Prognostic value of plasma exosomal levels of histone H3 protein in patients with heat stroke

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Abstract. Heat stroke (HS) is a condition that can lead to multiple organ dysfunction syndrome and death; however, there is no reliable method for stratifying mortality risk in HS. The abundance of exosomes in the circulation and their contents may be used as potential biomarkers of HS. The present study aimed to examine whether histone H3 levels in plasma exosomes could be used to determine HS prognosis. Blood samples were collected from patients with HS (36 survivors and 8 non-survivors) at admission to the intensive care unit and 4 days after admission. Blood samples were additionally collected from 15 healthy volunteers. Plasma exosomes were isolated using high-speed differential centrifugation. Correlation between histone H3 level and organ function and disease severity was examined. The results suggested differential expression and enrichment of histone H3 in the plasma exosomes of patients with HS (survivors, 249.3±04.6; non-survivors, 500.4±216.8; healthy controls, 161.1±52.49 pg/100 μg; P<0.05). The increased expression of histone H3 was associated with increased disease severity and duration. Plasma exosomal levels of histone H3 were significantly correlated with both organ dysfunction and disease severity (P<0.0001) and were significantly different between non-survivors and survivors (area under the receiver operating characteristic curve, 0.9668). A cutoff value of 307 pg/100 μg demonstrated optimized sensitivity (95%) and specificity (91.67%) for predicting mortality risk, suggesting that histone H3 levels in plasma exosomes may be a reliable biomarker for HS prognosis.

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Introduction

Heat stroke (HS) is a condition characterized by a core body temperature of >40˚C and neurological abnormalities. A less severe form of HS without neurological abnormalities is termed heat-induced illness. HS is recognized as a serious and prevalent disorder that may lead to secondary systemic inflammation and multiple organ dysfunction syndrome (MODS) (1,2). Epidemiological studies have shown that the average case fatality rate of HS in China is 10-15% and that the mortality rate of HS is as high as 40% (3). Therefore, early identification of critically ill patients and timely and effective interventions are crucial to improving the survival rate of patients with HS. However, there is currently no method for the evaluation of the severity and prognosis of HS, as current methods are sensitive but not specific and typically rely on comprehensive scores based on vital signs and routine biochemical tests (4,5), which may occur at later stages, meaning that treatment is initiated too late.

In recent years, many studies have shown that exosomes play an important role in the pathogenesis of various conditions, including pathogenic immune responses, inflammation, tumors and infection (6,7). Exosomes are vesicle-like structures measuring 30-150 nm in diameter and containing multiple bioactive molecules, including proteins, nucleic acids and lipids. According to previous studies, the levels and contents of plasma exosomes vary between healthy people and patients with various illnesses, such as sepsis, cardiogenic shock and alcoholic hepatitis (8-10), and the expression profiles of exosomal cargo are highly disease-related and disease-specific. Moreover, compared with freely-circulating substances in the plasma, exosomal contents, which are protected by the bilayer membrane of the exosome, are more stable and easier to transport within the circulatory system without being degraded. Therefore, the time window for their detection is wider (11-13). Taken together, these findings suggest that plasma exosomes and their contents can be used as novel and more reliable biomarkers of diseases (14).

Few studies have been conducted on exosomes and their relationship with HS. A previous study in dogs found that the plasma level of histone H3 significantly increased after HS occurrence and was correlated with multiple organ injury (5). Other nuclear proteins or other histone monomers may also

be released from the nucleus to the extracellular space which serve as danger signals in stressful conditions, as demonstrated in numerous disease models, such as sepsis, trauma and HS (15-17). In our previous study, proteomic analysis of HS induced exosomes was performed and histone H3 identified among the top 10 most upregulated proteins in HS-exosomes, with the highest fold change (14.62) among histone monomers and HMGB1 (18). The aim of the present study was to investigate whether the level of histone H3 in plasma exosomes in patients with HS could be considered as an early clinical indicator for disease severity, organ dysfunction and mortality risk. The study also aimed to investigate changes in plasma exosome levels of histone H3 in patients with HS and to assess their correlation with organ dysfunction and disease severity.

Materials and methods

Patient recruitment and clinical data collection. Between June 2016 and June 2019, 44 patients who were admitted to the intensive care unit (ICU) of the General Hospital of the Southern Theatre Command of the People's Liberation Army (PLA) of China, a tertiary hospital, within 24 h of HS occurrence were prospectively enrolled. Patients were diagnosed with HS according to the Expert Consensus on Diagnosis and Treatment of Heat Stroke in China (2019) (19), promulgated by the Expert Group on Prevention and Treatment of Heat Stroke and Critical Care Committee of the PLA of China. The diagnostic criteria were as follows: A medical history of i) exposure to high temperature and high humidity or ii) high intensity exercise; clinical manifestations of i) central nervous system dysfunction (e.g., coma, convulsion, delirium or abnormal behavior), ii) core body temperature >40°C and iii) multiple (≥2) organ dysfunction that could not be explained by other etiologies. Patients with malignant tumors, chronic liver or kidney diseases, chronic cardiac insufficiency (New York grades 3-4), chronic pulmonary insufficiency, underlying central nervous system disease or metabolic disorders were excluded. Patients were categorized as survivors (n=36) and non-survivors (n=8). Healthy subjects from the physical examination center (15) were enrolled as the control group. Baseline demographic characteristics were recorded on ICU admission (day 1). Body temperature was measured using an ear thermometer on day 1. Blood samples were collected on day 1 and after 4 days of treatment (day 4). Biochemical parameters (lactate, albumin, alanine aminotransferase, aspartate aminotransferase, urea nitrogen, creatine kinase, creatine kinase-myocardial band, creatinine, cardiac troponin I, fibrin degradation product, fibrin, international normalized ratio, myoglobin, partial pressure of arterial oxygen, procalcitonin, platelet, prothrombin time, total bilirubin, white blood cell count and D-dimer levels were assessed) reflecting organ function were collected from patients at the Laboratory of the General Hospital of the Southern Theatre Command of the People's Liberation Army (PLA) of China. Acute Physiology and Chronic Health Enquiry (APACHE) II (20) and Sequential Organ Failure Assessment (SOFA) (21) scores were also recorded. Written informed consent was obtained from all patients or their representatives. This study was approved by the Medical Ethics Review Committee of the General Hospital of the Southern Theater Command of the PLA of China.

