Original Article
Unfractionated heparin attenuates intestinal injury in mouse model of sepsis by inhibiting heparanase

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Abstract: Intestinal injury is a key feature in sepsis. Heparanase, a heparin sulfate-specific glucuronidase, mediates the onset of organ injury during early sepsis. Heparin has the function to attenuate inflammation and injury induced by multiple factors; however, whether unfractionated heparin (UFH) can attenuate the intestinal injury induced by sepsis as well as the underlying mechanism is still unknown. In the present study, the function of UFH in intestinal injury induced by sepsis was explored. Results of our study showed that after CLP operation, the inflammatory response and expression of heparanase were increased and NF-κB and MAPK P38 signaling pathways were activated. However, pretreatment with UFH will inhibit the expression and activation of heparanase, and reverse the activation of NF-κB and MAPK P38 signaling pathways, thus attenuating inflammatory responses induced by sepsis. These results suggest that UFH may be a promising therapeutic drug for intestinal injury caused by sepsis.

Keywords: Unfractionated heparin, heparanase, intestinal injury, sepsis, NF-κB, MAPK

Introduction

The mortality rate of patients with sepsis has remained high in spite of significant investigative efforts [1], which is partly because of an incomplete understanding of the underlying pathophysiology. Information suggests that the small intestine plays a central role in the pathophysiology of sepsis and it has been referred to as the "motor" of systemic inflammatory response syndrome (SIRS) [2]. Pathologic inflammation in the gastrointestinal tract and breakdown of the gut barrier function are key hallmarks in sepsis [3, 4]. Excessive inflammation may cause tissue damage and even organ failure. Failure to maintain intestinal epithelial barrier integrity could promote the translocation and invasion of bacteria and their toxins.

Heparanase is an endogenous glucuronidase capable of degrading both heparin sulfate (HS) and heparin glycosaminoglycan chains [5]. HS is ubiquitous polysaccharides found at the cell surface and in the extracellular matrix (ECM), playing important roles in ECM integrity and barrier function [6]. Heparanase was induced in several inflammatory condition, associated with degradation of HS, remodeling of the ECM, facilitation of inflammatory cells migration towards the injury sites and release of chemokines anchored within the ECM network and cell surfaces [7]. Sepsis is associated with activation of pulmonary heparanase, leading to degradation of the pulmonary endothelial glycocalyx with consequent endothelial dysfunction and inflammatory lung injury [8]. In chronic inflammatory bowel disease, such as ulcerative colitis, and Crohn's disease, heparanase was also up-regulated [9, 10].

Unfractionated heparin (UFH), as a potent anticoagulant, is a linear polysaccharide and composed of a polymer of alternating derivatives of D-glucosamine and uronic acid connected by glycosidic linkages, which is related to HS but has higher N- and O-sulfate contents [11]. Systematic reviews suggest that heparin may be beneficial in sepsis [12, 13]. Interestingly, besides being an effective anticoagulant, there is mounting evidence that heparin is also a potent modulator of inflammation. Heparin inhibits the expression and function of adhesion molecules, such as P-selectin and L-selectin [14, 15]. Moreover, heparin can

Table 1. Primers for quantitative real time PCR

Gene name	Forward primer	Reverse primer
TNF-α	TTCTACTGAACTTCGGGGTGAT	CACTTGGTGGTTTGCTACGA
IL-6	ACTTCCATCCAGTTGCCTTCTT	TCATTTCCACGATTTCCCAGA
IL-1β	TTTGAAGTTGACGGACCCC	ATCTCCACAGCCACAATGAGTG
heparanase	AAGCGTGAGTCCCTCGTTC	GGCTCAGACCTGCAAATATC
β-actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTCC

directly affect proinflammatory mediators, such as tumor necrosis factor-α (TNF-α) through the nitric oxide system [16]. Our previous report demonstrated that UFH attenuated coagulation and inflammation in endotoxemic mice [17]. However, whether UFH has a relieving function on intestinal injury caused by sepsis as well as the underlying mechanism is not yet clear. In the present study, effect of UFH on intestinal injury induced by sepsis was explored and the results indicate that UFH is a promising drug for sepsis intestinal injury treatment.

