ORIGINAL ARTICLE

Effect of unfractionated heparin on endothelial glycocalyx in a septic shock model

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The authors declare that there are no conflicts of interest.

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Background: The constituents of vascular endothelial glycocalyx, such as syndecan-1 and heparan sulphate (HS), can be detected in the plasma of patients and animals with septic shock. However, the dynamics of glycocalyx degradation and its association with inflammation remains largely unknown. In this study, we investigated the association between the biomarkers of acute endothelial glycocalyx degradation and inflammatory factors. We also evaluated the effect of unfractionated heparin (UFH) on glycocalyx shedding in a canine septic shock model.

Methods: Twenty adult beagle dogs were randomly allocated to one of the following four groups ($n = 5$): (1) a sham group; (2) a shock group [3.5×10^8 colony-forming unit (cfu) Escherichia coli (*E. coli*)/kg]; (3) a basic therapy group (sensitive antibiotics and 0.9% saline, 10 ml/kg/h); and (4) a heparin group (40 units/kg/h UFH plus basic therapy). After the onset of septic shock, systemic haemodynamic indices were measured. Endothelial glycocalyx degradation markers (i.e., syndecan-1, HS) and inflammatory factors [i.e., interleukin 6 (IL-6), tumour necrosis factor (TNF)-α], platelet count and activated partial thromboplastin time were measured at various time points.

Results: A lethal dose of *E. coli* induced a progressive septic shock model. We observed increased syndecan-1 and HS levels, which correlated with IL-6 and TNF-α in the septic shock model. The glycocalyx shedding was reduced by UFH, which might be regulated by the inhibition of inflammatory factors.

Conclusions: A therapeutic dose of UFH can protect glycocalyx from shedding by inhibiting inflammation. Additional studies with larger sample sizes are needed to confirm our conclusions.

Gram-negative bacillus septic shock is a common and high-mortality condition that is characterised by a systemic inflammatory response to infection, coagulation disorder and impaired vascular barrier function, which may ultimately result in extravasation of circulating cells.[1] Glycocalyx is a thin layer containing different components, such as anchor protein syndecan-1, hyaluronic acid, heparan sulphate (HS) and other glycosaminoglycans (GAGs), which serves as the first barrier of vascular endothelial surface layer (ESL) to cover the luminal surface of blood vessels.[2] The endothelial glycocalyx is a 0.2 to 1-μm thick layer that is negatively charged. It is an anti-adhesive and anticoagulant layer on the top of the endothelium that protects vascular endothelial cells and maintains vascular integrity.[3] Glycocalyx shedding has been observed in trauma and ischemia-

reperfusion models.[4,5] However, there are few studies of the dynamics of glycocalyx degradation and its relationship with inflammatory responses, particularly in a large animal model of septic shock induced by live bacteria.

Previous studies have shown that glycocalyx degradation involves the loss of HS and syndecan-1 because of the activation of endothelial heparanase by tumour necrosis factor-α (TNF-α)-dependent mechanisms.[6] We have previously observed that unfractionated heparin (UFH) alleviates sepsis-induced coagulation disorders and acute renal failure, with reduced mortality.[7,8] Furthermore, the Li et al.'s study showed that UFH could inhibit lipopolysaccharide-induced inflammatory responses by inhibiting Rho kinase and p-c-Scr kinase activity. Additionally, UFH blocked p38 mitogen-activated protein kinase and NF-κB activation in the endothelium in sepsis-associated acute lung failure.[9,10] Therefore, we hypothesise that UFH is a competitive antagonist of heparanase and prevents sepsis-induced glycocalyx loss by inhibiting inflammatory factors.

In the present study, we examined glycocalyx degradation markers at different time points in a novel *Escherichia coli* (*E. coli*) septic shock model. We also determined the correlation between glycocalyx degradation and several inflammatory factors. The second objective was to examine the protective effects of UFH on the endothelial glycocalyx in septic shock.

Materials and methods

Animals

Twenty adult beagle dogs (12 males and 8 females) weighing 13.31 ± 1.65 kg were purchased from the Laboratory Animal Centre of Dalian Medical University (Dalian, China) with the animal qualification number SCXX (Liao) 2008-0002. The experimental protocol conformed to the Guide for the Care and Use of Laboratory Animals, as promulgated by the Council of the American Physiologic Society (8th Edition, 2011). This study was approved by the Ethical Committee for Animal Research at China Medical University. The animals were transferred to the Animal Department of China Medical University and fed with standard dog food for a minimum of 10 days before beginning the study.

