ADLI

The lung W/D ratio and LPI were measured to detect the effect of Ulinastatin on lung endothelial permeability (Fig. 1). The W/D ratio and LPI were significantly increased in the F group ($P < 0.05$). Large-dose Ulinastatin (10×10^4 U/kg) markedly attenuated the increases in the W/D ratio and LPI induced by freshwater instillation ($P < 0.05$).

Effect of Ulinastatin on the histopathological changes in rabbits with freshwater instillation-induced ADLI

Histopathological staining showed that freshwater instillation-induced pulmonary oedema, the infiltration of inflammatory cells, alveolar collapse, bronchiole epithelia desquamation, and haemorrhage. As shown in Fig. 2, the F group exhibited histological changes typical of lung injury, but the administration of large-dose Ulinastatin (10×10^4 U/kg) reduced these histopathological changes induced by freshwater instillation. The Smith score can be used to quantify lung injury caused by different interventions. The results showed that (Fig. 2) the F group had a higher Smith score than the C group ($P < 0.05$). Compared with that of the F group, the Smith scores in Large-dose Ulinastatin treatment group was significantly decreased ($P < 0.05$). Thus, these results indicate that large-dose Ulinastatin administration could significantly reduce the lung injury induced by freshwater instillation.

Effect of Ulinastatin on inflammatory responses in rabbits with freshwater instillation-induced ADLI

Inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-10, play important roles in the inflammatory response in the lungs. Therefore, we detected the TNF-α (Fig. 3A), IL-6 (Fig. 3B), IL-1β (Fig. 3C), and IL-10 (Fig. 3D) content in the BALF to study the inflammatory response in lung tissues. After freshwater instillation, the TNF-α, IL-1β, IL-6, and IL-10 content was significantly increased ($P < 0.05$), and large-dose Ulinastatin (10×10^4 U/kg) treatment markedly inhibited the expression of proinflammatory cytokines (TNF-α, IL-1β, IL-6, $P < 0.05$) but increased the expression of an anti-inflammatory cytokine (IL-10, $P < 0.05$).

Effect of Ulinastatin on the protein and mRNA levels of HIF-1α/VEGF in the lung tissues of rabbits with freshwater instillation-induced ADLI

As shown in Fig. 4, freshwater instillation increased both the protein and mRNA levels of *HIF-1α/VEGF* in the lung tissue of rabbits ($P < 0.05$), while the administration of large-dose Ulinastatin (10×10^4 U/kg) markedly attenuated the increases in the protein and mRNA levels of *HIF-1α/VEGF* induced by freshwater instillation ($P < 0.05$). Therefore, these results suggest that Ulinastatin attenuates inflammatory reactions and injury in the lung through inhibiting the *HIF-1α* and *VEGF* pathways.

Discussion

Drowning refers to the injury of humans or other animals result from submersion in water or another liquid medium. A common cause of accidental injury in humans, drowning has a high mortality. Some of the liquid medium can be absorbed into the blood circulation, which changes blood osmotic pressure and causes electrolyte disturbance and tissue damage. The most serious consequence of drowning is

respiratory and cardiac arrest. As the lung is the first organ to be damaged by drowning, it is key in the treatment of drowning [18, 19]. The most common liquid media involved in drowning are seawater and freshwater. Although the physical and chemical properties of seawater and freshwater are quite different, the mechanism of lung injury caused by seawater and freshwater can be summarized as follows: Drowning may cause lung inflammation and type II alveolar epithelial cell damage. This damage may result from perturbation of the physiological function of epithelial cells and the inhibition of surfactant synthesis and secretion [20]. Furthermore, this event provokes alterations in lung ventilation/perfusion ratios [21, 22], which may ultimately result in ALI/ARDS. An inflammation cascade is then caused by the release of large quantities of inflammatory cytokines and chemokines [23–26]. This abnormal hyperactive inflammatory response may promote and/or aggravate ALI/ARDS [19]. Following the inhalation of water, the change in osmotic pressure within alveoli can inflict cell damage and result in the loss of normal physiological function [27].

Lin and his colleagues showed that VEGF increased the permeability of endothelial cells by 50 percent in ARDS [28]. Further study found that the expression of VEGF and its receptor in lung tissues was significantly enhanced in an animal study of chest trauma combined with saltwater lung injury, and plasma VEGF levels were positively correlated with pulmonary vascular permeability [29]. Some studies have demonstrated that enhanced expression of VEGF can be induced by hypoxia [30]. Meanwhile, the instillation of a large amount of hyperosmotic fluid in the interstitial space induced tissue oedema and had a synergistic effect on the aggregation of inflammatory cells, which released inflammatory mediators, resulting in the further aggravation of tissue damage.