Materials and methods

Animals

6-8 week-old male C57BL/6 mice weighing 22-25 g were obtained from the Experimental Animal Centre of China Medical University (Shenyang, China). They were housed in plastic cages containing wood shaving and maintained in a room with a 12-h light cycle with free access to food and water. The animal study protocol was reviewed and approved by the Animal Experimental Committee of China Medical University.

Sepsis model

Mice were subject to cecal ligation and puncture (CLP) according to the method of Rittirsch *et al* [18], but with some changes. Mice were anesthetized with 10% chloral hydrate, and a 1 cm ventral midline abdominal incision was made aseptically through the skin and linea alba. The cecum was located, exteriorized, and ligated with 4 silk just distal to the ileocecal valve, and was then punctured with a 23G needle. The cecum was gently squeezed to extrude a small amount of stool and then replaced into the abdomen, which was then closed in layers. Sham mice were treated identically, except that the cecum was neither ligated nor punctured. Animals were humanely killed at 4 h and 24 h for functional studies.

Groups

Forty C57BL/6 mice were randomly divided into four groups (n = 10 mice per group): Sham, CLP, CLP + UFH and CLP + NAH. Mice in the CLP group were treated with CLP operation. Mice in the Sham group were externalized without ligation or puncture. Mice in the CLP + UFH group were intravenously injected with 8 U of UFH (Heparin sodium injection, Qianhong Biopharm, Changzhou, China) diluted in 200 µl sterile saline 0.5 h before and 12 h after CLP. Mice in the CLP + NAH group were subcutaneously injected with 150 µg NAH (ZZStandard, Shanghai, China) diluted in 200 µl sterile saline 2 h before CLP. 4 h and 24 h after CLP, five animals were sacrificed respectively and the blood samples were immediately centrifuged, then serum was stored at -80°C respectively. Intestinal tissue samples in each group were collected for histopathology examination, Immunohistochemistry, cytokines detection. Intestinal tissues were frozen in liquid nitrogen for Western blot and quantitative real time polymerase chain reaction (PCR).

Enzyme linked immunosorbent assay (ELISA)

Concentration of myeloperoxidase (MPO) in intestinal tissue was measured with MPO Detection Kit (JianchengBio, Nanjing, China); tumor necrosis factor-α (TNF-α), iInterleukin-6 (IL-6) and interleukin-1β (IL-1β) in serum were determined with corresponding ELISA kits (Boster, Wuhan, China) and heparanase in serum was determined with Heparanase Detection Kits (USCN, Wuhan, China) according to the manufacturer's instructions.

Histopathology examination

Intestinal tissues were fixed in 4% paraformaldehyde and embedded in paraffin. 5 µm thick sections were obtained for routine hematoxylin and eosin (H&E) staining and light microscopic examination.

Quantitative real time PCR (RT-PCR)