Tolerance and lethal dose study

Nine beagle dogs (5 males and 4 females) were used to study *E. coli* tolerance and to determine the lethal dose. These animals were also used to determine the responses to fluid and UFH. All dogs were sacrificed 12 h after being administered *E. coli* (serotype O79, 3.5×10^8 colony-forming unit [cfu]/kg, the lethal dose used in the subsequent studies). The dose range for fluid resuscitation and UFH were determined by measuring systemic haemodynamic indices [e.g., mean artery pressure (MAP), cardiac index (CI), urine output (UO) and oxygenation index (PaO_2/FiO_2)] and pathological changes (e.g., lung, liver, kidney, data not shown).

Animal procedures

The animals were fasted overnight prior to the study, but had free access to water. The animals were anaesthetised by administering intraperitoneally pentobarbital (25 mg/kg). The intravascular catheters were placed into the femoral artery and external jugular vein. Sedation and analgesia were maintained by continuous intravenous infusion of pentobarbital (5–6 mg/kg/h) combined with fentanyl (0.3–0.4 g/kg/h) through the jugular vein. All dogs were orally intubated (7.5#) for mechanical ventilation (Servo ventilator 900C, model: PC, Pi: 12–16cmH_2O, RR: 12–14bpm, FiO_2:23–25%, Siemens-Elema, Munchen, Bavaria, Germany) to maintain pulse oxygen saturation > 95%, partial pressure of oxygen > 80 mmHg and partial pressure of carbon dioxide at 28–35 mmHg. A pulse indicator continuous cardiac output (PICCO) catheter (Piccoplus Pc8100, PULSION Medical System AG, Munich, Bavaria, Germany) was used for MAP, CI, stroke volume index, systemic vascular resistance index, maximal *intraventricular* developed pressure (dpmax) measurements. Blood was collected from the femoral artery. A cystostomy with a self-retaining adult urinary catheter (14F) was performed through a small suprapubic laparotomy to connect a precise urine-storage bag for recording the UO. A decrease in systemic blood pressure was corrected by administering 0.9% saline before injecting the *E.*

coli. The animals' core body temperatures were maintained at 36–37°C via a heating pad.

Systemic haemodynamic index measurements

A total of 3.5×10^8cfu *E. coli*/kg was infused continuously through the external jugular vein for 2 h. This period was designated as T-0h to T-2h. The MAP, CI and PaO_2/FiO_2 were measured at different time points, including T-0, 1, 2, 3, 4, 6, 8, 12, 18 and 24 h. The PICCO parameters were adjusted by injecting 5 ml of ice-cold saline three times every 6 h. UO was recorded hourly.

Blood collection

A 5.5-ml blood sample was collected at T-0, 6, 12, 18 and 24 h for routine analysis and coagulation testing at the department of Clinical Laboratory of the First Hospital of China Medical University. To measure the concentration of interleukin 6 (IL-6), TNF-α, syndecan-1 and HS, 3.8-ml blood sample was collected at T-0, 1, 2, 3, 6, 12 and 24 h and anti-coagulated with ethylenediaminetetraacetic acid. The blood was centrifuged at 3000*g* for 30 min, and aliquots of 1.0 to 1.2-ml plasma were saved in polypropylene tubes and stored at −70°C. The concentrations of IL-6, TNF-α and syndecan-1 were determined using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. To measure HS, the plasma samples were concentrated with 10-kd cut-off ultra-filter units (Millipore, Billerica, MA, USA), and aliquots of 0.1 ml were analysed using an ELISA kit (Seikagaku Corporation, Tokyo, Japan).

Animal treatment

Twenty canines were randomly allocated to the following four groups ($n = 5$): (1) a sham group (0.9% saline at 3 ml/kg/h); (2) a shock group (3.5×10^8 cfu/kg *E. coli* at 3 ml/kg/h); (3) a basic therapy group. The animals were administered 0.9% saline for fluid resuscitation (10 ml/kg/h) when MAP decreased by 20% of the baseline (45–75 min after the *E. coli* infusion). Ceftriaxone sodium was administered at 0.5 g/12 h intravenously 2 h after *E. coli* infusion (T-4h). The basic therapy strategies aimed to maintain MAP and UO of septic dogs at an acceptable level; (4) a heparin group. The animals were treated in the same manner as the basic therapy group and were simultaneously infused with UFH at 40 IU/kg/h (heparin sodium, Shanghai No. 1 Biochemical & Pharmaceutical Co. Ltd., Shanghai, China, molecular weight 12,000 D, USP 12,500 units/2 ml). The animals were given 250 units/ml heparin sodium or the same volume of 0.9% saline in a 50-ml syringe. The treatment was infused at 0.16 ml/kg/h in the heparin or basic therapy groups. All groups were blinded to the researchers to prevent bias. At the end of the study, the animals were sacrificed with an overdose of potassium chloride for pathologic post-mortem analysis.

