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Heparin defends against the toxicity of circulating histones in sepsis

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TABLE OF CONTENTS

1. ABSTRACT

Although circulating histones were demonstrated as major mediators of death in septic mice models, their roles in septic patients are not clarified. The present study sought to evaluate the clinical relevance of the circulating histone levels in septic children, and the antagonizing effects of heparin on circulating histones. Histone levels in the plasma of septic children were significantly higher than healthy controls, and positively correlated with disease severity. Histone treatment could activate NF-κB pathway of the endothelial cells and induce the secretion of large amount of cytokines that further amplify inflammation, subsequently leading to organ damage. Co-injection of low dose heparin with lethal dose histones could protect mouse from organ damage and death by antagonizing circulating histones, and similar effects were also observed in other septic models. Collectively, these findings indicated that circulating histones might serve as key factors in the pathogenesis of sepsis and their levels in plasma might be a marker for disease progression and prognosis. Furthermore, low dose heparin might be an effective therapy to hamper sepsis progression and reduce the mortality.

2. INTRODUCTION

Histones are cationic proteins that associate with DNA in nucleosomes and are critically involved

in chromatin remodeling and regulation of gene transcription. Despite histones are usually located in nucleus, they can also be found in the cytoplasm or extracellular milieu by secretion and incorporation into neutrophil extracellular traps (NETs) by activated neutrophils to defend pathogens(1), or by passive release from dying cells into the extracellular milieu(2). Recent studies in septic mice models have identified that circulating histones in the plasma could sever as major mediators of endothelial damage, organ failure and death in sepsis, and a neutralizing antibody against histone H4 could significantly reduce the mortality(3). Furthermore, circulating histones can promote platelet aggregation and thrombus formation, which may contribute to disseminated intravascular coagulation (DIC) in septic patients. Circulating histones can also induce kidney injury by directly activating and killing renal endothelial and tubular cells(2), or induce fatal liver injury through Toll-like receptors(4), similar to multiple organ dysfunction syndrome (MODS) in severe sepsis.

So far, several reagents have been shown to be able to antagonize circulating histones *in vitro* or *in vivo*. Circulating histone-induced cytotoxicities to endothelium or in septic mice models can be effectively inhibited by anti-histone antibodies or activated protein C (APC) by direct neutralization or cleavage (3). Furthermore, circulating histone-induced platelet aggregation and thrombus formation can be completely abolished by APC and heparin (5,6). Recently, C-reactive protein (CRP) has also been demonstrated to interact with histones and alleviate histone-induced endothelial cell damage, permeability increase and platelet aggregation, which would be a generic mechanism of host defense in humans (7).

Although findings in mice models identified circulating histones as the major mediator of death and may serve as a potential taget in sepsis, to date, few studies have discussed their relevance in septic patients. In the present study, we sought to investigate the levels of circulating histones in septic children and the correlation with the disease severity. In addition, outcome of treatment to septic mouse with low dose heparin, an anticoagulant that has been shown to bind histones more tightly than DNA(8) and efficiently inhibit histone-induced platelet aggregation(6), was analyzed. The underling mechanisms of histone-induced cytotoxicities and protective effects of heparin were also studied in both *in vitro* and *in vivo* models.

3. MATERIALS AND METHODS

3.1. Ethics statement

This study conformed to the principles outlined in the Declaration of Helsinki for use of human blood and was approved by the Ethics Committee of Experimental Research of Shanghai Jiaotong University School of Medicine. All clinical studies were approved by the Institutional Review Board of Shanghai Children's Medical Center Affiliated to Shanghai Jiaotong University School of Medicine under an informed consent protocol. Patients admitted to Shanghai Children's Medical Center between January 2009 and June 2013 and diagnosed with sepsis were recruited using a clinical database. All human blood samples were obtained from patients who provided written informed consents which were obtained from guardians on behalf of the minors/children enrolled in study.

Male C57BL/6 mice of 6–12 week of age were used for histone/LPS/cecal ligation and puncture (CLP)-induced septic models, and were carried out in strict accordance with international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, with the protocol being approved by the Institutional Animal Care and Use Committee of Shanghai Children's Medical Center Affiliated to Shanghai Jiaotong University School of Medicine.

3.2. Patients

Patients admitted to Shanghai Children's Medical Center between January 2009 and June 2013 and diagnosed with sepsis were recruited using a clinical database. The diagnosis of sepsis and severe sepsis met the criteria recommended by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference (9).

3.3. Mouse Models

Male C57BL/6 mice of 6–12 week of age were used for the three types of septic models, caused by direct histone or LPS tail vein injection and CLP operation, respectively,. Histones and LPS were injected directly through the tail vein at indicated concentrations alone as described previously (3,10), or co-injected with heparin (10 mg/kg) or argatroban (1 mg/kg). CLP model was also performed as described (3,10). Briefly, male C57BL/6 mice of 6–12 week of age were fasted for 16 hours and anesthetized by intraperitoneal injection of phenobarbital (5mg/kg). The cecum was exposed after mid–line laparotomy and ligated right below the ileo–cecal valve without causing intestinal obstruction. After being punctured twice with an 18G needle, the cecum was placed back in the peritoneal cavity, and the abdominal wall was closed in 2 layers. The mice were then treated with cefotaxime (100 mg/kg) and/or heparin (3 mg/kg) or argatroban (1 mg/kg) by tail vein injection 4 hours after the CLP operation for 3 days. The day on which the operation or injection was performed was considered as day 0, and every following-24-hour was counted as 1 day. The condition of the treated mice was monitored at 8am, 2pm and 8pm everyday after treatment. To accurately determine the effects of the drugs and to minimize the artifact, no analgesics or anaesthetics was

applied after operation. Survival was used as primary endpoint in these models (11), and the survival duration was calculated right after the treatment. All the survived mice were euthanized by lethal dose of phenobarbital 7 days after treatment.

3.4. Reagents and cell culture

Calf thymus histones and LPS from Salmonella typhimurium were purchased from Sigma, and cell apoptosis detection kit was from BD. Human endothelial cell line HUVEC was purchased from ATCC and cultured in M200 supplemented with LGGS (Invitrogen) according to the manual.

Primary human endothelial cells HUVECs were incubated with calf thymus histones at various concentrations or for different time periods, and the percentage of viable cells was determined by flow cytometry after PI and FITC-annexin V double staining.