Total RNA was extracted from intestinal tissue using RNA simple Total RNA Kit (Tiangen, Beijing, China) according to the manufacturer's instruction. The concentration and purity of the RNA in each sample were determined using a
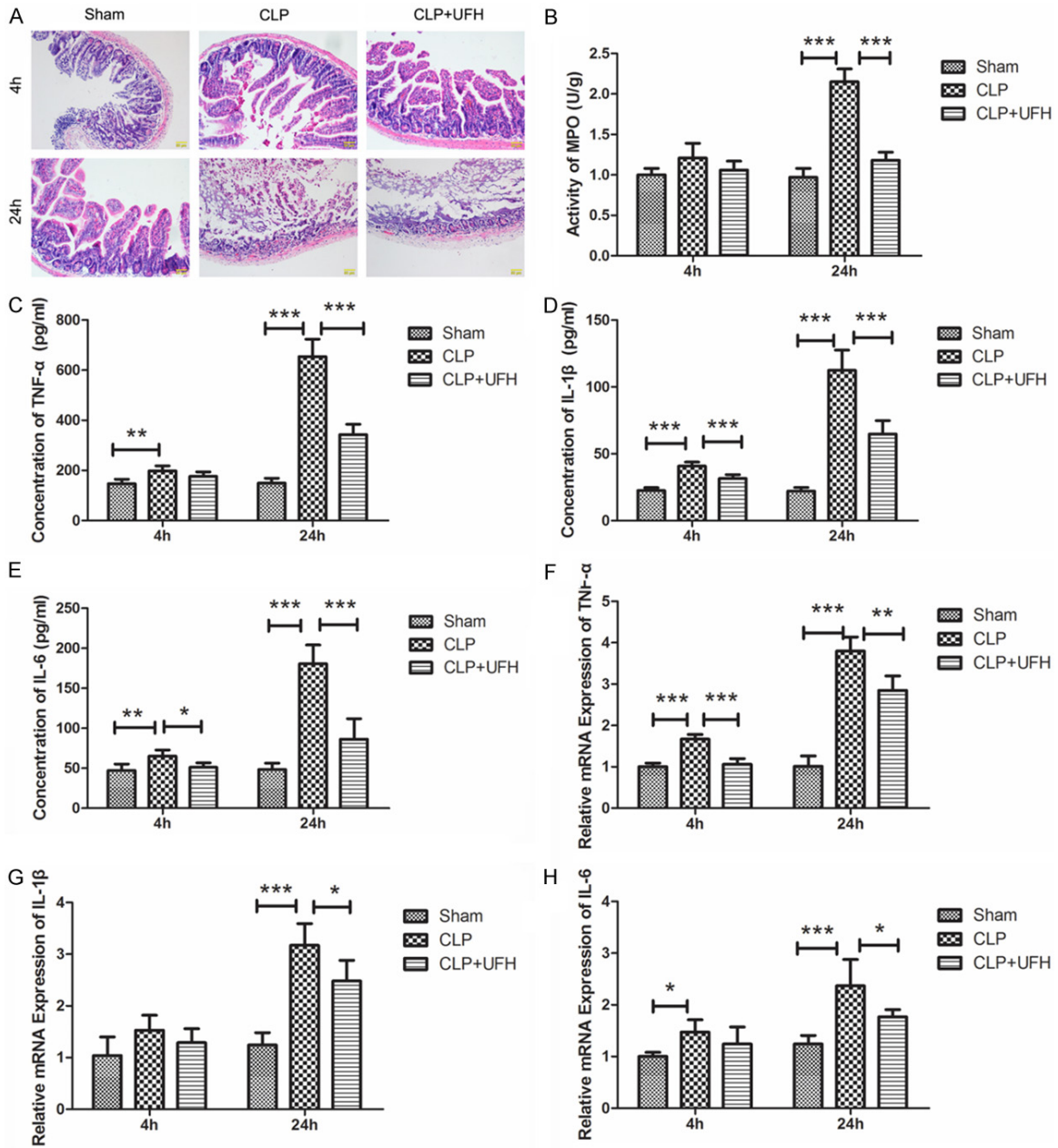
Figure 1. UFH attenuates intestinal injury induced by CLP. A. H&E staining for intestinal tissues of each group. B. MPO activity in intestinal tissues of each group. C-E. The concentrations of TNF-α, IL-1β and IL-6 in serum of each group were measured by ELISA. F-H. The relative mRNA levels of TNF-α, IL-1β and IL-6 in intestinal tissues of each group were detected by RT-PCR. The results were presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. Typical results are presented.

spectrophotometer. Reverse transcription was performed on 1 μg of total RNA from each sample using Super M-MLV (BioTeke, Beijing, China) and oligo(dT)$_{15}$. Then the mRNA levels of TNF-α, IL-6, IL-1β and heparanase were measured using RT-PCR (SYBR GREEN method) with cDNA as template and primers in **Table 1**. Mouse β-actin was served as an internal control. The relative mRNA level was calculated using $2^{-\Delta\Delta CT}$ method [19].