Statistical analysis

All data are expressed as the mean ± standard deviation. The haemodynamic and biochemical data were calculated from samples collected at different time points. The data comparisons over time and between treatments were conducted with a two-way analysis of variance. When the initial analysis showed a statistically significant difference, a Dunnett's post hoc test was performed. The survival rate was calculated with Kaplan–Meier survival analysis. The data were analysed with SPSS 20.0 software (IBM SPSS Statistics, Chicago, IL, USA). A *P*-value of < 0.05 was considered to be significant.

Results

Lethal septic shock model

All animals in the sham group survived until the end of the study (T-24h), and all animals in the shock group died. Two of the five canines in the basic therapy group and four of the five canines in the heparin group survived to T-24h. The mean survival time was not significantly longer in the heparin group (22.8 ± 1.1 h) than the basic therapy group (20.9 ± 1.5 h) ($P > 0.05$) (Fig. 1).

Systemic haemodynamic index

Two hours after the *E. coli* infusion (T-2h), the MAP declined by 40%. In the shock group, the MAP slowly decreased until T-8h and then sharply

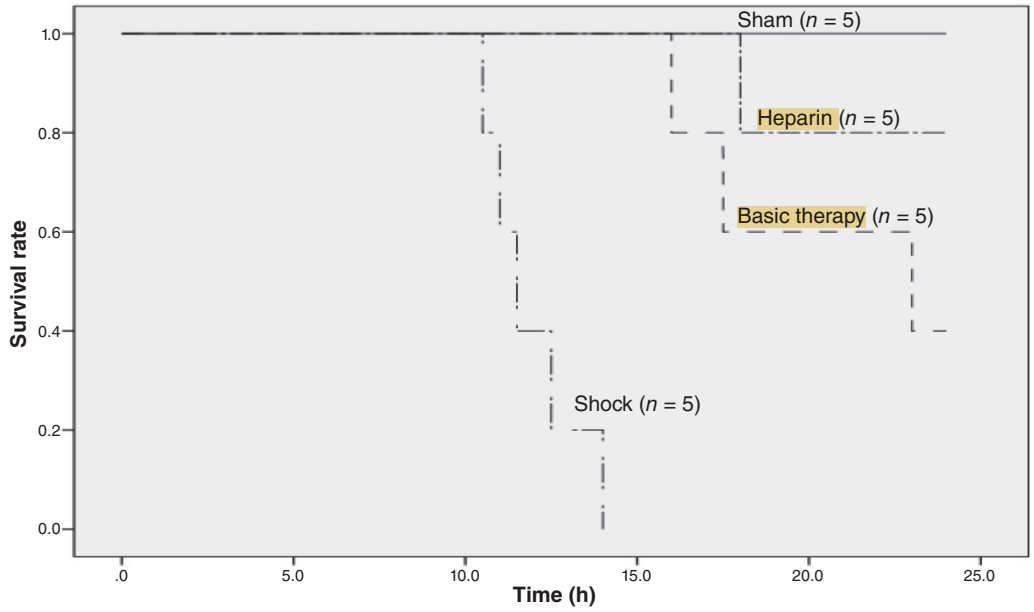

Fig. 1. The effects of lethal concentrations of *E. coli*, basic therapy strategies and unfractionated heparin plus basic therapy on survival rate 3.5×10^8 cfu/kg *E. coli* were infused i.v. from T-0h to T-2h.