3.5. ELISA

Extracellular histone levels in the plasma from patients or mice were measured using a sandwich enzyme-linked immunosorbent assay (Cell Death Detection ELISA plus Kit; Roche Diagnostics) as previously described(12,13) and according to the manufacture instructions. Briefly, 20 μL citrated plasma was diluted 1:4 with phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), 0.5.% Tween-20, and 1 mM ethylenediamine tetra-acetic acid (EDTA), added to streptavidin-coated microtiter plates containing biotinylated mouse anti-histone antibody and peroxidase conjugated anti-DNA antibodies, and incubated at room temperature for 2 hours. Peroxidase activity was measured with spectrophotometer at 405 nm after incubation with the substrate ABTS (2, 2'-azino-di (3-ethylbenzthiazoline-sulfonate)). Cytokines released into the cell media were measured by the ELISA-based Bioplex multiplex cytokine measurement system (Bio-Rad) as previously described(12,13) and according to the manufacture instructions.

To investigate whether they also play pivotal roles in septic patients, totally 40 healthy controls and 80 septic children admitted into our hospital from January 2009 to June 2013 were enrolled in the present study, in which 40 patients were with severe sepsis. Sepsis and severe sepsis, were diagnosed according to the criteria recommended by the American College of Chest Physicians and the Society of Critical Care Medicine Consensus Conference (9). Whole blood samples of these children were collected upon admission, and the levels of circulating histones in the plasma were determined by ELISA.

3.6. Flow cytometry

5×10^5 Human umbilical vein endothelial cells (HUVECs) were seeded into 6-well plate and cultured in M200 supplemented with LGGS (Invitrogen) overnight before being treated with histones at various concentration or for different time period as indicated in OPTI-MEM (Invitrogen). Treated HUVECs were detached with 0.2.5% trypsin/EDTA, washed with PBS and incubated in binding buffer containing FITC–anti-annexin V and/or propidium iodide (BD Biosciences) for 15 minutes at room temperature. Percentage of viable cells (annexin V negative and propidium iodide negative cells) was determined by an FACS Calibur flow cytometer (BD Biosciences).

3.7. Western blotting

Activation of downstream signaling pathway was analyzed by standard Western blot after being stained with anti–IκB-α, anti–phospho p38 or anti–total p38 antibodies (Cell Signaling Technology), developed by ImageQuant LAS 4000 mini (GE Healthcare) and quantified with AlphaView SA (Cell Biosciences). Density of IκB-α was normalized to that of b-actin in the same sample, reflecting the activation extent of NF-kB pathway, and the ratio of vehicle-treated cells was considered as 1. Similarly, the ratio of phospho p38 to total p38, which reflected the activation extent of mitogen activated protein kinase (MAPK) pathway, was also calculated, with that of vehicle-treated cells being considered as 1.

3.8. Transmission electron microscopy and scanning electron microscopy

Treated HUVECs or lung tissue specimens from septic mice were doubled stained with saturated 3% (w/v) uranyl acetate in 50% (v/v) alcohol and lead citrate after fixation, and then examined using a transmission electron microscope (Philips CM120). To further confirm the membrane morphology of histone-treated HUVECs, the fixed cells were examined using a scanning electron microscopy (Quanta 250).

3.9. Statistical Analysis

Survival studies were analyzed using the log-rank test in the program Prism (GraphPad). Other data were expressed as the mean ± SD. Comparisons between two groups were performed by two-tailed student t test. Differences with p value < .05 were considered as statistically significant.

4. RESULTS

4.1. The levels of circulating histones in the septic children were correlated with the severity of sepsis

Circulating histones have been demonstrated as major mediators of death in septic mice models(3), but few studies have discussed their relevance in septic patients. As shown in Figure 1A, histone levels in the plasma of the children diagnosed with mild sepsis were significantly higher than healthy controls, and histone levels in the severe septic children were even higher

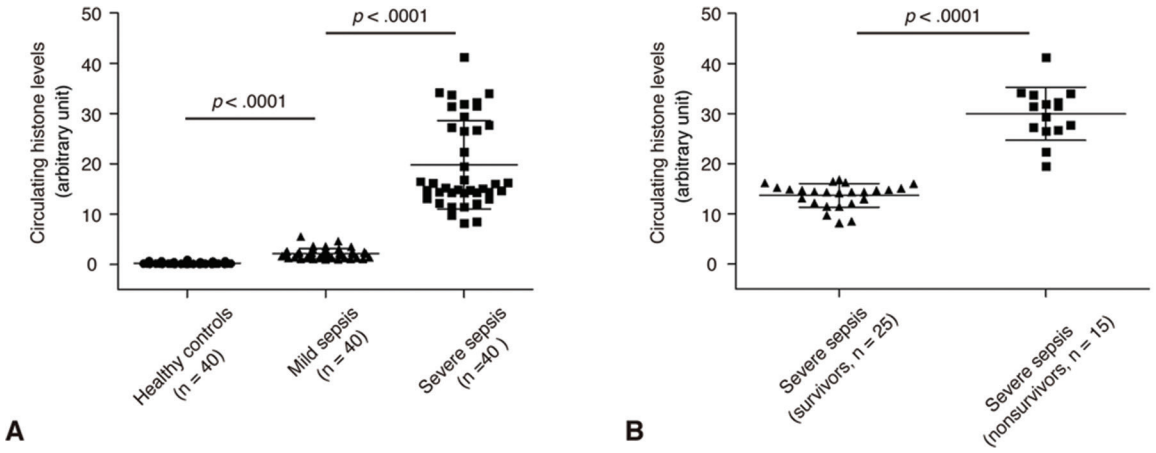

Figure 1. Circulating histone levels in septic children were correlated with the severity of sepsis. Whole blood samples were collected from 80 septic children (40 were with mild sepsis and the others were severe sepsis) upon admission and 40 healthy children, and histone levels in the plasma were determined by ELISA. The data were present as the mean ± SD. (A) Histone levels in severe septic children were significantly higher than those with mild sepsis, and both were significantly higher than healthy controls ($p < .001$). (B) Among all 40 severe septic children, histone levels in the nonsurvivors were significantly than the survivors ($p < .001$).