Western blotting

Protein in each sample was extracted using RIPA lysis buffer (Beyotime, Shanghai, China) or Nucleoprotein Extraction Kit (Beyotime). After measurement of protein concentration, equal amount of protein was loaded onto a sodium dodecyl sulfate polyacrylamide gel for electrophoresis after the concentration of total protein was adjusted. The separated protein was trans-
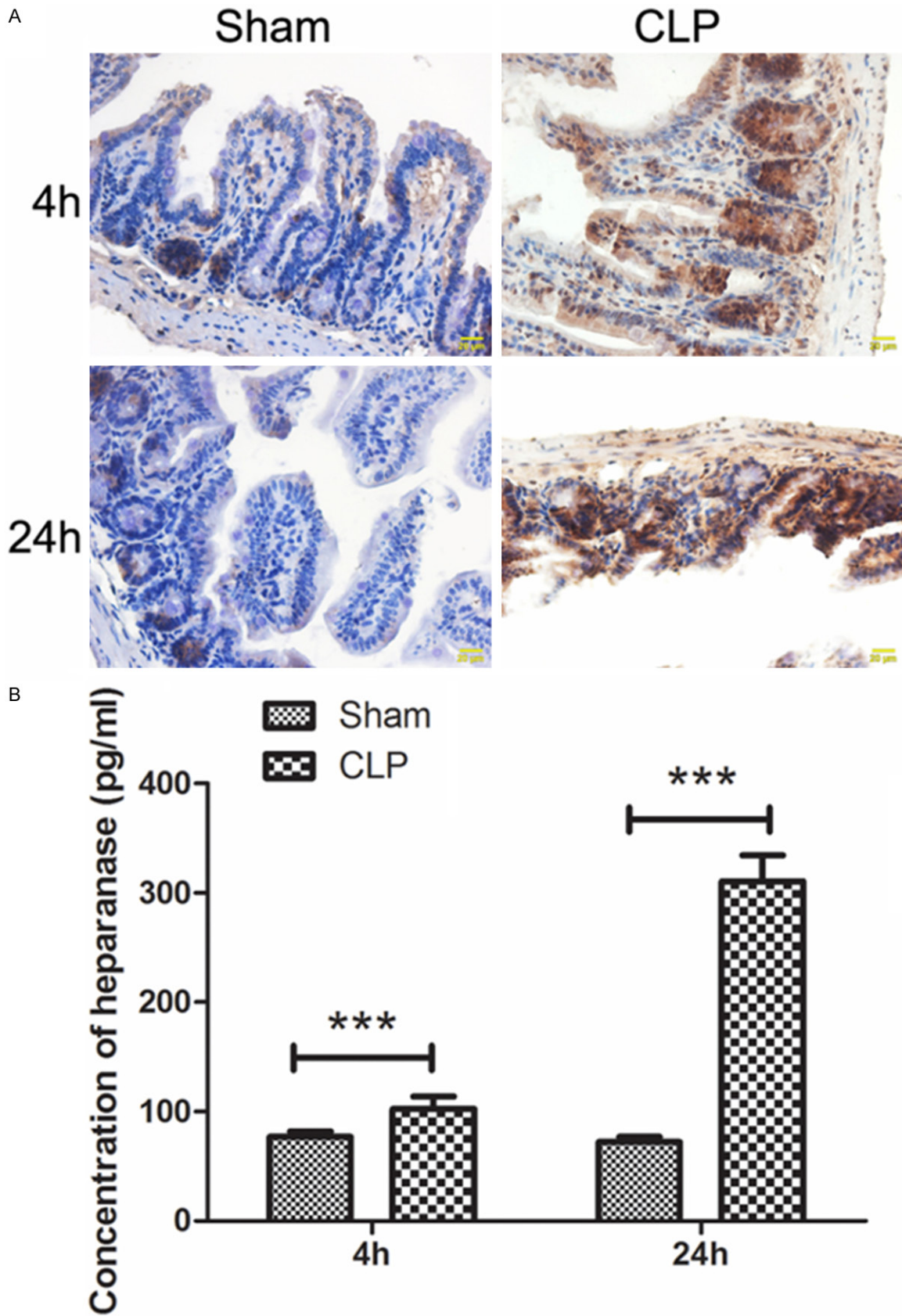
Figure 2. Heparanase is increased by CLP operation. A. The heparanase levels in intestinal tissues of each group were detected by immunohistochemistry. B. The concentration of heparanase in serum of each group was detected by ELISA. The results were presented as mean ± SD. ***$P < 0.001$. Typical results are presented.