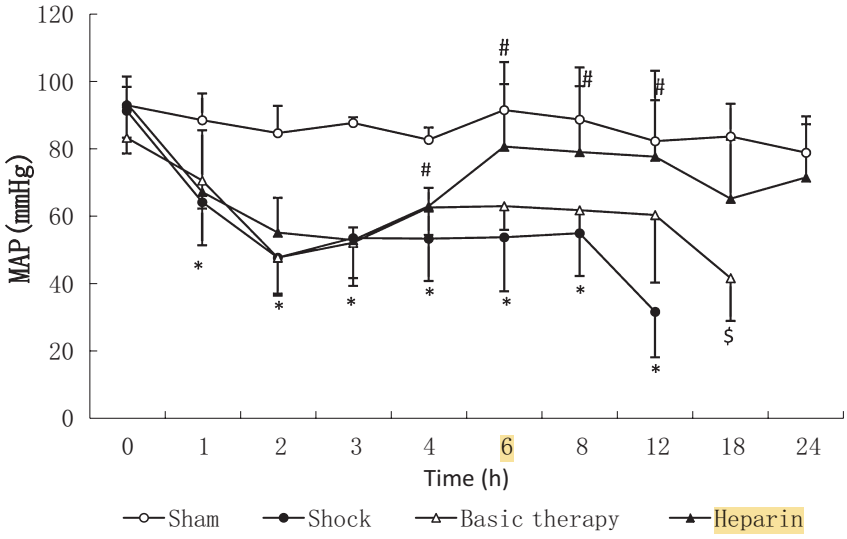

Fig. 2. The effect of unfractionated heparin on mean artery pressure (MAP) in septic shock. n = 5/Group; *$P < 0.05$ relative to T-0h; #$P < 0.05$ the heparin group compared with the shock group; \$$P < 0.05$ the heparin group compared with the basic therapy group.

decreased. The MAP recovered partially after T-4h in the basic therapy group and returned significantly in the heparin group. After T-12h, the MAP declined significantly in the basic therapy group, whereas MAP recovered to the baseline level in the heparin group ($p < 0.05$) (Fig. 2).

In the shock group, CI reduced sharply after T-8h. In the basic therapy group, CI was higher than the baseline level (1.59 ± 0.29 l/min/m^2) to T-6h, but decreased to 1.57 ± 0.55 l/min/m^2 at T-12h. There was no significant difference of CI between the basic therapy and heparin groups ($P > 0.05$) (data not shown).

Compared with the sham group, the UO was significantly decreased by 68–78% 1 h following the *E. coli* administration in the other three groups ($P < 0.05$). In the two therapy groups, we observed decreased UO and decreased MAP. After T-4h, we observed recovered MAP and increased UO in the basic therapy and heparin groups. The UO increased more significantly in the heparin group ($P = 0.039$) (Fig. 3).

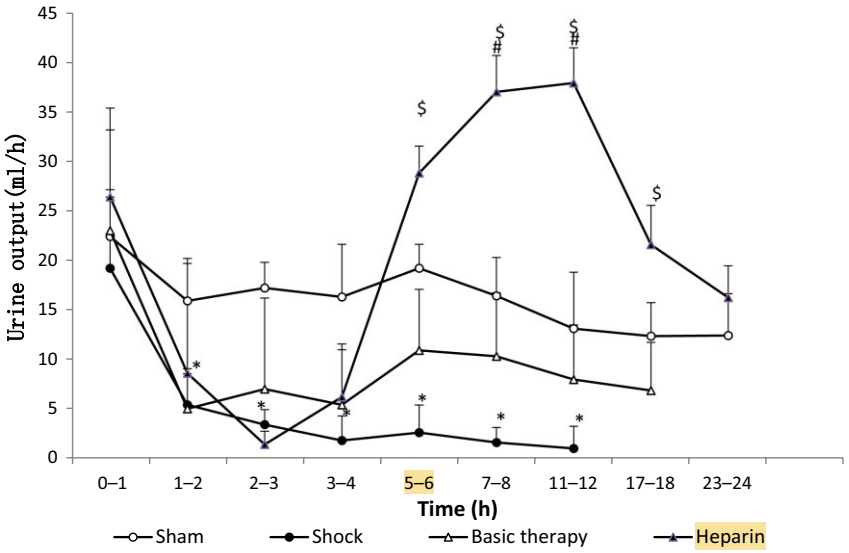

Fig. 3. The effect of unfractionated heparin on urine volume per hour in septic shock. *$P < 0.05$ relative to T-0h; #$P < 0.05$ compared with the shock group; \$$P < 0.05$ compared with the basic therapy group.