Blood sample collection and exosome isolation. A 10 ml volume of blood was collected from the peripheral vein of each patient using ethylenediaminetetraacetic acid anticoagulant tubes. Blood collection tubes were left standing vertically at 22-27°C for 30 min. Whole blood was then centrifuged at 2,500 x g at 4°C for 10 min to isolate the plasma. Each plasma sample was diluted with the equivalent volume of phosphate-buffered saline (PBS) and subjected to three rounds of centrifugation at 4°C (2,500 x g for 30 min, 12,000 x g for 45 min and 110,000 x g for 2 h). Finally, the precipitate (exosomes) was resuspended in 50-200 μl of PBS and stored at -80°C, as previously described (12).

Observation of exosome morphology using transmission electron microscopy. Exosome samples were obtained from a healthy individual, a patient with mild HS (HS without multiple organ failure), and a patient with a severe HS (HS complicated with multiple organ failure) (randomly selected) and prepared for transmission electron microscopy (TEM) evaluation. Exosomes were precipitated in 50-100 μl 2% paraformaldehyde (diluted in PBS at 4°C). A 5 μl volume of exosome suspension was added to a formvar-coated copper grid (Mecalab Ltée), incubated for 30 min, washed in 100 μl of PBS, fixed in 2% paraformaldehyde for 10 min and then stained with 2% uranyl acetate dissolved in 50% ethanol for 15 min (all at 4°C). The samples were then visualized using a Philips CM10 transmission electron microscope (model no. JEM-2100F; Philips Healthcare).

Nanoparticle tracking analysis. To determine the level and size distribution of the isolated exosomes, nanoparticle tracking analysis (NTA) was performed using the NanoSight NS3000 (Malvern Panalytical; Spectris plc), according to the manufacturer's instructions. Briefly, the exosome samples were diluted to a final ratio of 1:5,000 in sterile PBS, and each sample was analyzed three times, each for 60 sec, using the NanoSight's automatic analysis settings.

Western blot analysis. Proteins extracted from the plasma exosomes using RIPA lysis buffer (Biosharp Life Sciences; cat. no. BL504A) with a protease inhibitor (Biosharp Life Sciences; cat. no. BL612A) and phenylmethanesulfonyl (Biosharp Life Sciences; cat. no. BL507A) at a ratio of 100:1:1. The protein concentration were determined using a BCA test kit (Biosharp Life Sciences; cat. no. BL521A). Protein (10 μg/lane) was loaded and isolated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred to a 0.2 μm nitrocellulose membrane. After blocking the nonspecific binding sites with bovine serum albumin (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at 25°C, the membrane was incubated with primary antibodies (diluted 1:1,000) at 4°C overnight and then incubated with HRP-conjugated secondary antibodies (Goat Anti-Mouse IgG H&L; cat. no. ab6789; 1:5,000) at 25°C for 1 h. The antibodies used were the following: Anti-CD63 (cat. no. ab216130), anti-CD9 (cat. no. ab223052), anti-CD81 (cat. no. ab155760), anti-Tsg-101 (cat. no. ab30871) and anti-GAPDH (cat. no. ab8245) and were all obtained from Abcam.

Histone H3 assay. Histone H3 levels in the plasma exosomes were measured using a commercial ELISA kit (Human

Table I. Comparison of patient characteristics measured on day 1 (admission) and day 4.

Characteristic	Healthy controls (15)		Survivors (36)		Non-survivors (8)		P-value
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	
Temperature (˚C)	36.42±0.46	36.78±0.26	38.58±1.62[b]	36.80±0.79[d]	39.11±1.81[b]	36.61±0.94[e]	<0.001
APACHE II score	0.47±0.83	0.40±0.83	9.11±7.46[b]	6.33±8.21[c]	16.13±530[b]	15.75±3.20[d]	<0.001
SOFA score	0.6±0.63	0.33±0.49	5.94±2.30[a]	7.42±6.99[d]	10.88±4.32[b]	16.5±2.98[d,f]	<0.001

APACHE, Acute Physiology and Chronic Health Evaluation; SOFA, Sequential Organ Failure Assessment. [a]P<0.05, [b]P<0.001 vs. day 1 in healthy controls; [c]P<0.05, [d]P<0.001 vs. day 4 in healthy controls; [e]P<0.001 vs. day 1 in non-survivors; [f]P<0.001 vs. day 4 in non-survivors.

Histone H3 ELISA kit; cat. no. MM-51796H; Jiangsu Enzyme Free Industrial Co., Ltd.). The protocol provided by the manufacturer was followed without modifications.