(0.2.6 ± 0.1.9 vs. 2.1.6 ± 0.9.8 vs. 19.8.0 ±8.6.7, p < .0001). Among the children diagnosed with severe sepsis, the levels of circulating histones in the nonsurvivors were significantly higher than those in the survivors (29.9.7 ± 5.1.1 vs. 13.7.0 ± 2.3.2, p < .0001, Figure 1B). The above results clearly indicate that the levels of circulating histones in the septic children correlate positively with the disease severity. Since the basic characteristics of the study subjects are comparable in all groups (Table 1 and 2), our findings suggest that circulating histones might be indicators for disease progression and play pivotal roles in septic patients.

4.2. Heparin could effectively inhibit histone-induced HUVEC death

Since vascular endothelium injury, the key factor in sepsis that could gradually lead to MODS, was also observed in histone-treated mice(3), to further investigated the effects of circulating histones on endothelium and the underlying mechanism. As shown in Figure 2, histones induced HUVEC death in a dose- and time-dependent manner, which can be effectively inhibited by heparin (Figure 2A-C). In addition, heparin showed a step-wise decrease of inhibitory effect when added at different time points after histone treatment (Figure 2D), indicating that earlier usage of heparin could provide better efficacy. Histone treatment also resulted in formation of the pores in the plasma membrane of HUVECs to disrupt the cells and condensation of the nuclear chromatin, as observed under electron microscope (Figure 2E and F). Further studies demonstrated significant NF-κB and MAPK activation in HUVECs after histone treatment, as evident by IκB-α degradation and p38 phosphorylation (Figure 3A), which subsequently led to the secretion of a range of important pro-inflammatory cytokines, such as TNF-α and IL-6 (Figure 3B). All the effects induced by histone treatment could be effectively inhibited by heparin. Therefore, it is possible that high levels of histones in the plasma activate NF-κB pathway to induce the secretion of large amount of cytokines that further amplify inflammation. Meanwhile, circulating histones also destroy the endothelium, subsequently leading to organ damage.

4.3. Heparin protects septic mice from organ damage and death

To demonstrate the protective effects of heparin against histones *in vivo*, we injected 75 mg/kg histones intravenously into mice in the absence or presence of 10 mg/kg heparin. Consistent with previous studies(3,7,14), all mice (n = 5) died within one hour after injection with 75 mg/kg histones, exhibiting severe multifocal alveolar hemorrhage, release of myeloperoxidase (MPO) to alveoli, and vacuolization in endothelial cells (Figure 4). Given that MPO could enhance the production of hypochlorous acid from hydrogen peroxide and chloride anion, which kills bacteria but also damages host cells, increased MPO release may further contribute to lung injury, including alveolar endothelial cell damage. Co–injection of heparin (10 mg per kg) rescued all of the mice (n = 5) challenged with the same lethal dose of histones (Figure 4A). After heparin intervention, alveolar hemorrhage in lung tissues significantly reduced, and much less neutrophils infiltrated into the lungs as evident by anti-MPO immunohistochemical staining of the lungs (Figure 4B, C).

To further confirm the protective roles of heparin in sepsis, CLP-induced sepsis models were performed. Histone levels in the plasma of CLP mice were significantly higher than controls, and all mice (n = 10) died within 4 days if without any drug treatment (Figure