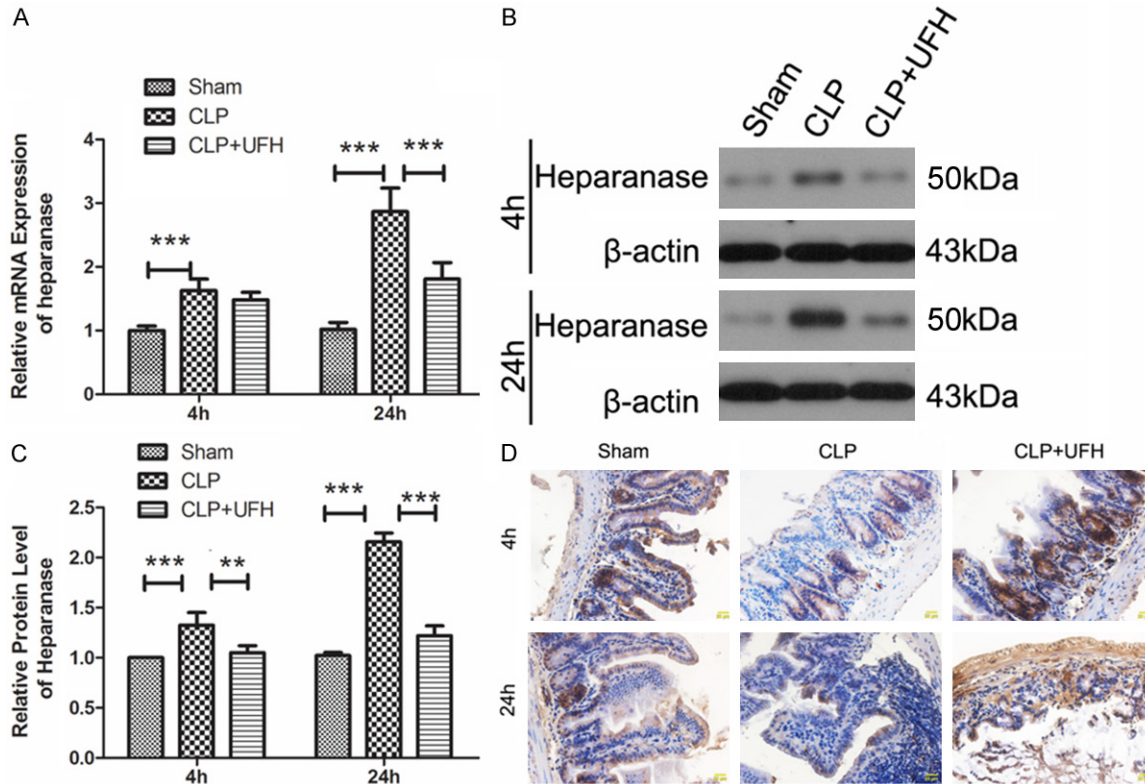
Figure 3. UFH inhibits the up-regulation of heparanase expression and activity induced by CLP. A. The mRNA level of heparanase in intestinal tissues of each group was detected by RT-PCR. The relative mRNA level was calculated using $2^{-\Delta\Delta CT}$ method. B, C. The protein level of heparanase in intestinal tissues of each group was measured by Western blot using β-actin as internal reference. D. The activity of heparanase in intestinal tissues of each group was evaluated by HS degradation using immunohistochemistry. The results were presented as mean ± SD. **$P < 0.01$, ***$P < 0.001$. Typical results are presented.