Statistical analysis. A kurtosis test was used to check the data for normality. Normally distributed data are presented as the mean ± standard deviation, while non-normally distributed data were presented as medians ± interquartile ranges. Each statistical analysis involving two groups was performed using a two-tailed Student's t-test for normally distributed data, Kruskal-Wallis H test for non-normal data and Fisher's exact test for categorical data. Mixed two-way ANOVA with both within-subjects (day) and between-subjects (patient group) factors with a post hoc test (simple main effects with a Bonferroni correction) was utilized to compare more than two groups. For categorical data, McNemar's test was used for comparisons within groups. Correlations were analyzed using the Spearman's rank correlation test. The discriminated ability of survivors and non-survivors by plasma exosome histone H3 was examined by receiver operator characteristic (ROC) curve analysis with comparisons to plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST). A P-value of <0.05 was considered statistically significant. All tests were performed using GraphPad software version 7.0 (GraphPad Software, Inc.).

Results

Comparison of baseline characteristics between healthy controls and patients with HS. Blood samples from 44 patients clinically diagnosed with HS and from 15 healthy controls were collected. All patients with HS and healthy controls were male and had few underlying diseases. The mean patient age was comparable between healthy controls and HS patients 25±6 (range 17-40) vs. 21±4 (range 16-38) years]. Clinical and laboratory data are summarized in Tables I-III. The overall 28-day mortality rate in patients with HS was 18.2% (8/44). The duration of ICU stay was significantly longer in non-survivors than in survivors (median: 10 vs. 7 days; P<0.001). Body temperatures on admission were similar between the HS survivor and non-survivor groups (38.58±1.62 and 39.11±1.81˚C, respectively) and were higher in these groups than in healthy controls (36.42±0.46˚C; both P<0.001). The majority of the patients were cooled prior to admission and the cooling strategies are presented in Table II. On day 4, significant resolution of hyperthermia was observed in both survivors and non-survivors (both P<0.001 vs. day 1; Table I).

Based on the results of routine biochemical examinations performed on day 1, few of the routine biochemical parameters reflecting organ dysfunction were significantly different between non-survivors and survivors. These were hemodynamic instability (as measured by an increased need for vasoactive drugs and increased lactate levels), respiratory impairment (an increased need for mechanical ventilation), need for continuous renal replacement therapy and presence of coagulation abnormalities [prolonged international normalized ratio (INR), increased fibrinogen degradation product (FDP) levels and decreased fibrinogen levels], rhabdomyolysis (increased creatine kinase and myoglobin levels) and central nervous dysfunction [decreased Glasgow coma scale (GCS) score]. Meanwhile, there were no significant differences between survivors and non-survivors in terms of heart rate (HR), mean arterial pressure, PaO_2/FiO_2 ratio, liver injury indices [total bilirubin (TBil) level and ALT]/AST ratio], renal function [creatinine levels, blood urea nitrogen (BUN) levels, and urine output], prothrombin and D-dimer levels, cardiac injury index [cardiac troponin-I (cTnI)], and levels of the inflammatory marker procalcitonin. Myoglobin levels were significantly higher in HS survivors than in healthy controls but were lower in non-survivors than in survivors. Notably, TBil levels, renal parameters, and D-dimer and cTnI levels did not appear to be significantly different between non-survivors and survivors until day 4. On day 1, both the APACHE II and SOFA scores, which were used to assess overall disease severity, were not significantly different between non-survivors and survivors, whereas SOFA scores were higher among non-survivors only on day 4. Hemodynamic indices, mechanical ventilation (MV) ratios, TBil levels, and renal parameters in non-survivors deteriorated from day 1 to day 4, while the remaining indicators of organ function remained unchanged. Among survivors, most variables were not significantly different between day 1 and 4, except for white blood cell count and procalcitonin levels (Tables I-III).

Characterization of plasma exosomes isolated from healthy subjects and patients with HS. TEM was performed for the isolated plasma exosome samples obtained from healthy controls and surviving and non-surviving patients with HS. Double-membrane vesicle-like structures of ~100 nm in diameter were observed in the samples from each group (Fig. 1A). According to the NTA results, the level of plasma exosomes was higher in both the survived and non-survived HS group than in the control group ($6.10x10^9$ and $3.37x10^9$ vs.

Table II. Comparison of patient characteristics measured at a single time point.

Characteristic	Healthy controls (15)	Survivors (36)	Non-survivors (8)	P-value
Median length of ICU stay/days (IQR)	N/A	7 (5-8.65)	10 (3-17.45)	<0.001
Cooling strategy				
Alcohol wipe, n (%)	N/A	33 (91.2)	8 (100)	0.643
Ice pack, n (%)	N/A	36 (100)	8 (100)	0.987
Water bath, n (%)	N/A	1 (2.8)	2 (25)	<0.01
Cooled saline infusion, n (%)	N/A	30 (83.3)	7 (87.5)	0.345
Cooled saline gastric lavage, n (%)	N/A	1 (2.8)	1 (12.5)	<0.05
CRRT with cooled dialysate, n (%)	N/A	2 (5.6)	2 (25)	<0.01
Time lag between HS onset and ICU admission	N/A	1.28±0.14	1.25±0.3	0.931

ICU, intensive care unit; IQR, interquartile range; CRRT, continuous renal replacement therapy; HS, heat stroke. Time lag was assessed using the Kruskal-Wallis H test.

0.66x10^9 particles/ml), while the diameter distribution was comparable between the two groups, the peak value was 76 nm in the control group vs. 78 nm for the survived HS group and 67 nm for the non-survived HS group; the proportion of particles with a diameter of 30-200 nm was 94.7% in the control group vs. 78.2% for the survived HS group and 97.1% for the non-survived HS group (Fig. 1B). The proportion of particles with a diameter >200 nm was much higher in the HS group. It can be hypothesized that this may be due to the increased presence of contaminant microparticles/microvesicles in the HS group as a result of increased cell injury/death. Western blot analysis indicated that the characteristic exosomal surface markers CD9, CD63 and CD81 (22) were positively expressed in both the control and survived and non-survived HS groups (Fig. 1C). Therefore, the characteristics of the extracted vesicles were consistent with those of plasma exosomes.