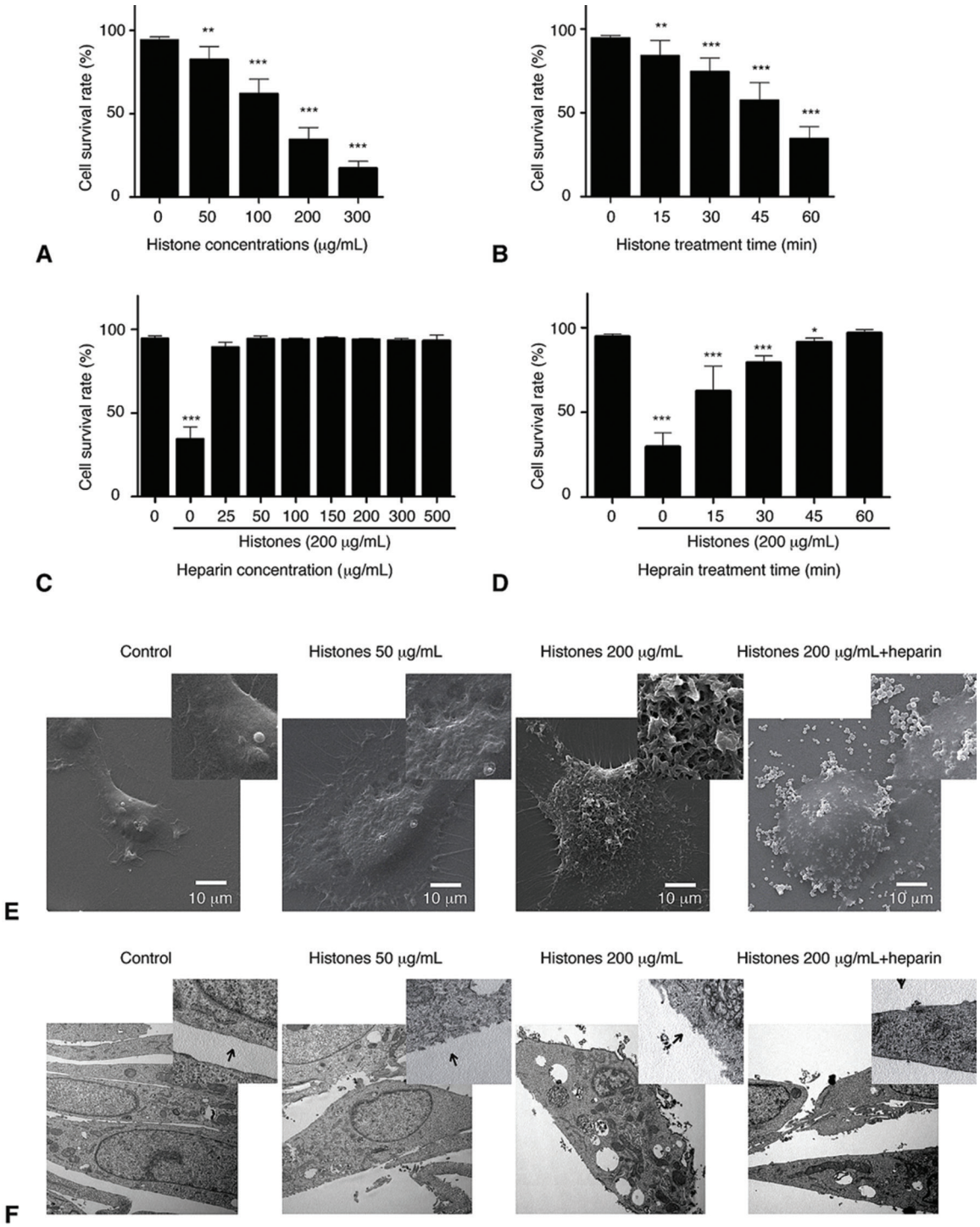

Figure 2. Heparin could efficiently inhibit histone-induced cytotoxicities to HUVECs. (A-D) 5×10^5 HUVECs seeded in 6-well plate and cultured overnight were treated with various concentrations of histones for 60 min (A), or treated with 200 μg/mL histones for various time periods (B), or treated with various concentrations of heparin (C) or 50 μg/mL heparin for different time periods (D) in the presence of 200 μg/mL histones. Treated cells were double stained with FITC-Annexin V and propidium iodide, and detected by flow cytometry. Cells negative for both Annexin V and propidium iodide were considered as viable cells, and the cell survival rate was calculated from 3-8 independent experiments and presented as the mean ± SD. **, $p < .01$; ***, $p < .001$. (E, F) Treated cells were either fixed and examined using a scanning electron microscopy (E) or doubled stained with saturated 3% (w/v) uranyl acetate in 50% (v/v) alcohol and lead citrate after fixation and examined using a transmission electron microscope (F).

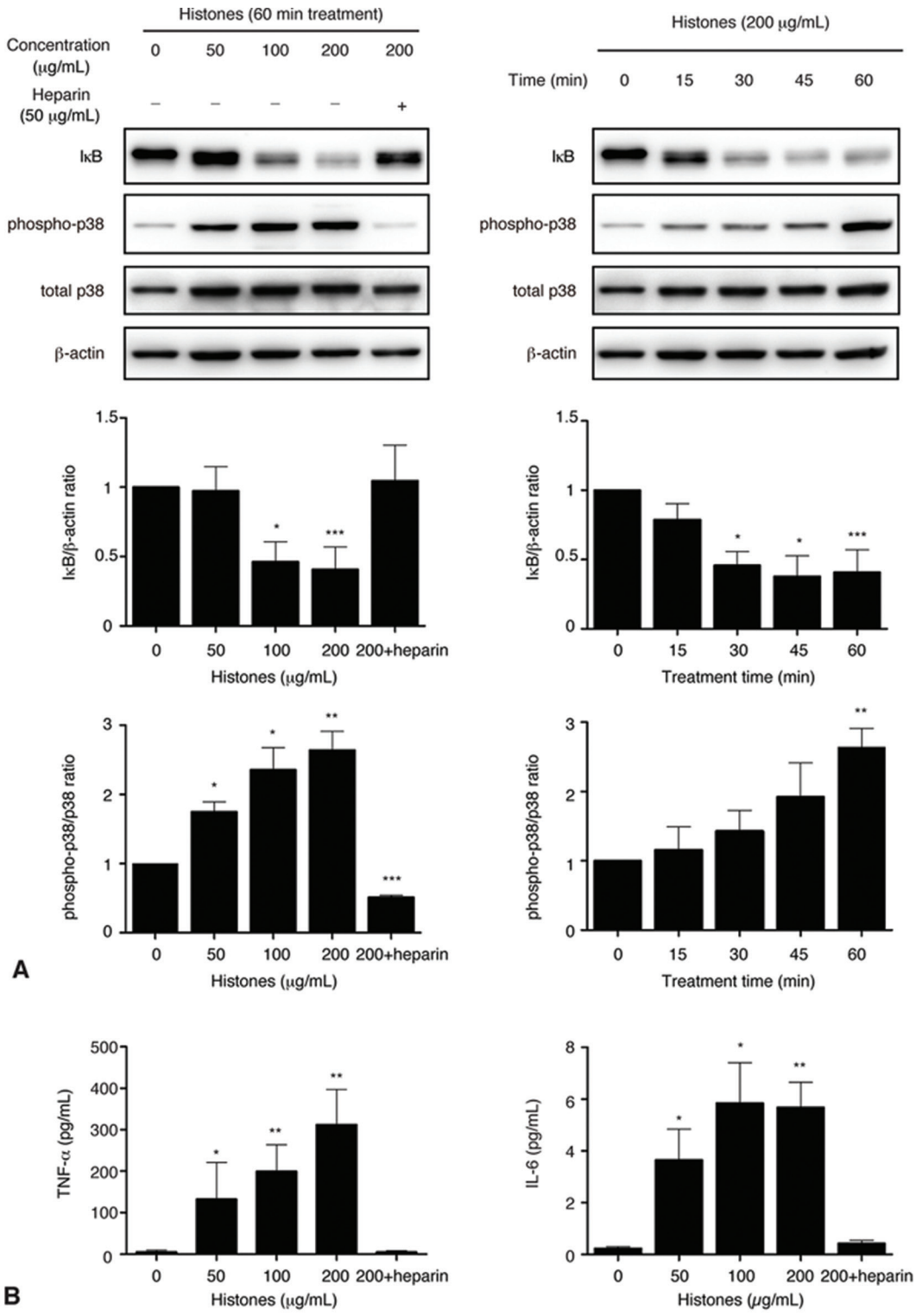

Figure 3. Heparin could efficiently inhibit histone-induced HUVEC activation and cytokine release. 5×10^5 HUVECs seeded in 6-well plate and cultured overnight were treated with histones at various concentrations or for various time periods. (A) Treated cells were lysed in 1% Triton X-100 lysis buffer, and the cell lysates were separated on 10% Tris-glycine SDS-PAGE, transferred to PVDF membrane and stained for IκB-α, phospho-p38, total p38 or β-actin. Band density was quantified with AlphaView SA, and normalized to untreated cells (considered as 1). The ratio was calculated from 3-4 independent experiments and presented as the mean ± SD. (B) Culture media were collected from treated cells, and levels of TNF-α and IL-6 in the media were determined by Bioplex multiplex cytokine measurement system. The data were calculated from 4 independent experiments and presented as the mean ± SD. *, $p < .05$; **, $p < .01$; ***, $p < .001$.