ferred to polyvinylidene fluoride (PVDF) membranes. After blockade, the membranes were incubated with primary antibody against heparanase (1:300, Bioss, Beijing, China), P38, p-P38, NF-κB P65, IκB, Lamin A, β-actin (1:1000, Wanleibio, Shenyang, China) overnight at 4°C. After washing with Tris buffered saline with 0.05% Tween-20 (TBST), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Beyotime) at 37°C for 45 min. The membranes were washed three times with TBST and detected using Enhanced ECL Detection System (Beyotime).

Immunohistochemistry

Intestinal tissues were fixed, processed for embedding in paraffin, and cut into 5 μm sections. The sections were dewaxed in xylene and hydrated, and antigen retrieval was done by sodium citrate treatment at 40°C for 10 min. After inactivation of endogenous peroxidase by 3% H_2O_2 and blockade with goat serum, the sections were incubated with primary antibody against heparanase (1:100, Bioss) and heparin sulfate (HS) (1:100, Boster) overnight at 4°C. After thoroughly washed with phosphate buffered saline (PBS), the corresponding secondary antibody (biotin labeled) and avidin (HRP labeled) were applied and incubated at room temperature for 30 min. Reaction products were visualized by incubation with 3,3'-diaminobenzidine (DAB) and then counterstained with hematoxylin.

Data analysis

The results were presented as means ± SD. Differences between groups were conducted using a one-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison. The processing of the data and figures was performed using Graphpad Prism 5.0 software. $P < 0.05$ was considered to be significant.
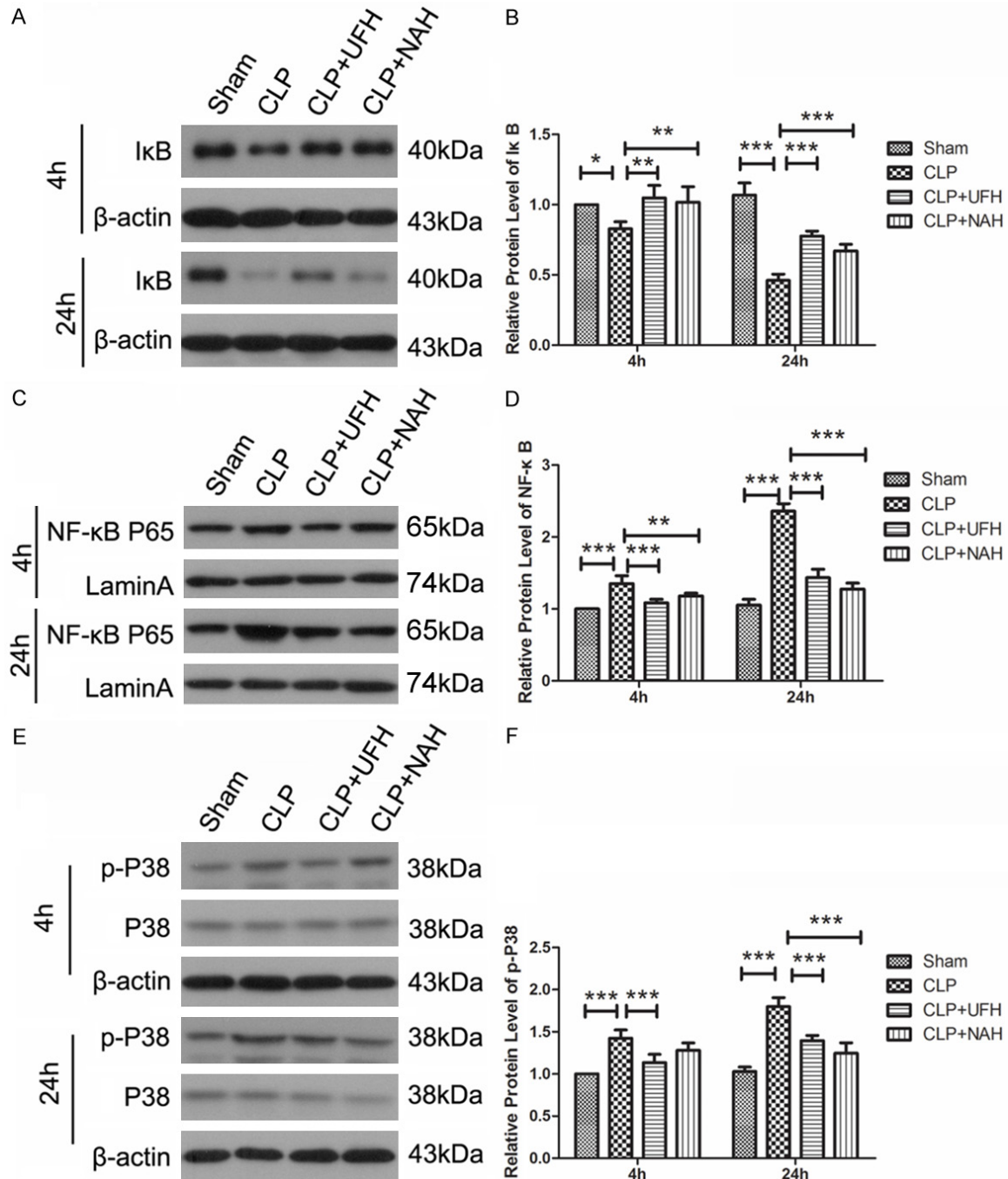
Figure 4. UFH inhibits the activation of NF-κB and MAPK P38 signaling pathways induced by CLP. A, B. The protein level of IκB was detected by Western blot with β-actin as internal reference. C, D. The protein level of NF-κB in nucleus was detected by Western blot. Lamin A was used as an internal reference. E, F. The protein levels of P38 and phosphorylated P38 (p-P38) were detected by Western blot with β-actin as internal reference. The results were presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Typical results are presented.