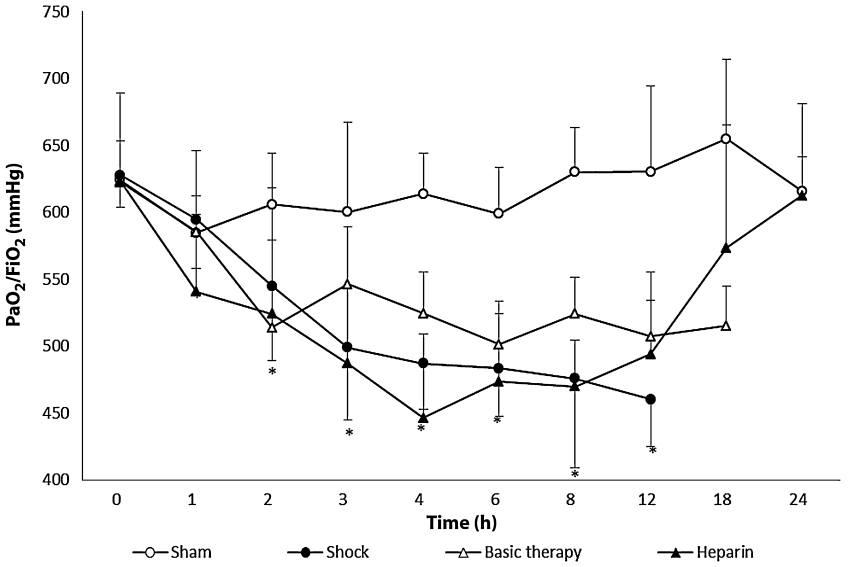

Fig. 4. The effect of unfractionated heparin on oxygenation index in septic shock. n = 5/Group; *$P < 0.05$ relative to T-0h.

We observed a constant decrease in PaO_2/FiO_2 in the shock and basic therapy groups after the infusion of *E. coli*. In the heparin group, we observed a transient decline until T-4h and then a return to baseline at T-24h. There was no significant difference observed in PaO_2/FiO_2 among the three shock groups (Fig. 4).

Plasma syndecan-1, HS, IL-6 and TNF-α levels

In the shock group, the plasma syndecan-1 level increased sharply at T-1h and then increased slowly between T-2h and T-12h. The levels reached 20.02 ± 6.51 ng/ml (twofold greater than the baseline) at T-12h (Fig. 5).

HS increased significantly at T-1h and then progressively increased from T-2h to T-24h in the shock group (Fig. 6).

IL-6 level increased sharply at T-1h and then progressively rose from T-2h to T-12h. The IL-6 level finally reached 175.43 ± 10.61 ng/ml (threefold compared with baseline) at T-12h (Fig. 7). The TNF-α level increased significantly from T-2h to T-6h and then declined slowly (Fig. 8).

The presence of glycocalyx degradation products in plasma showed a positive correlation

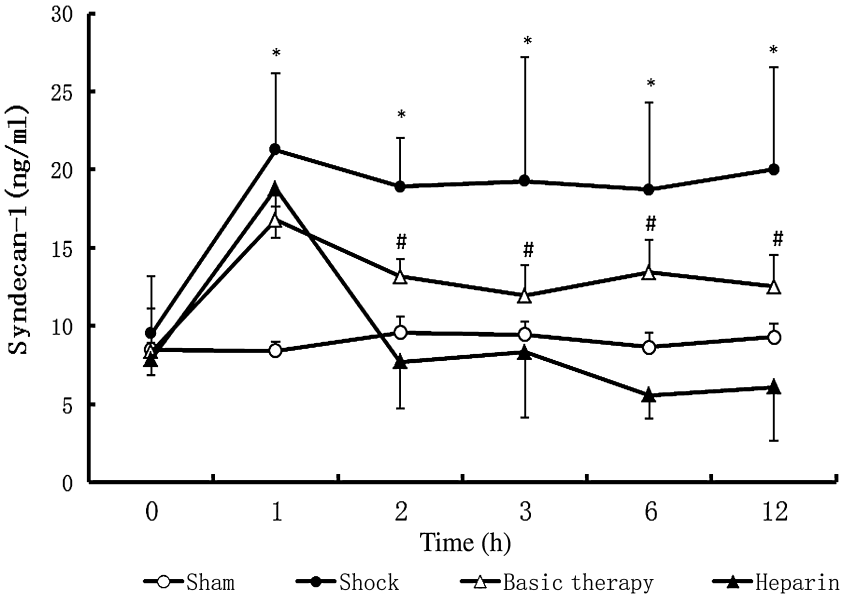

Fig. 5. The effect of unfractionated heparin on syndecan-1 shedding in septic shock. n = 5/Group; *P < 0.01 relative to the T-0h; #P < 0.05 compared with the heparin group.