Additionally, further investigation revealed that high levels of secreted HIF-1α are positively correlated with VEGF reaction in the lung tissues of an animal model of drowning generated with freshwater, similar to the results of in vitro studies that reported HIF-1α expression in alveolar epithelial cells induced by freshwater damage. In addition, our research verified that the expression of both HIF-lα and VEGF is involved in the pathological process of freshwater drowning-induced ALI, and the finding that VEGF expression was affected by HIF-1α regulation helped to clarify its the pathogenic mechanism of drowning. Expression of the gene encoding HIF-1α, the core upstream regulator of VEGF, could restore stability to the intracellular environment under hypoxic conditions, and HIF-1α was found to regulate its transcription through binding various DNA effectors to advance hypoxic tolerance by promoting oxygen supplementation and reducing a cell's oxygen requirement, protecting the cell [31, 32]. Therefore, hypertonic freshwater drowning resulted in intense HIF-1α expression in lung tissues and increased pulmonary vascular permeability induced by VEGF upregulation. These results suggest that VEGF overexpression was regulated by HIF-1α, both of which are involved in the pathological process of ALI caused by drowning [33, 34], consistent with our research data.

Ulinastatin is a broad-spectrum protease inhibitor that can inhibit the activities of trypsin, chymotrypsin, neutrophil elastic protease, fibrinolytic enzymes and other proteases, thereby exerting a protease-related biological effect on damaged tissue [35]. In addition, it can stabilize the lysosomal membrane, improve microcirculation and tissue perfusion and remove oxygen free radicals [36]. In-depth studies focused on

the application of Ulinastatin in clinical practice discovered positive therapeutic efficacy of Ulinastatin in critical diseases [37]. In the present study, we found that large-dose Ulinastatin intervention alleviated freshwater drowning lung injury and simultaneously significantly reduced the mRNA and protein expression of HIF-1a and VEGF. In particular, Ulinastatin may have anti-inflammatory properties through reducing the associated activating inflammatory mediators (TNF-α, IL-1β, and IL-6) in the BALF and increasing IL-10, an anti-inflammatory cytokine. Likewise, the expression of HIF-1α/VEGF and Smith score (indicating lung injury) in the LU group was different from those of the MU and SU groups. Gao and her colleagues [31] also suggested that Ulinastatin reduced the plasma TNF-α and IL-6 content to achieve thoracic destruction mimicking uncontrolled haemorrhagic shock/recovery in a rat model of ALI. In addition, unlike previous studies, we found that large-dose Ulinastatin exhibited a positive effect that was manifested by the release of these inflammatory mediators. The dosages of Ulinastatin in large-dose Ulinastatin treatment group was 10×10^4 U/kg, which was determined according to a previous study [9]. Song *et al.* also illustrated the ultra-early protective effect of Ulinastatin of 100 KU/kg on rabbit acute lung injury induced by paraquat [38]. Besides, the reported study exhibited infusion of Ulinastatin of 25,000 U/kg were protected from LPS-induced lung injury in Rabbits [39].

Limitation

Although Ulinastatin could reduce the expression of inflammatory factors related to lung injury induced by freshwater drowning and alleviate lung tissue injury, improvements in the P/F were not observed. This may have been because these indicators are affected by many factors, and intravenous administration of Ulinastatin alone was not enough to improve these indicators. In addition, the observation duration in this experiment was short, and Ulinastatin was applied in a single dose, so observation of its sustainable effect was not possible. Furthermore, the experimental results were extrapolated to 6 h, so it was impossible to observe any possible changes at follow-up. Moreover, changes in the relevant experimental indicators were not observed dynamically, as only the results collected at the end-point of the experiment were compared. Moreover, although our results indicated that large dose of Ulinastatin prevents freshwater instillation-induced ADLI animal model by inhibiting the HIF-1α/VEGF expression, we did not explore if the treatment would be effective in the presence of HIF-1α/VEGF pathway downregulation. Future studies need to investigate the exact relationship between Ulinastatin and HIF-1α/VEGF pathway in freshwater instillation-induced ADLI.

Conclusion

The present animal experimental study confirms that large-dose Ulinastatin treatment could markedly attenuate changes in the lung endothelial permeability, histopathology, and expression of inflammatory cytokines induced by freshwater instillation by inhibiting the HIF-1α/VEGF pathway. This finding partially elucidates the mechanism of Ulinastatin in the treatment of freshwater drowning-induced ALI.