Results

UFH attenuated intestinal injury in mouse model of sepsis

Acute intestinal injury occurred early in sepsis. To explore the effect of UFH, a histopathology

examination was performed. As shown in **Figure 1A**, in the Sham group, the intestinal tissue showed a normal structure and epithelium under a light microscope. However, in the CLP group, intestinal tissue lost its normal structure, and widespread epithelium destruction

and inflammatory cell infiltration was discovered at 24 h after CLP. In CLP + UFH group, the histopathology changes of the intestine were attenuated compared to those in the CLP group. These results suggested that UFH could attenuate intestinal injury in mouse model of sepsis. To estimate the extent of neutrophil infiltration into the intestine, MPO in intestinal tissue was detected. After CLP operation, the concentration of MPO was increased significantly (**Figure 1B**, $P < 0.001$). Whereas, pretreated with UFH reversed the upheaval of MPO concentration. These results demonstrated that pretreatment with UFH attenuated intestinal injury in mouse model of sepsis.

To further validate whether UFH could attenuate intestinal injury in mouse model of sepsis, inflammatory cytokines, such as TNF-α, IL-6 and IL-1β, were detected by ELISA and RT-PCR. Consistent with the results of histopathology, after CLP operation, the expression levels of TNF-α, IL-6 and IL-1β in intestinal tissue and serum were both increased. However, pretreated with UFH, the expression levels of TNF-α, IL-6 and IL-1β in intestinal tissue and serum were both lower than that of CLP group (**Figure 1C-H**). These results further demonstrated that UFH could attenuate intestinal injury.

Heparanase was increased after CLP operation

Heparanase plays an important role in degradation of extracellular matrix and it was reported to be important to the formation of injury in multiple organs. The expression level of heparanase in intestinal tissue was detected by immunohistochemistry. As shown in **Figure 2A**, the expression of heparanase was increased within endothelium and epithelium of intestine after CLP operation comparing with the Sham group. Similar result was also discovered in serum heparanase level detected by ELISA: after CLP operation, the heparanase level in serum was also significantly increased (**Figure 2B**, $P < 0.001$). These results demonstrated that heparanase level, both in intestinal tissue and in serum, was increased after CLP operation.

UFH inhibited the up-regulation of heparanase expression and activity in mouse model of sepsis

The expression of heparanase in intestinal tissue was detected by RT-PCR and Western blot.

As shown in **Figure 3**, after CLP operation, heparanase expression level was increased both in mRNA level and in protein level. However, pretreated with UFH, the up-regulation of heparanase was inhibited (**Figure 3A-C**). As HS degradation is a marker of heparanase activity, the level of HS in intestinal was detected using immunohistochemistry. Coincident with the increased expression level of heparanase in intestinal after CLP operation, the HS level was decreased significantly after CLP. Whereas, pretreated with UFH, the level of HS in intestinal was increased compared with that of CLP group (**Figure 3D**), which indicated the inhibition of heparanase activity. These results demonstrated that pretreatment with UFH inhibited the up-regulation of heparanase expression and activity induced by CLP.