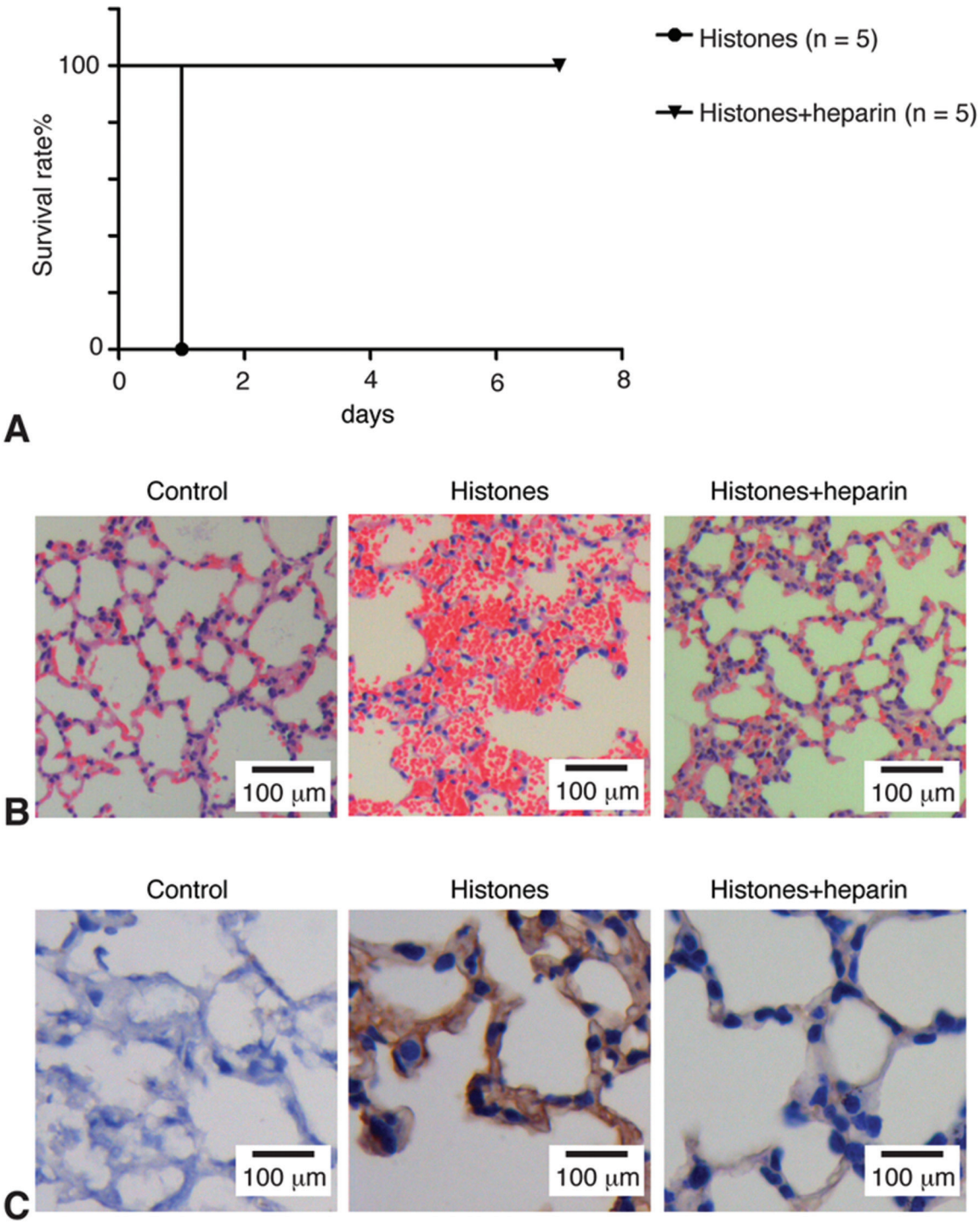

Figure 4. Heparin protects mice from organ damage and death in a histone-treated model. A. Survival rates of mice injected intravenously of histones (75 mg/kg) with or without heparin (10 mg/kg). 5 mice were included in each group. (B) Hematoxylin and eosin (H&E) staining of the lung tissues from untreated mice (control) and mice treated with histones (75 mg/kg) with or without heparin (10 mg/kg). Intra-alveolar hemorrhage was observed in the lungs after histone treatment, which was completely inhibited by heparin. (C) Anti-MPO antibody staining of the lung tissues from untreated mice (control) and mice treated with histones (50 mg/kg) with or without heparin (10 mg/kg) 48 hours after treatment. Significant neutrophil infiltration was observed in the lungs after histone treatment, which was completely inhibited by heparin.

Table 1. Basic characteristics of patients

Patient characteristics	Healthy controls (n=40)	Mild sepsis (n=40)	Severe sepsis (n=40)
Age, years	2.4.9±1.3.3	2.3.6±2.0.8[p1]	2.3.2±1.6.4[p2]
Gender, males	40, 20	40, 23[p3]	40, 25[p4]
PCIS[a]	-	86.8.5±3.4.3	72.1.0±5.4.2[p5]

[a]PCIS, pediatric critical illness score, determined as described (26).
p values, p1=0.6.2 compared to healthy controls; p2=0.9.1 compared to mild sepsis; p3=0.0.8 compared to healthy controls; p4=0.1.6 compared to mild sepsis; p5<0.0.001 compared to mild sepsis

Table 2. Basic characteristics of patients

Patient characteristics	Severe sepsis (survivors, n=25)	Severe sepsis (nonsurvivors, n=15)	p
Age, years	2.0.2±1.4.0	2.8.3±1.8.6	0.1.4
Gender, males	25,16	15,9	0.7.3
PCIS[a]	75.5.2±3.0.6	66.8.±4.1.8	<0.0.001

[a]PCIS, pediatric critical illness score, determined as described (26)

5A, B). Administration of cefotaxime alone could improve the survival rate of CLP mice to 30% when determined at day 7 after operation. Treatment with heparin alone after operation did not induce apparent bleeding and could improve the survival rate to 40% (Figure 5A). Notably, when cefotaxime and heparin were injected in combination, circulating histone levels were significantly reduced, and 80% of the mice survived to day 7 without apparent organ damage and neutrophil infiltration into the lungs as evident by anti-MPO immunohistochemical staining (Figure 5). These findings clearly indicated that heparin could effectively protect septic mice from organ damage and significantly improve the survival rate of these mice. Similar effects were also observed in LPS-treated mice (Figure 6).