Abbreviations

ARDS: acute respiratory distress syndrome; ADLI: acute drowning lung injury; ALI: acute lung injury; VEGF: Vascular endothelial growth factor; HIF-1α: Hypoxia inducible factor-lα; PaO2: arterial partial pressure of oxygen; FiO2: fraction of inhaled oxygen; LPI: lung permeability index; TNF-α: tumour necrosis factor-α; IL-6: interleukin-6; IL-1β: interleukin-1β.

Declarations

Authors' contributions

SSX, ZFL, MYD designed the study. SSX, LH, HYW, YZ performed the experiments. Data were collated by LH and the results of data were discussed by HYW, YZ and WH. SSX prepared the figures. SSX and LH wrote the first draft of the manuscript. All authors have given their final approval for the version to be published and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

Competing interests

The authors declare no conflict of interest.

Availability of data and materials

The dataset supporting the conclusions of this article is included in the article.

Ethics approval

All experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Second Military Medical University (SCXK2012-0007) and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 8023, revised 1978).

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Figures

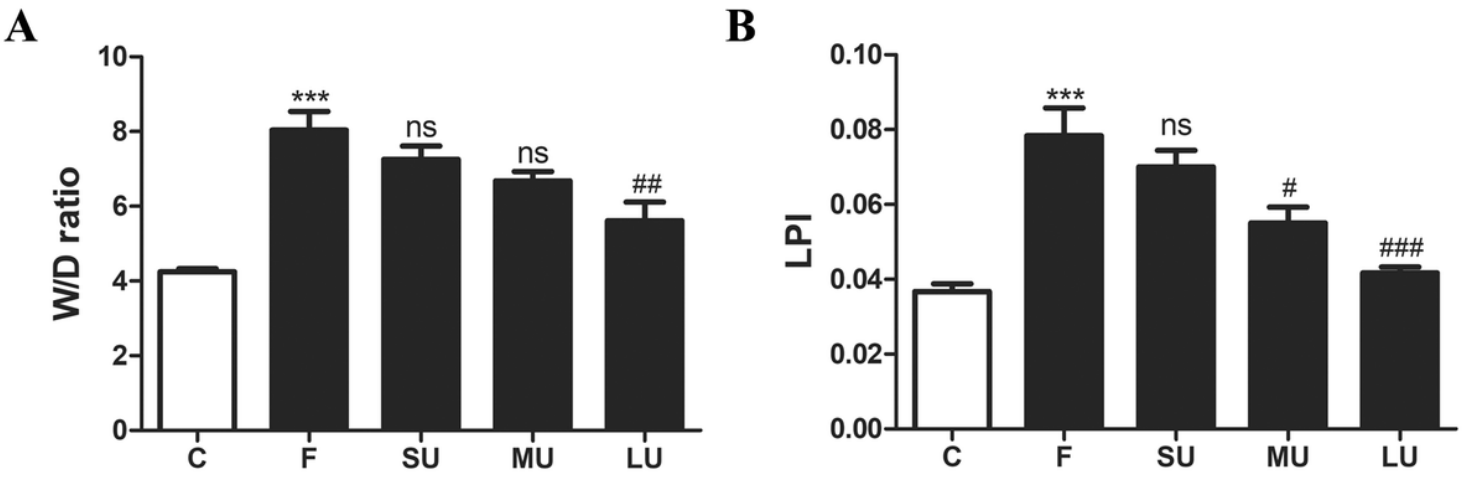

Figure 1

Effect of Ulinastatin on lung wet/dry weight ratios (A) and permeability index (B) in freshwater instillation-induced ADLI of rabbits. All data are representative of the mean ± standard deviation (SD) (n=6). ***P□0.001 versus control group (C); #P□0.05, ##P□0.01 and ###P□0.001 versus freshwater instillation group (F).