Enrichment of histone H3 in plasma exosomes in patients with HS. The levels of histone H3 in exosomes and free histone H3 in plasma were detected by ELISA. Histone H3 levels in plasma exosomes on days 1 and 4 after admission were significantly higher in HS non-survivors than in both HS survivors and healthy controls (all P<0.001). On admission, a 2.0-fold increase in histone H3 levels was observed in non-survivors in comparison with survivors. However, there were no significant changes in plasma exosome levels of histone H3 between survivors and healthy controls (P>0.05). Additionally, on day 4, histone H3 levels in plasma exosomes significantly increased in non-survivors (P<0.001) but decreased, though not significantly in survivors (P>0.05), compared with levels on day 1. Moreover, levels of free histone H3 in plasma on days 1 and 4 were significantly higher in HS non-survivors than in healthy controls (all P<0.001). However, no significant changes in plasma exosomal levels of histone H3 were observed between survivors and healthy controls (P>0.05) on both days 1 and 4. On day 4, levels of free histone H3 in plasma were elevated in non-survivors (P<0.001) but remained unchanged in survivors (P>0.05), compared with levels on day 1 (Fig. 2).

Correlation of histone H3 levels in plasma exosomes with organ function and disease severity in HS patients. The histone H3 levels in plasma exosomes on ICU admission were significantly positively correlated with numerous organ function parameters, including HR, lactate levels, ALT levels, creatinine levels, BUN levels, prothrombin levels, INR, and D-dimer levels, as well as APACHE II and SOFA scores on days 1 and 4; AST, TBil, myoglobin, and troponin-I levels on day 1; and FDP levels on day 4. By contrast, the plasma exosomal levels of histone H3 were negatively correlated with PaO$_2$/FiO$_2$ ratios, urine output, fibrinogen levels, platelet counts, and GCS scores on both days 1 and 4 and with albumin levels on day 4 (Table IV). However, all correlations between plasma exosomal levels of histone H3 and organ function indicators on day 1 were poor (all r<0.6), except for that with D-dimer levels (r=0.78), whereas the correlations between plasma exosomal levels of histone H3 and organ function indicators improved on day 4.

The area under the receiver operating characteristic (ROC) curve of plasma exosomal levels of histone H3 for discriminating between non-survivors and survivors was 0.9668 [95% confidence interval (CI), 0.9231-1.014; P<0.001]. At a cutoff value of 307 pg/100 μg, the sensitivity and specificity for predicting mortality risk were 95 and 91.67%, respectively (Fig. 3). The area under the ROC of exosome histone H3 was higher than both plasma AST (0.7882; 95% CI 0.5602-1.016; P=0.01158) and similar with ALT (0.9028; 95% CI, 0.8121-0.9935; P<0.001; Fig. 3).

Discussion

The present study aimed to evaluate changes in plasma exosomal levels of histone H3 in HS patients and their correlation with organ function and disease severity. The mortality of patients with severe HS was as high as 18.2% and non-survivors were characterized by a prolonged ICU stay, more severe hyperthermia on admission and a higher incidence of subsequent multiple organ dysfunction. Histone H3 was enriched in the plasma exosomes of patients with HS and was expressed at higher levels in non-survivors than in survivors. The abundance of histone H3 in plasma exosomes also decreased during the course of the disease in survivors but increased in non-survivors. There was a significant

Table III. Comparison of clinical and laboratory values of patients with heat stroke and healthy controls according to day of admission and outcome status.