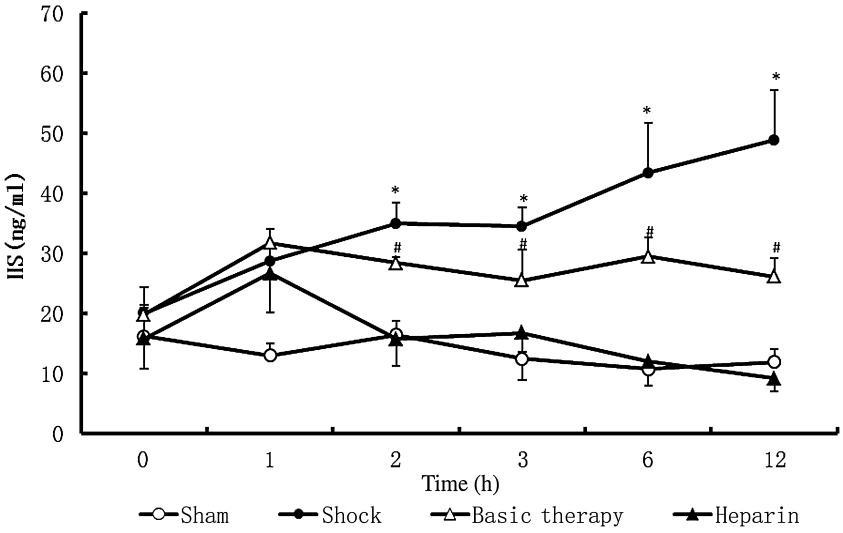

Fig. 6. The effect of unfractionated heparin on heparan sulphate (HS) shedding in septic shock. n = 5/Group; *P < 0.01, relative to the T-0h; #P < 0.01 compared with the herapin group.

with inflammatory factors (P < 0.01) (syndecan-1 vs. IL-6: r = 0.876; HS vs. IL-6: r = 0.899; syndecan-1 vs. TNF-α: r = 0.449; HS vs. TNF-α: r = 0.541).

Effect of UFH on glycocalyx degradation and inflammation

In contrast to the shock group, the syndecan-1 level decreased slowly from T-1h to the endpoint in the treated group. The level remained higher than baseline in the basic therapy group. The syndecan-1 level declined significantly compared with the basic therapy group after UFH administration (P = 0.012) (Fig. 5).

The HS level declined more noticeably in the heparin group (P < 0.01) than in the basic therapy group (Fig. 6).

The plasma levels of IL-6 and TNF-α decreased in the basic therapy and heparin groups, especially in the heparin group from T-3h to T-24h (Figs 7 and 8).

The normal platelet count was approximately 260 × 10^9/l and decreased to < 100 × 10^9/l at T-12h following the *E. coli* administration. In the heparin group, the platelet count was greater than that in

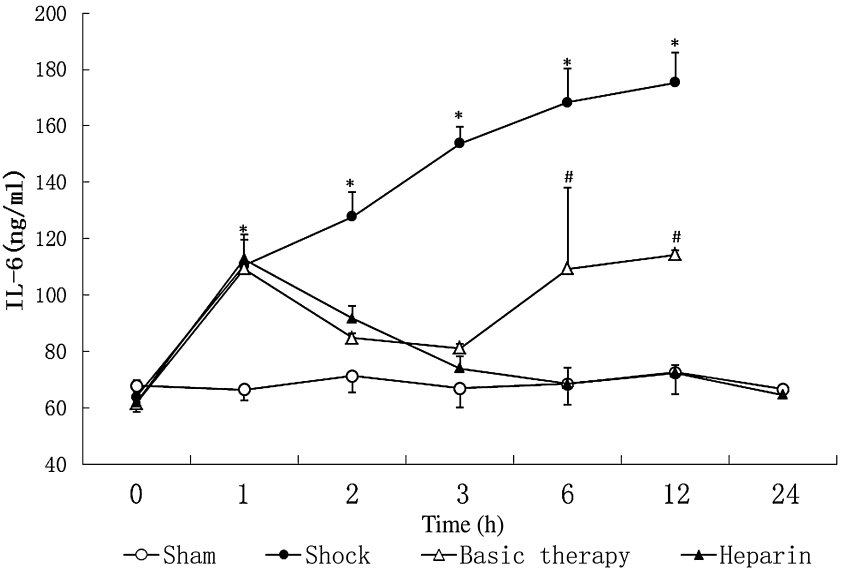

Fig. 7. The effect of unfractionated heparin on inflammatory factors interleukin-6 (IL-6). *n* = 5/Group;*$P < 0.05$ relative to the T-0h; #$P < 0.01$ compared with the herapin group.