UFH inhibited the activation of NF-κB and MAPK P38 signaling pathways induced by CLP

The above results indicated that UFH may attenuate intestinal injury by inhibiting heparanase, so we introduced a commercial heparanase inhibitor N-desulfated re-N-acetylated heparin (NAH) which was similar to the hypothetical function of UFH. NF-κB signaling pathway and MAPK P38 signaling pathway have close relationships with inflammation, and the activation of NF-κB and MAPK P38 signaling pathway was detected in the present study. After CLP treatment, the protein level of IκB was decreased (**Figure 4A** and **4B**) and the level of NF-κB in nucleus was up-regulated (**Figure 4C** and **4D**), which indicated the activation of NF-κB signaling pathway. An increase in phosphorylation of P38 was also discovered (**Figure 4E** and **4F**) after CLP, which indicated the activation of MAPK P38 signaling pathway. However, pretreatment with UFH reversed the changes of IκB, nuclear NF-κB and phosphorylation level of P38. These results demonstrated that the activation of NF-κB and MAPK P38 signaling pathways induced by CLP was inhibited by pretreatment with UFH. Coincident results were also discovered in CLP + NAH group. This suggested that the function of UFH was similar to NAH and UFH attenuate CLP-induced intestinal injury by inhibiting heparanase.

Discussion

In the present study, we explored the effect of UFH on intestinal injury in mouse model of sepsis and found that UFH inhibited the expression

and activity of heparanase induced by CLP, inhibited the activation of NF-κB signaling pathway and MAPK P38 signaling pathway, thus attenuating the inflammation of intestines in mouse model of sepsis.

Heparin is primarily used as an anticoagulant. Recent studies showed that heparin also has an abundance of anti-inflammatory activities [12, 13, 20], and it is applied in burn injury adjuvant therapy [21, 22]. In our study, heparin was also found to attenuate inflammation of intestines induced by CLP and down-regulate the apophysis of inflammatory cytokines. Consistent with our result, heparin was found to prevent acute lung injury induced by sepsis in rats [12], inhibit lipopolysaccharide (LPS) induced inflammation [13] and attenuate intestinal dysfunction caused by ischemia and reperfusion [23].

In the present study, an increase in heparanase level in both intestinal tissues and serum was discovered after CLP operation. Heparanase is an endogenous glucuronidase capable of degrading both heparin sulfate and heparin glycosaminoglycan chains. Heparin sulfate plays important roles in the integrity of extracellular matrix and its barrier function [6], Heparin also regulates the activity of a serious of cytokines and growth factors and has multiple functions in inflammation and disease [5, 24, 25]. Heparanase mediates renal dysfunction during sepsis [26] and heparanase plays important roles in bowel diseases [10], so heparanase may also play important roles in intestinal injury during sepsis. If so, inhibiting of heparanase will be a promising therapeutic for intestinal injury of sepsis.

The up-regulation of heparanase induced by CLP operation was inhibited by UFH in the present study. These results prompt us to the hypothesis that UFH performed its function to attenuate intestinal injury of sepsis by inhibiting heparanase. NF-κB is an important transcription factor that modulates the generation pro-inflammatory cytokines. Previous studies indicate that LPS-induced NF-κB activation in lung tissue is associated with cytokines and chemokines production and microvascular epithelial permeability changes [13, 27]. NF-κB and MAPK P38 signaling pathways are important signaling pathways associated with heparanase function [28-30]. So we introduced a

heparanase inhibitor NAH and detected the influence of UFH and NAH on NF-κB and MAPK P38 signaling pathways. Results of our study showed that UFH inhibited the activation of NF-κB and MAPK P38 signaling pathways, which was in accordance with the effect of NAH. This indicates that UFH, acting as an inhibitor of heparanase, inhibited the expression and activity of heparanase, thus inhibiting that activation of NF-κB and MAPK P38 signaling pathways, attenuating intestinal injury induced by sepsis. However, how exactly UFH performed its function of heparanase inhibitor is still unclear and more explorations are needed.

In conclusion, the present study demonstrated that UFH functioned as an inhibitor of heparanase inhibiting the activation of NF-κB and MAPK P38 signaling pathways, thus attenuating intestinal injury induced by sepsis. The protective function of UFH suggests that UFH may be a promising therapeutic drug for intestinal injury caused by sepsis. This study laid theoretical foundation for the development of sepsis therapeutic, however, more explorations about the functions and mechanisms of UFH are still needed.

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Disclosure of conflict of interest

None.

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