Characteristics	Healthy controls (n=15)		Survivors (n=36)		Non-survivors (n=8)		P value
	Day 1	Day 4	Day 1	Day 4	Day 1	Day 4	
Hemodynamic data							
HR (beats/min)	74.3±7.74	73.80±4.93	86.67±26.79[a]	91.28±18.49	91.38±35.50	95.13±18.34	0.0092
MAP (mmHg)	77.6±6.99	77.53±7.62	75.53±15.45	77.08±16.48	76.13±19.79	65.00±14.32	0.3998
Vasoactive drug, n (%)	0 (0)	0 (0)	6 (16.7)[b]	5 (13.9)[e]	3 (37.5)[i,c]	4 (50)[o,f,j]	<0.001
Lactate (μmol/l)	1.07±0.47	0.91±0.48	1.91±1.93	2.11±2.28[d]	3.39±1.97[b,g]	3.78±1.84[d]	0.0012
Ventilatory data							
PaO$_2$/FiO$_2$	378.7±72.25	394.3±31.99	319.7±53.59	294.0±80.74	313.1±71.52[c]	302.9±72.66[d]	0.3158
MV, n (%)	0 (0)	0 (0)	7 (19.4)[a]	12 (33.3)[e]	5 (62.5)[h,c]	7 (87.5)[n,f,j]	<0.001
Inflammation data							
WBC (x10^9 cells/l)	9.42±3.14	6.60±1.42	11.34±5.48	7.08±3.01[g]	11.72±5.92	7.80±3.64	<0.001
PCT (ng/ml)	0.34±0.34	0.38±0.29	2.88±3.63	5.50±16.56[g]	3.27±2.32	16.92±27.79[e]	0.0211
Hepatic data							
ALT (U/l)	25.51±13.7	19.9 ±8.94	414.6±1437	727.4.4±1294	1832±2463[a]	1788±1190[d]	0.0013
AST (U/l)	22.4±13.8	24.07±9.04	354.9±1323[c]	329.9±442.8	3905±5177[c]	2261±4160[f]	<0.001
TBil (μmol/l)	9.29±4.45	14.29±4.79	36.28±73.123	55.70±72.45	39.81±28.63	225.2±218.3[f,o,l]	<0.001
ALB (g/l)	40.79±5.36	43.03±3.31	39.26±3.63	40.69±5.73	35.94±3.77	37.00±1.90	0.0046
Renal data							
Cr (μmol/l)	95.4±27.28	70.8±12.2	125.1±55.72	114.9±79.13	162.4±100.44	190.0±87.00[f,m]	<0.001
BUN (mmol/l)	5.51±2.19	5.13±1.51	6.84±5.30	7.29±5.09	6.76±2.52	16.05±9.97[f,o,k]	<0.001
Urine output (ml/d)	2680±727.2	2521±530.7	2570±1131	1863±1488	1939±1044	122.9±107.1[f,n,j]	<0.001
CRRT, n (%)	0 (0)	0 (0)	12 (33.3)[a]	6 (16.7)	6 (75)[e]	8 (100)[d,n]	<0.001
Coagulation data							
PT (s)	13.39±0.93	13.53±0.96	19.76±12.16	18.01±7.71	24.73±7.69[a]	24.50±7.03[d]	0.0024
INR	1.02±0.09	1.087±0.11	1.80±1.72	1.70±1.26	2.18±0.87[i,e]	3.08±1.27[e]	0.0031
Fib (g/l)	3.53±0.66	3.78±0.76	2.23±0.67[b]	3.39±1.90	2.01±0.53[h]	2.13±1.42[d]	<0.001
PLT (x10^9/l)	219.6±65.05	195.7±74.14	139.0±57.47[b]	154.8±74.85	85.13±40.83[c]	80.00±18.31[f]	<0.001
D-dimer	1.46±1.32	0.56±0.35	6.46±7.55[c]	3.93±5.80	16.14±6.00[c]	16.46±5.85[o,f]	<0.001
FDP	6.8±2.88	4.19±0.98	32.55±64.79	27.73±41.21	133.2±276.3[g,b]	86.90±52.09	0.0044
Rhabdomyo lysis data							
CK (μg/l)	54.3±23.52	63.6±24.85	1582±2432	1042±1680	7999±7799[i,c]	5185±2444[f,n]	<0.001
MYO (μg/l)	48.15±24.91	25.39±24.85	8876±1024[b]	285.0±634.8[a]	1050±516.7[g]	896.9±1299	<0.001
Cardiac data							
CK-MB	2.54±1.45	1.73±1.11	14.51±18.07	9.12±20.50	51.55±76.75[a]	47.26±102.4[d]	0.0028
cTnI	12.76±9.94	4.74±2.69	424.9±935.1	65.13±115.3	459.6±398.0	1349±3497[d,m]	0.0257
CNS data							
GCS score	15±0	15±0	12.03±4.05	11.86±3.74[d]	8.0±4.50[g,c]	7.38±3.46[m,f]	<0.001

ALB, albumin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; CK-MB, creatine kinase-myocardial band; CNS, central nervous system; Cr, creatinine; cTnI, cardiac troponin I; FDP, fibrin degradation product; Fib, fibrin; FiO$_2$, percentage of inspired oxygen; GCS, Glasgow Coma Scale; HR, heart rate; INR, international normalized ratio; MAP, mean arterial pressure; MV, mechanical ventilation; MYO, myoglobin; PaO2, partial pressure of arterial oxygen; PCT, procalcitonin; PLT, platelet; PT, prothrombin time; TBil, total bilirubin; WBC, white blood cell count. [a]P<0.05, [b]P<0.01, [c]P<0.001 vs. day 1 in healthy controls; [d]P<0.05, [e]P<0.01, [f]P<0.001 vs. day 4 in healthy controls; [g]P<0.05, [h]P<0.01, [i]P<0.001 vs. day 1 in survivors; [j]P<0.05, [k]P<0.01, [l]P<0.001 vs. day 1 in non-survivors; [m]P<0.05, [n]P<0.01, [o]P<0.001 vs. day 4 in survivors.