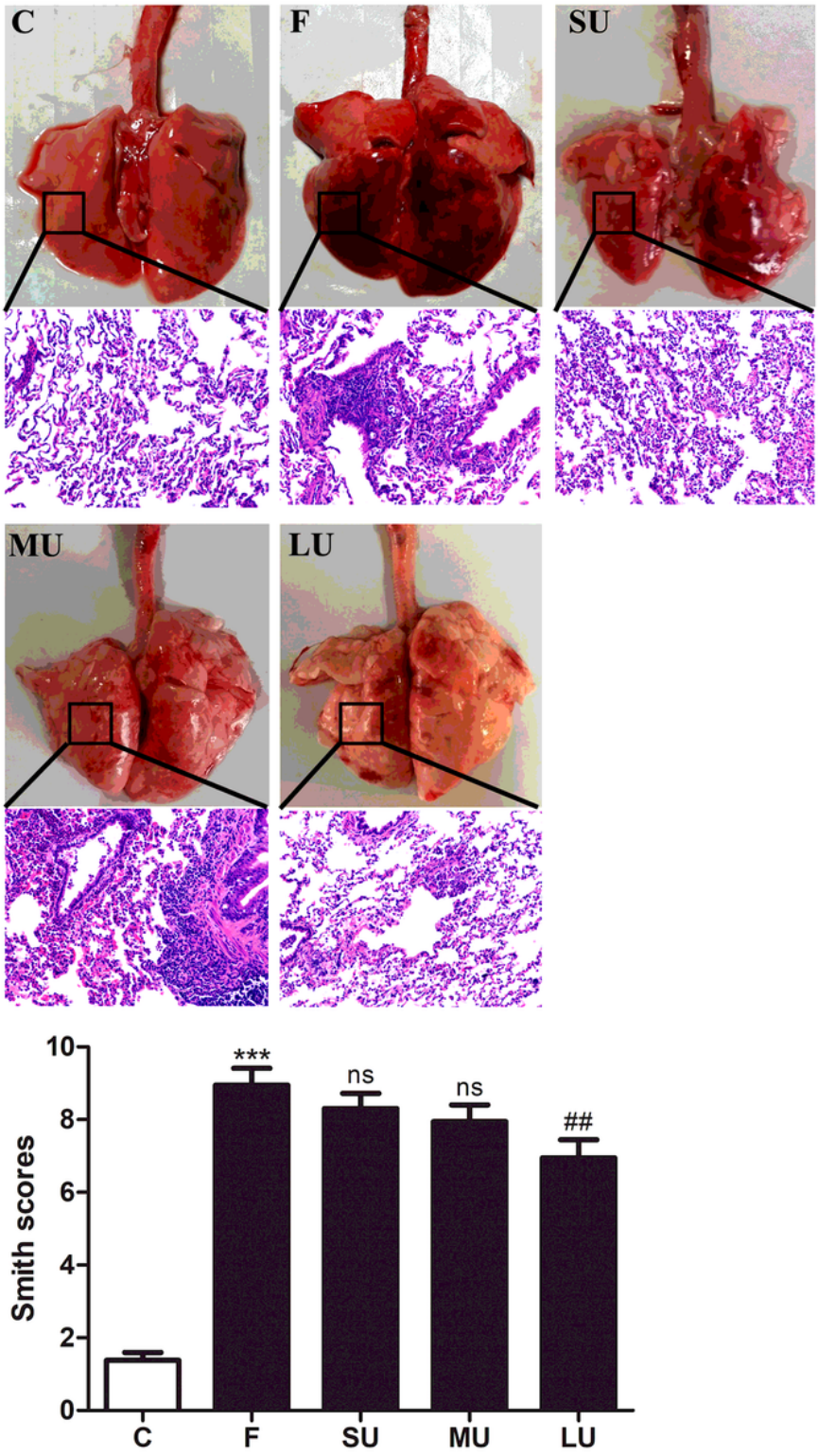

Figure 2

Ulinastatin administration reduce the lung injury histopathological changes induced by freshwater instillation. C: Control group; F: Freshwater instillation group; SU: Freshwater instillation + Small doses (2.5×104 U/kg) Ulinastatin treatment group; MU: Freshwater instillation + Medium dose (5×104 U/kg) treatment group; LU: Freshwater instillation + Large doses (10×104 U/kg) Ulinastatin treatment group. All

data of Smith scores are representative of the mean ± standard deviation (SD) (n=6). ***P0.001 versus control group (C); #P0.05, ##P0.01 and ###P0.001 versus freshwater instillation (F) group.