4.4. Protective effects of heparin against circulating histones do not depend on its anti-coagulant function

Since heparin is considered as an effective anticoagulant by interfering with thrombin pathway(15,16), to distinguish whether the protective effects of heparin are by antagonizing circulating histones or by inhibiting coagulation, mice were treated with argatroban, another small molecule anti-coagulant directly inhibiting thrombin(17,18). Mice co-injected with argatroban (1 mg/kg) and lethal dose of histones died within one hour (data not shown). This is in line with the observation that argatroban (up to 80 μg/mL) could not inhibit histone-induced HUVEC death *in vitro* (Figure 7A). All these findings suggested that heparin may protect mice from histone-induced cytotoxicities by directly antagonizing circulating histones, rather than its well-known anticoagulant function. To further confirm this, CLP mice were treated with argatroban as histones treatment. In contrast to heparin treatment, argatroban in combination with cefotaxime did not improve the survival rate of CLP mice; 7 out of 10 mice died at day 7 (Figure 7B). This is consistent with the observation that argatroban (up to 1 mg/kg) could not inhibit histone-induced mice death *in vivo*. All these findings suggested that heparin protected septic mice from organ damage and death by antagonizing histones, rather than anticoagulation.

5. DISCUSSION

Histones are highly alkaline proteins that package DNA into nucleosomes and regulate gene transcription inside the nucleus, but extracellular histones elicit toxic and pro-inflammatory effects to various organs(2,4,19). Although Xu *et al* reported extracellular histones as major mediators of death in septic mouse models (3), no definitive evidence on the roles of circulating histones in septic patients has been provided. In the present study, we report that the levels of circulating histones in septic children were significantly higher than controls and correlated with the severity of sepsis (Figure 1). Therefore, elevated circulating histones level may be regarded as a clinical biomarker in sepsis for disease progression and prognosis.

The pathophysiology of sepsis involves a highly complex, integrated response including the activation of a number of cell types, inflammatory mediators, and the haemostatic system. A crucial tissue involved in sepsis pathogenesis is the endothelium. Endothelial cells critically participate in hemodynamics, immunity and coagulation pathways, the three main cornerstones of septic response (20). NETs and histones have been shown to induce epithelial and endothelial cells death *in vitro*(3,14,21), but the mechanism remains poorly studied. Our results showed that histone treatment triggered the activation of NF-κB and p38, resulting in the release of a range of important pro-inflammatory cytokines and chemokines, such as TNF-α and IL-6, which would further amplify inflammation (Figure 3). On the other hand, histones disrupted cell membranes and directly caused endothelial death, subsequently leading to organ damage (Figure 2 and 4).

To further confirm the protective effects of heparin against circulating histones, three different septic mice models were utilized in the present study. We have observed a significant decrease in circulating histone levels in CLP mice models when heparin was injected 4 hours after operation (Figure 5). In addition, in all three models, treating the septic mice with low dose of heparin could alleviate the symptoms of the mice including alveolar hemorrhage and endothelial damage, and significantly improve the survival rates (Figure 4 and 5). Under these conditions, heparin may take effects

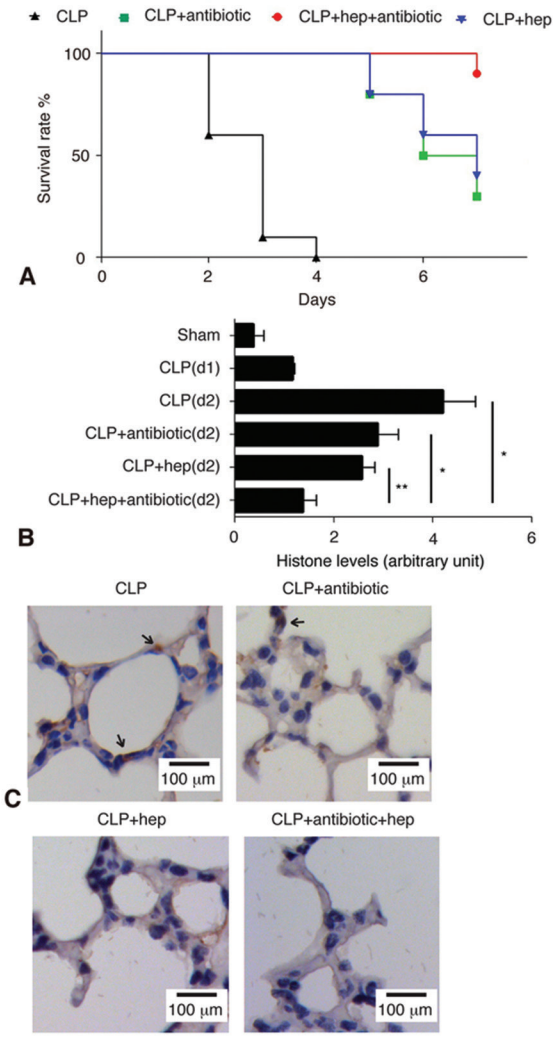

Figure 5. Heparin treatment protects mice from organ damage and death in a CLP model. (A) Survival rate of mice subjected to CLP and various treatments. The mice were either treated with cefotaxime (100 mg/kg) and/or heparin (3 mg/kg) by tail vein injection 4 hours after the CLP operation for 3 days or normally fed without any treatment after CLP operation. 10 mice were included in each group and the number of survival mice was recorded every 24 hours for 7 days. (B) Histone levels in the plasma of various treated mice as determined by ELISA. The data were calculated from 3 mice and presented as the mean ± SD. *, $p < .05$; **, $p < .01$; d1, 24 hours after CLP operation; d2, 48 hours after CLP operation. (C) Lung tissues from various treated mice were stained with anti-MPO antibody after fixation, and examined using a microscopy. Neutrophil infiltration was indicated by arrows. hep, heparin.