correlation between plasma exosomal levels of histone H3 with both organ dysfunction (as assessed by SOFA scores) and disease severity (as assessed by APACHE II scores) and the histone H3 levels in plasma exosomes were significantly different between non-survivors and survivors (area under the ROC curve: 0.9250). The sensitivity and specificity for
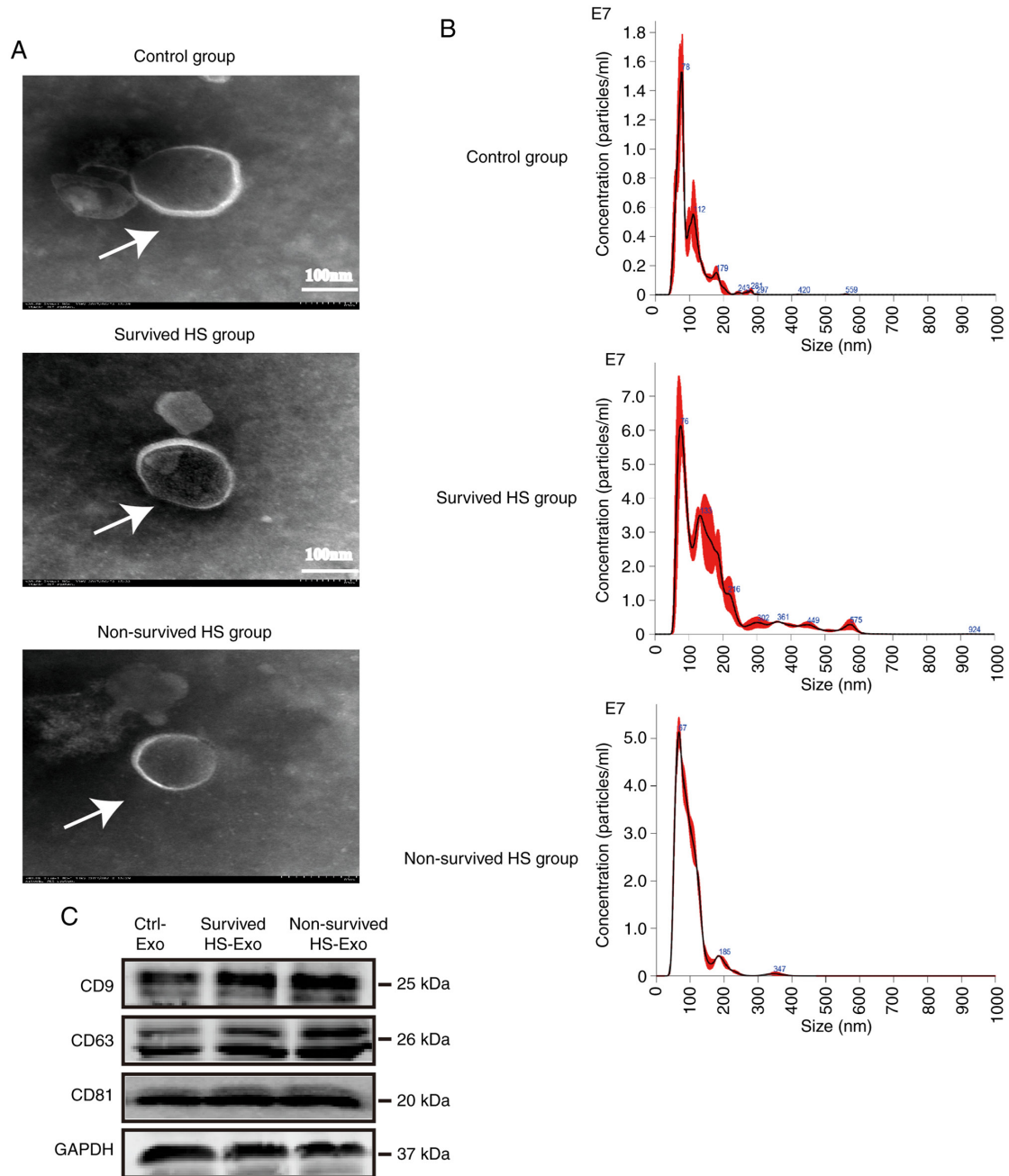
Figure 1. Characterization of plasma exosomes in healthy controls and surviving and non-surviving patients with HS. (A) Morphology of plasma exosomes visualized under transmission electron microscopy. White arrow indicates the bilayer round vesicles of ~100 nm in diameter (scale bar=100 nm). (B) Size distribution of exosomes detected by nanoparticle tracking analysis. (C) Expression of the characteristic exosomal surface markers CD9, CD63 and CD81 following western blot analysis. All experiments were repeated three times. HS, heat stroke.

predicting mortality risk were optimized at a histone H3 cutoff value of 307 pg/100 μg.

Despite the early introduction of intensive cooling strategies to treat HS, the mortality of patients with severe HS in the present study remained as high as 18.2% and was often associated with MODS. Such a high mortality rate may be partly due to the fact that this study was conducted in a tertiary hospital, which receives a high number of critically ill patients transported from local clinics and emergency departments.

Currently, the methods for accurately assessing the severity and prognosis of HS are limited (1). Circulating biochemical markers have been proposed to indicate organ failure, facilitate an accurate diagnosis and indicate prompt treatment in patients