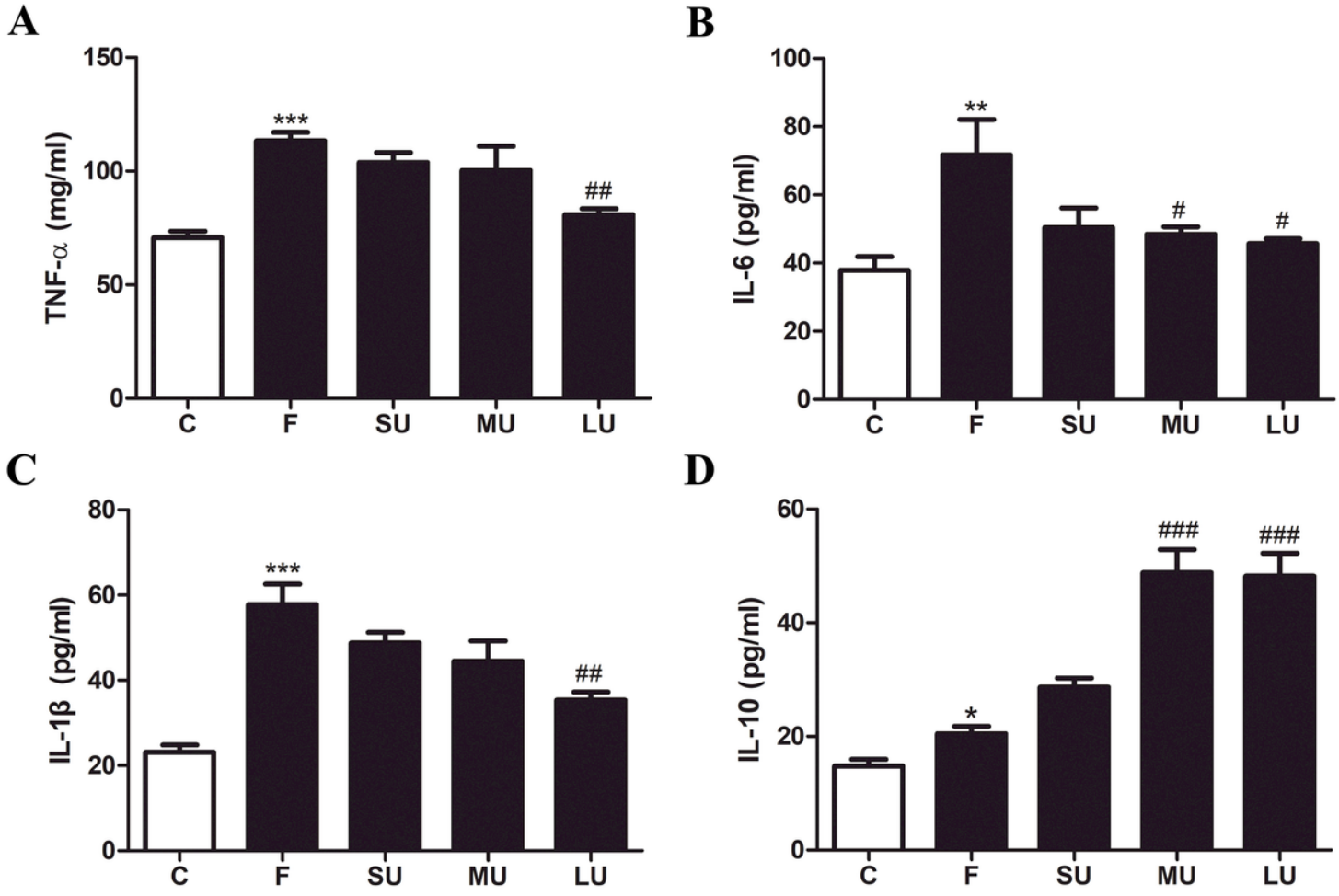

Figure 3

Effects of Ulinastatin on inflammatory cytokines in BALF after freshwater instillation. TNF-α (A), IL-6 (B), IL-1β (C), and IL-10 (D) content in BALFs was assessed by ELISA. C: Control group; F: Freshwater instillation group; SU: Freshwater instillation + Small doses (2.5×104 U/kg) Ulinastatin treatment group; MU: Freshwater instillation + Medium dose (5×104 U/kg) treatment group; LU: Freshwater instillation + Large doses (10×104 U/kg) Ulinastatin treatment group. All data are representative of the mean ± standard deviation (SD) (n=6). **P0.01, and ***P0.001 versus control group (C); #P0.05, ##P0.01 and ###P0.001 versus freshwater instillation (F) group.