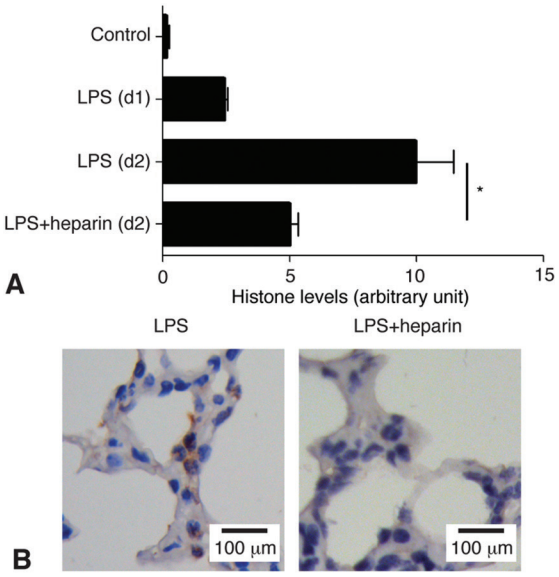

Figure 6. Heparin protects mice from organ damage in a LPS-treated model. Mice were injected intravenously with LPS (50 mg/kg) with or without heparin (10 mg/kg), and peripheral blood and lung tissues were collected at indicated time point. (A) Histone levels in the plasma of various treated mice as determined by ELISA. The data were calculated from 3 mice and presented as the mean ± SD. *, $p < .05$; d1, 24 hours after LPS treatment; d2, 48 hours after LPS treatment. (B) Anti-MPO antibody staining of the lung tissues from untreated mice (control) and mice treated with LPS (50 mg/kg) with or without heparin (10 mg/kg) 48 hours after treatment. Significant neutrophil infiltration was observed in the lung after LPS treatment, which was completely inhibited by heparin.

primarily by antagonizing circulating histones rather than anticoagulation, as evident by the lack of such effects when treating the septic mice with another anticoagulant argatroban (Figure 6), which is also supported by the recent study that non-anticoagulant heparin could prevent histone-mediated cytotoxicities(11).

Several reagents have been shown to antagonize circulating histones in animal models, such as anti-histone antibodies and APC(3). However, despite APC can cleave histones into non-toxic fragments thus inhibit histone-induced cytotoxicities *in vitro* and *in vivo*, clinical trials showed that the septic patients did not benefit from APC treatment (22). In contrast to APC, heparin has been suggested to treat sepsis by the Surviving Sepsis Campaign Management Guidelines Committee(23,24). A retrospective, propensity matching, multi-center cohort study showed that early septic shock patients intravenously injected with low dose heparin had decreased 28 d mortality rates, especially in patients with severe disease. In addition, injection of heparin can improve the success rate of removing the ventilator and vasoactive drugs (25).

The potential bleeding risk is a major concern of treating septic children with heparin, but our *in vitro* findings on HUVECs indicated a high efficacy of heparin against histones, since heparin could completely inhibit 200 μg/mL histone-induced cytotoxicities at concentration as low as 25 μg/mL (i.e. 3 U/mL). Therefore, usage of low dose heparin may be effective to treat sepsis, without causing bleeding, as evident in our mice studies. Another concern is that the patients will

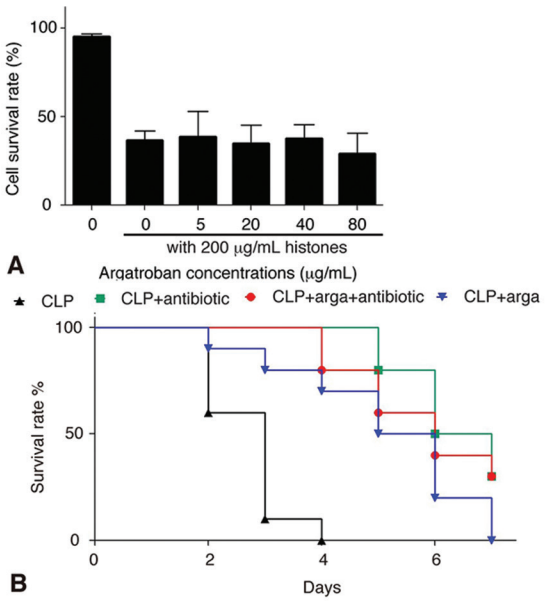

Figure 7. Protective effects of heparin in CLP models are independent of its anticoagulant function. (A) Cell survival rate of histone-treated HUVECs in the presence or absence of argatroban. 5×10^5 HUVECs seeded in 6-well plate and cultured overnight were treated with 200 μg/mL histones and various concentrations of argatroban. Treated cells were double stained with FITC-Annexin V and propidium iodide, and detected by flow cytometry. Cells negative for both Annexin V and propidium iodide were considered as viable cells, and the cell survival rate was calculated from 3 independent experiments and presented as the mean ± SD. There was no difference on the survival rate of 200 μg/mL histone-treated HUVECs in the presence or absence of argatroban. (B) Survival rate of mice subjected to CLP and various treatments. The mice were either treated with cefotaxime (100 mg/kg) and/or argatroban (1 mg/kg) by tail vein injection 4 hours after the CLP operation for 3 days or normally fed without any treatment after CLP operation. 10 mice were included in each group and the number of survival mice was recorded every 24 hours for 7 days. arga, argatroban.

be under different stages when admitted to the hospital, and it might not be possible to treat the patients as soon as they develop sepsis. Our *in vitro* study demonstrated that usage of heparin at different time points after histone treatment could still hamper further histone-induced cytotoxicities (Figure 3D), suggesting that the septic patients could benefit from heparin treatment even after disease progresses.

In conclusion, our results have demonstrated that the levels of circulating histones were significantly higher in septic children and correlated with disease severity, suggesting that they might serve as a good biomarker for sepsis progression and prognosis. Heparin could significantly improve the survival rate of septic mice, possibly by antagonizing circulating histones to prevent histone-mediated cytotoxicities. Further multicenter prospective studies might be required to validate the efficacy and safety of the low dose heparin therapy to septic patients.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A: Neutrophil extracellular traps kill bacteria. *Science* 303(5663),1532-1535 (2004)
DOI: 10.1126/science.1092385

2. Allam R, Scherbaum CR, Darisipudi MN, Mulay SR, Hägele H, Lichtnekert J, Hagemann JH, Rupanagudi KV, Ryu M, Schwarzenberger C, Hohenstein B, Hugo C, Uhl B, Reichel CA, Krombach F, Monestier M, Liapis H, Moreth K, Schaefer L, Anders HJ: Histones from dying renal cells aggravate kidney injury via TLR2 and TLR4. *J Am Soc Nephrol* 23(8),1375-1388 (2012)
DOI: 10.1681/ASN.2011111077