with HS. These biomarkers include high-mobility group box protein 1 (15), neutrophil gelatinase-associated lipocalin (also known as 24p3, uterocalin, or neu-related lipocalin) (23), cTnI (24), the ratio of urinary heat shock protein 72 to urinary creatinine (25), histones (5) and cryptdin-2 peptide (an intestinal α-defensin) (26). However, these biomarkers are still at the experimental stage and have not been clinically evaluated or approved. As novel diagnostic biomarkers, exosomes and their contents have gained attention for several reasons (27). Exosomes are superior to freely-circulating substances in plasma as they contain highly specific cell, organ and disease-related substances, due to their bioactive roles in sorting and transporting exosomal cargo in response to different stimuli
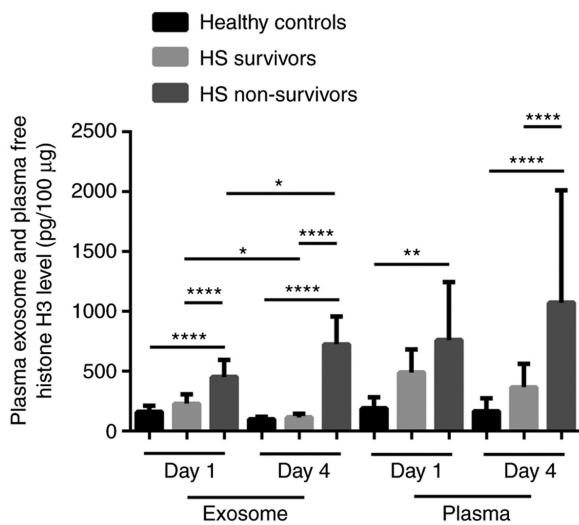
Figure 2. Histone H3 levels in plasma exosomes and free plasma of healthy controls and patients with HS (survivors and non-survivors) on days 1 and 4. Plasma exosomal levels of histone H3 were significantly higher in non-survivors than in healthy controls and survivors on both days 1 and 4. Plasma exosomal levels of histone H3 in non-survivors were significantly higher on day 4 than on day 1. Plasma histone H3 levels on day 1 and 4 were significantly higher in the HS non-survivors than healthy controls. There were no significant changes in plasma exosome histone H3 levels between survivors and healthy controls both on day 1 and 4. On day 4, plasma free histone H3 levels were elevated in non-survivors and remain unchanged in survivors compared to day 1. HS, heat stroke. *P<0.05, **P<0.01, ****P<0.0001.
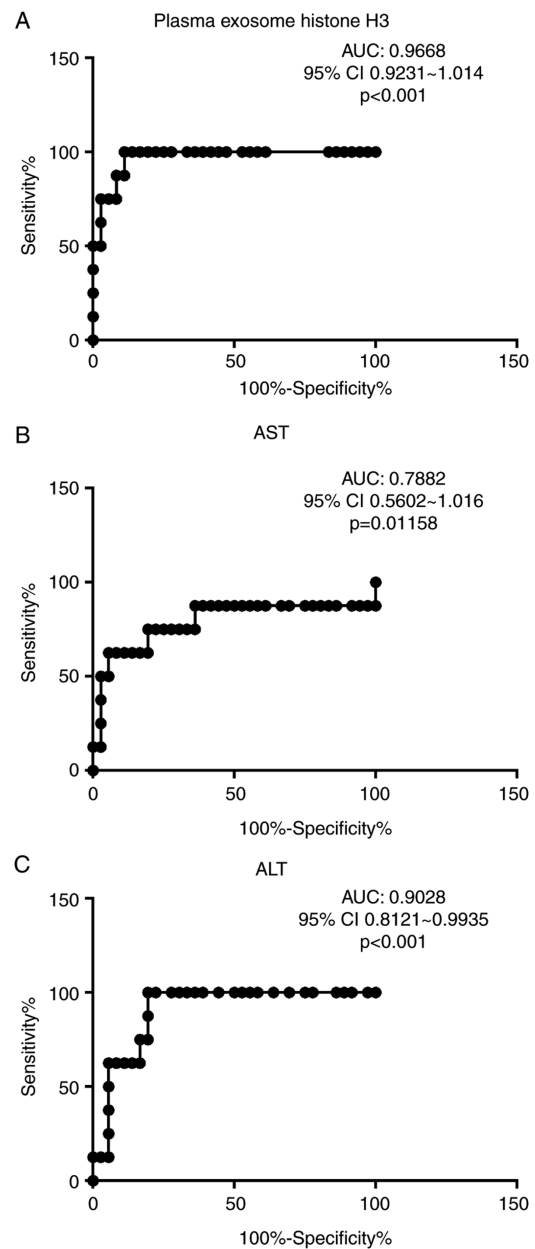

Figure 3. ROC curves for the discrimination of survivors and non-survivors among patients with HS. (A) ROC curve of plasma exosomal levels of histone H3 for discriminating between survivors and non-survivors among patients with HS. (B) ROC curve of plasma AST for the discrimination of survivors and non-survivors among patients with HS. (C) ROC curve of plasma ALT for the discrimination of survivors and non-survivors among the patients with HS. ROC, receiver operator characteristic; HS, heat stroke; AST, aspartate aminotransferase; ALT, alanine aminotransferase; AUC, area under the curve; CI, 95% confidence interval.

or insults (27). Moreover, the active components within the exosome's double-membrane surface are protected from degradation, allowing for the preservation of biological substances in various environments and thereby providing a wider time window for detection (6). Conversely, the diagnostic value of active components directly exposed to plasma is significantly reduced, as a result of chemical instability, resulting in a short half-life. This narrows the time window for detection and increases the rate of false negatives. Furthermore, efforts to discover valuable and novel serum or plasma biomarkers are often impeded by the abundance of background blood components (13). In this regard, isolated exosomes could be preferred as alternative biomarkers, as they have bioactive roles within the cell and are more stable and resistant to degradation, which could increase the sensitivity of diagnosis.

Serum histones have been identified as biomarkers of the severity of HS in dogs (5). In the present study, the value of plasma exosomal levels of histone H3 as a potential prognostic indicator for severe HS were clinically verified. Compared with extracellular forms of histones, which are passively released as a consequence of cell death and destruction of chromatin induced by severe injury (28), the current authors hypothesized that the release of exosomes may be a relatively early event that occurs in the initial phase of injury through an active mode independent of cell death. In a previous experiment from our team (data not shown), histones in HS hepatic exosomes were not indicated to be derived from dead cells and inhibition of apoptosis or necrosis did not significantly affect the number of exosomes released by the HS hepatocytes nor the levels of the exosomal histone H3. This would render exosomal histone levels more sensitive to detection than many other proposed markers. In the present study, experiments

were not performed to determine the cell origin of plasma exosomes. In our previous study, proteomic analysis of HS stimulated hepatocyte exosomes suggested that histone H3 was among the top 10 most upregulated proteins with a fold change of 14.62. It could be plausible to hypothesize that the exosome H3 comes from the liver (18).

Histones are highly conserved intranuclear proteins that traditionally serve to maintain the structural conformation and stability of chromatin. They have also been recognized to function as endogenous damage-associated molecular pattern molecules upon their release into the extracellular

Table IV. Correlation between day 1 plasma exosome histone H3 levels and laboratory indicators on days 1 and 4.