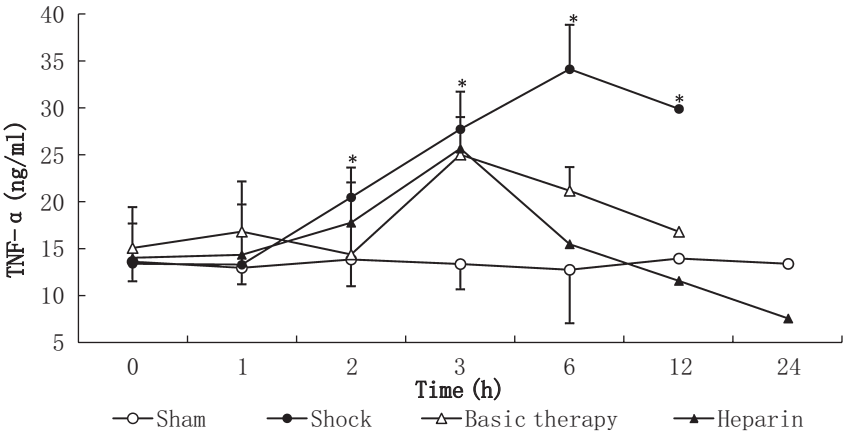

Fig. 8. The effect of unfractionated heparin on inflammatory factors tumour necrosis factor (TNF)-α. *n* = 5/Group; *$P < 0.05$, relative to the T-0h.

the shock group and decreased slowly until T-24h (Fig. 9). The activated partial thromboplastin time (aPTT) value remained relatively low (mean, 27.3 ± 1.97 s) in the basic therapy group. In the heparin group, the aPTT level significantly increased to fourfold the baseline level at T-6h ($P < 0.01$) and then declined. However, the aPTT value in the heparin group remained higher than that in the basic therapy group at T-12h and T-18h (Fig. 10).

Discussion

In this study, we observed improvements in several systemic haemodynamic indices, including the MAP, UO, CI and oxygenation index. Septic shock has been shown to cause failure in multiple organs through hypercoagulability, hyper-inflammation, microcirculatory abnormalities and endothelial injury, which may ultimately lead to increased mortality.[1] Accumulating evidence indicates endothelial glycocalyx plays an important role in the pathophysiology of sepsis.[11]

Several studies have examined the effect of UFH on endothelial glycocalyx, but they have demonstrated inconsistent results.[12] In the novel canine septic shock model, we observed increased glycocalyx degradation markers (i.e., syndecan-1 and HS) and positive correlation with several inflammatory factors (i.e., IL-6 and TNF-α). The UFH treatment decreased glycocalyx shedding, which might be regulated by inhibiting inflammation. Proteoglycans are the main components of glycocalyx and provide structural support for the gly-

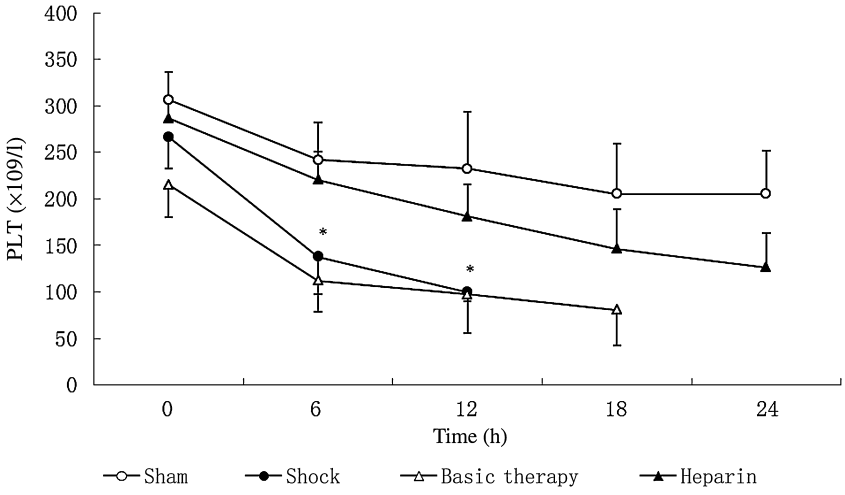

Fig. 9. The effect of unfractionated heparin on platelet counts in septic shock. *$P < 0.05$, relative to T-0h. PLT, platelet count.