3. Xu J1, Zhang X, Pelayo R, Monestier M, Ammollo CT, Semeraro F, Taylor FB, Esmon NL, Lupu F, Esmon CT: Extracellular histones are major mediators of death in sepsis. *Nat Med* 15(11),1318-1321 (2009)
DOI: 10.1038/nm.2053

4. Xu J, Zhang X, Monestier M, Esmon NL, Esmon CT: Extracellular histones are mediators of death through TLR2 and TLR4 in mouse fatal liver injury. *J Immunol* 187(5): 2626-2631 (2011)
DOI: 10.4049/jimmunol.1003930

5. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD Jr, Wrobleski SK, Wakefield TW, Hartwig JH, Wagner DD: Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci U S A* 107(36),15880-15885(2010)

DOI: 10.1073/pnas.1005743107

6. Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL, Esmon CT:Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood* 118(7),1952-1961 (2011) DOI: 10.1182/blood-2011-03-343061
7. Abrams ST, Zhang N, Dart C, Wang SS, Thachil J, Guan Y, Wang G, Toh CH: Human CRP defends against the toxicity of circulating histones. *J Immunol* 191(5), 2495-2502(2013) http://dx.doi.org/10.4049/jimmunol.1203181
8. Pal PK, Starr T, Gertler MM:Neutralization of heparin by histone and its subfractions. *Thromb Res* 31(1),69-79(1983) DOI: 10.1016/0049-3848(83)90008-7
9. Levy MM, Fink MP, Marshall JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G; SCCM/ESICM/ACCP/ATS/SIS:2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Crit Care Med* 31(4),1250-1256(2003) DOI: 10.1097/01.CCM.0000050454.01978.3B
10. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA :Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* 4(1), 31-36(2009) DOI: 10.1038/nprot.2008.214
11. Wildhagen KC1, García de Frutos P, Reutelingsperger CP, Schrijver R, Aresté C, Ortega-Gómez A, Deckers NM, Hemker HC, Soehnlein O, Nicolaes GA: Nonanticoagulant heparin prevents histone-mediated cytotoxicity *in vitro* and improves survival in sepsis. *Blood* 123(7), 1098-1101 (2014) DOI: 10.1182/blood-2013-07-514984
12. Kutcher ME1, Xu J, Vilardi RF, Ho C, Esmon CT, Cohen MJ:Extracellular histone release in response to traumatic injury: implications for a compensatory role of activated protein C. *J Trauma Acute Care Surg* 73(6),1389-1394 (2012) DOI: 10.1097/TA.0b013e318270d595
13. Keller M, Ruegg A, Werner S, Beer HD :Active caspase-1 is a regulator of unconventional protein secretion. *Cell* 132(5),818-831(2008) DOI: 10.1016/j.cell.2007.12.040
14. Abrams ST, Zhang N, Manson J, Liu T, Dart C, Baluwa F, Wang SS, Brohi K, Kipar A, Yu W, Wang G, Toh CH:Circulating histones are mediators of trauma-associated lung injury. *Am J Respir Crit Care Med* 187(2),160-169 (2013) DOI: 10.1164/rccm.201206-1037OC
15. Bjork I, Lindahl U :Mechanism of the anticoagulant action of heparin. *Mol Cell Biochem* 48(3): 161-182(1982) DOI: 10.1007/BF00421226
16. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ; ACCP/SCCM Consensus Conference Committee: Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. 1992. Chest 136 (5 Suppl),e28(2009) Doi not found.
17. Di Nisio M, Middeldorp S, Buller HR: Direct thrombin inhibitors. *N Engl J Med* 353(26), 1028-1040 (2005) DOI: 10.1056/NEJMra044440
18. Berry CN, Girardot C, Lecoffre C, Lunven C :Effects of the synthetic thrombin inhibitor argatroban on fibrin- or clot-incorporated thrombin: comparison with heparin and recombinant Hirudin. *Thromb Haemost* 72(3), 381-386(1994) Doi not found.
19. Chaput C, Zychlinsky A :Sepsis: the dark side of histones. *Nat Med* 15(11), 1245-1246(2009) DOI: 10.1038/nm1109-1245
20. Boos CJ, Goon PK, Lip GY:The endothelium, inflammation, and coagulation in sepsis. *Clin Pharmacol Ther* 79(1), 20-22(2006) DOI: 10.1016/j.clpt.2005.10.004
21. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, Lohmeyer J, Preissner KT:Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS One* 7(2), e32366(2012) DOI: 10.1371/journal.pone.0032366
22. Marti-Carvajal AJ, Sola I, Gluud C, Lathyris D, Cardona AF: Human recombinant protein C for severe sepsis and septic shock in adult and paediatric patients. *Cochrane Database Syst Rev* 12,CD004388 (2012)

Doi not found.

23. American College of Chest Physicians/ Society of Critical Care Medicine Consensus Conference: definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. *Crit Care Med* 20(6) 864-874(1992)
DOI: 10.1097/00003246-199206000-00025

24. Dellinger RP, Carlet JM, Masur H, Gerlach H, Calandra T, Cohen J, Gea-Banacloche J, Keh D, Marshall JC, Parker MM, Ramsay G, Zimmerman JL, Vincent JL, Levy MM; Surviving Sepsis Campaign Management Guidelines Committee:Surviving Sepsis Campaign guidelines for management of severe sepsis and septic shock. *Crit Care Med* 32(3),858-873(2004)
DOI: 10.1097/01.CCM.0000117317.18092.E4

25. Zarychanski R1, Doucette S, Fergusson D, Roberts D, Houston DS, Sharma S, Gulati H, Kumar A:Early intravenous unfractionated heparin and mortality in septic shock. *Crit Care Med* 36(11), 2973-2979(2008)
DOI: 10.1097/CCM.0b013e31818b8c6b

Abbreviations: NETs: neutrophil extracellular traps; DIC: disseminated intravascular coagulation; MODS: multiple organ dysfunction syndrome; APC: activated protein C; CRP: C-reactive protein; CLP: cecal ligation and puncture; EDTA: ethylenediamine tetra-acetic acid; HUVECs: Human umbilical vein endothelial cells; MPO: myeloperoxidase

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