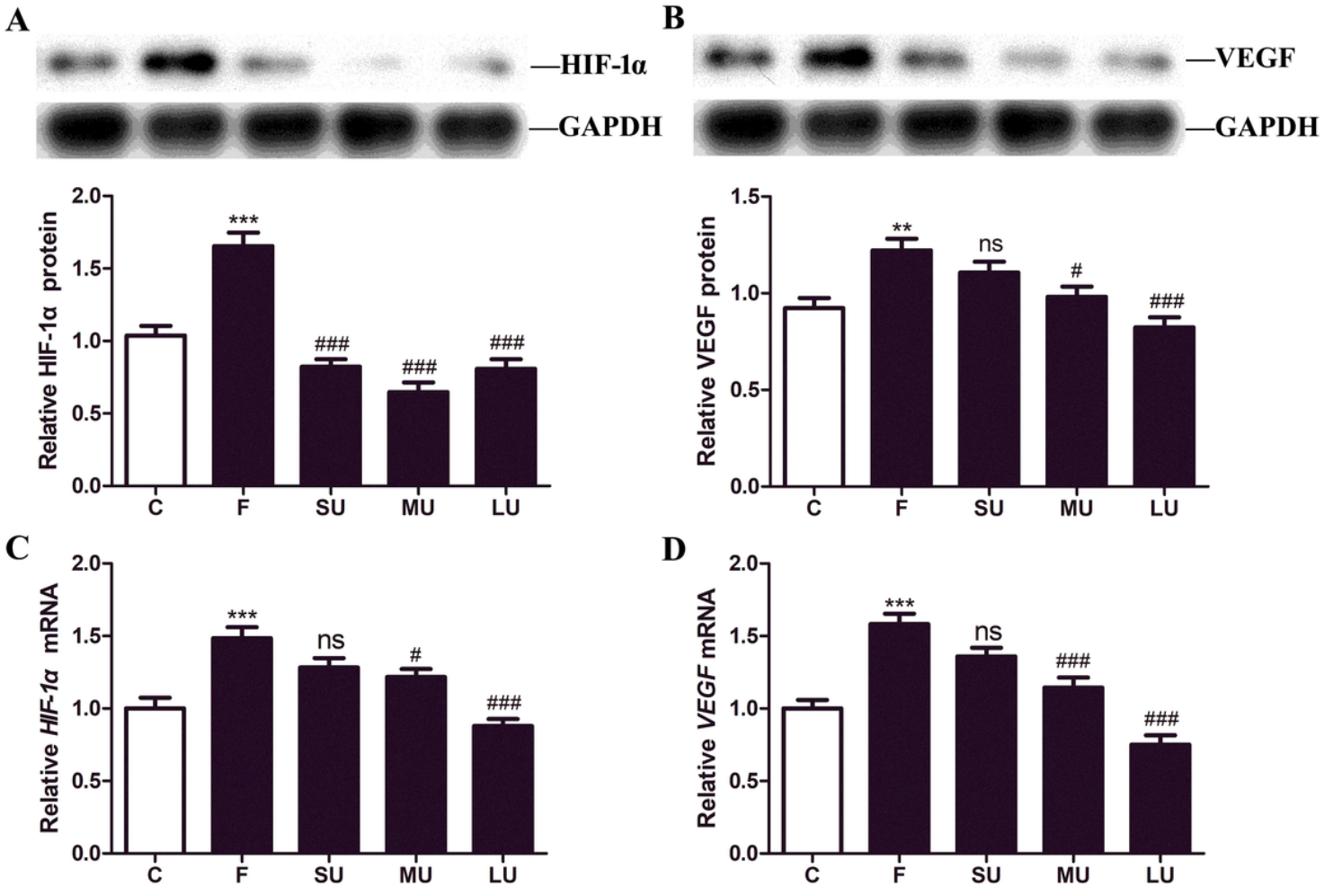

Figure 4

Effects of Ulinastatin on protein and mRNA levels of HIF-1α/VEGF in lungs. After instillation of freshwater, protein levels of HIF-1α (A) and VEGF (B) of lung tissue were detected by Western blotting, meanwhile the mRNA levels of HIF-1α (C) and VEGF (D) of lung tissue were detected by Q-PCR. Densitometric analysis of the blots was performed and results normalized to GAPDH (A, B). All data are representative of the mean ± standard deviation (SD) (n=6). **P□0.01 and ***P□0.001 versus control group; #P□0.05 and ###P□0.001 versus freshwater instillation group. C: Control group; F: Freshwater instillation group; SU: Freshwater instillation + Small doses (2.5×104 U/kg) Ulinastatin treatment group; MU: Freshwater instillation + Medium dose (5×104 U/kg) treatment group; LU: Freshwater instillation + Large doses (10×104 U/kg) Ulinastatin treatment group.