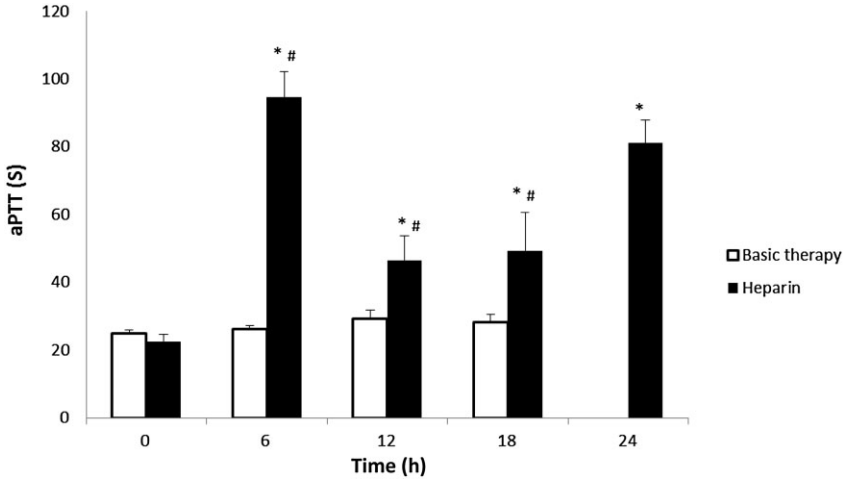

Fig. 10. Blood coagulation variable activated partial thromboplastin time (aPTT) in the basic theraphy group compared with the herapin group. *$P < 0.01$ relative to T-0h; #$P < 0.05$ compared with the basic theraphy group.

cocalyx. The glycocalyx consists of the core protein syndecan-1, which has attached GAGs.[13] Sepsis-induced glycocalyx degradation causes the exposure of adhesion molecules for circulating cells in plasma, which leads to platelet aggregation and neutrophil extravasation. These changes transfer albumin and fluid from plasma to tissue spaces, which aggravates the inflammatory response and tissue oedema.[14] Additionally, endothelial glycocalyx degradation may be linked to consumption coagulopathy. One recent study reported the early loss of glycocalyx-enhanced local thrombin generation, fibrinolysis and profound endothelial cell damage, as demonstrated by higher sTM4.[4]

In this study, we examined glycocalyx degradation at various time points in a novel septic shock model. Syndecan-1 was detectable in plasma in our septic shock model. We observed obvious protective effects of UFH on syndecan-1 shedding in septic shock. In a previous animal study, HS was shown to covalently conjugate to specific core proteins, such as heparan sulphate proteoglycans (HSPGs), which is a glycocalyx transmembrane that is complex and is mainly composed of syndecan-1.[15] One possible mechanism underlying the protective function of UFH, which is an HS analogue, may reconstitute cell surface by mobilising an intercellular pool of syndecan-1, which can reconstruct the protective network of proteoglycans to re-establish an effective vascular endothelial barrier.[16]

Several recent studies have demonstrated that in pathogen-induced sepsis, the pulmonary microvascular endothelial cells rapidly activate endogenous stores of heparanase. The heparanase

cleaves HS from endothelial glycocalyx and induces a rapid thinning of the ESL.[6,17] HS degradation can result in the increased release of pro-inflammatory mediators within the ESL, which aggravates the inflammatory response in septic shock.[18] The inhibition of heparanase activity has been regarded as a potential therapy for sepsis. In this study, the plasma concentration of HS decreased in the heparin group. Inflammatory factors, such as TNF-α and reactive oxygen species, have been implicated in glycocalyx degradation in sepsis or ischaemia-reperfusion models.[19,20] However, we observed increased TNF-α 1 h later than syndecan-1 and HS in this study. This result might be related to the regulatory function of inflammatory mediation by glycocalyx degradation products. It was reported that shed syndecan-1 ectodomains alleviated inflammation by suppressing the amplification of systemic cytokine storm and T-cell-mediated inflammatory tissue injury in an HS-dependent manner. In vitro studies have shown that soluble syndecan-1 ectodomains bind and regulate various inflammatory factors through their HS moiety in Gram-positive toxic shock.[16]

We did not observe bleeding in skin incisions and mucosa after the UFH administration. There was also no obvious platelet count decrease in the heparin group, which suggests that UFH is safe in this model.

We also recognise that our study has some limitations. First, our sample size is relatively small, and we did not perform a power analysis for experimental design (i.e., the correct number of samples or repeats). Thus, our results should be interpreted with caution. Second, this study describes a short (24 h) septic shock model; a long-term model is needed to better understand the dynamics of endothelial glycocalyx degradation.

Conclusion

We observed increased endothelial glycocalyx degradation, as assessed by syndecan-1 and HS. These degradation products were correlated with inflammation mediators, such as IL-6 and TNF-α in the septic shock model. We observed a protective effect of UFH on glycocalyx shedding, which might be due to reduced inflammation, without serious side effects (e.g., bleeding). More studies using larger sample sizes are needed to confirm our conclusion.

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