Laboratory indicators	Day 1		Day 4	
	R-value	P-value	R-value	P-value
Hemodynamic data				
HR (beats/min)	0.2968	0.0225	0.5417	<0.001
MAP (mmHg)	-0.3292	0.0109	-0.2744	0.0354
Lactate (μmol/l)	0.3443	0.0076	0.3903	0.0022
Ventilatory data				
PaO$_2$/FiO$_2$	-0.2493	0.0569	-0.3503	0.0065
Inflammatory data				
WBC (x10^9 cells/l)	-0.0005	0.9971	0.08678	0.5134
PCT (ng/ml)	0.3667	0.0043	0.4608	<0.001
Hepatic data				
ALT (U/l)	0.3674	0.0042	0.4426	<0.001
AST (U/l)	0.3832	0.0027	0.2844	0.0291
TBil (μmol/l)	0.2526	0.0536	0.5034	<0.001
ALB (g/l)	-0.3465	0.0072	-0.3632	0.0075
Renal data				
Cr (μmol/l)	0.3693	0.0040	0.6858	<0.001
BUN (mmol/l)	0.1343	0.3107	0.5436	<0.001
Urine output (ml/day)	-0.2157	0.1009	-0.7205	<0.001
Coagulation data				
PT (s)	0.4661	<0.001	0.4916	<0.001
INR	0.4056	0.0014	0.6214	<0.001
Fib (g/l)	-0.4179	0.0010	-0.4945	<0.001
PLT (x10^9/l)	-0.4627	<0.001	-0.5376	<0.001
D-Dimer	0.6767	<0.001	0.7310	<0.001
FDP	0.2662	<0.001	0.6268	<0.001
Rhabdomyolysis data				
CK (μg/l)	0.4830	<0.001	0.7936	<0.001
MYO (μg/l)	0.4611	<0.001	0.4851	<0.001
Cardiac data				
CK-MB	0.3649	0.0053	0.2486	<0.001
cTnI	0.3232	0.0126	0.1850	0.1606
CNS data				
GCS score	-0.6999	<0.001	-0.7474	<0.001
APACHE II score	0.6909	<0.001	0.7738	<0.001
SOFA score	0.6888	<0.001	0.8111	<0.001

ALB, albumin; ALT, alanine aminotransferase; APACHE, Acute Physiology and Chronic Health Evaluation; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CK, creatine kinase; CK-MB, CK-myocardial band; CNS, central nervous system; Cr, creatinine; cTnI, cardiac troponin I; FDP, fibrin degradation product; Fib, fibrin; FiO$_2$, percentage of inspired oxygen; GCS, Glasgow Coma Scale; HR, heart rate; INR, international normalized ratio; MAP, mean arterial pressure; MV, mechanical ventilation; MYO, myoglobin; PaO$_2$, partial pressure of arterial oxygen; PCT, procalcitonin; PLT, platelet; PT, prothrombin time; SOFA, Sequential Organ Failure Assessment; TBil, total bilirubin; WBC, white blood cell.

space under different types of pathological stress, such as injury or infection (28), to promote inflammation and tissue damage (16). Specifically, histones trigger sterile inflammation, resulting in cell death and organ injury, by interacting with toll-like receptors (TLRs), including TLR2, TLR4 and TLR9. The administration of a sublethal dose of histones to mice resulted in an intra-alveolar hemorrhage in the lung along with neutrophil margination and accumulation (17). Xu *et al* (29) also suggested that extracellular histones could be released and act as major mediators of endothelial dysfunction and organ failure in response to inflammatory processes such as sepsis. Moreover, in concanavalin A and acetaminophen liver toxicity models, histones were released and shown to be critical mediators of liver cell death through TLR2

or TLR4 (30). Conversely, in animal models of acute organ injury, neutralization of circulating histones was a protective factor against mortality (31).

In the present study, the elevation of exosomal histone levels in patients with HS and its correlation with disease severity also suggest that histones may be potential targets for alleviating HS-induced injuries. There are currently three therapeutic strategies that antagonize the deleterious effects of histones: Blocking the release of histones, neutralizing circulating histones and inducing competing intracellular signal transduction (32). These strategies have been shown to be beneficial in reducing acute organ injury related to sepsis, trauma and toxicity, among others, in animal models (33,34). However, the drugs administered in these strategies are directed to target circulating histones or neutrophil extra-cellular trap-associated histones, whereas interfering with intracellular signaling pathways may disrupt DNA structure or function, which could cause catastrophic adverse effects (35). The results of the present study suggest that the blockade of histones in exosomes may provide new therapeutic targets. Inhibition of the production of exosomes by GW4869 has been shown to confer protection against cardiac injury from sepsis (36).

The present study has a number of limitations. There is currently no standard method for isolating exosomes. The current preferred method is based on ultracentrifugation, which has limited accessibility and reproducibility in clinical practice. The possibility of acquiring purer exosomes remains the main obstacle in research as well as in clinical practice (6). Ultracentrifugation applied in the present study was not able to 100% exclude contaminant microparticles/microvesicles or apoptotic bodies. Purer EV preparations may be obtained by using density gradients or size exclusion chromatography, which may be less accessible and more expensive in clinical practice (6). This study had a relatively small sample size, which makes it difficult to generalize the results to different populations. Furthermore, patients were enrolled from only one hospital, which might lead to selection bias. Mechanisms by which H3 localize to exosomes is a subject of investigation in future experiments.

In conclusion, the present study demonstrated that histone H3 in plasma exosomes may be an innovative and effective marker for risk stratification and prognosis in patients with severe HS and thus, may have potential clinical applications. Further studies with larger sample sizes are required to validate these findings.

Acknowledgments

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HT and LS contributed to the study design and confirmed the authenticity of all the raw data. YL collected and interpreted the patient data and mainly contributed to writing the manuscript. XS performed the statistical analyses. ZL obtained ethics approval from the hospital and performed the characterization of exosomes. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Review Committee of the General Hospital of the Southern Theater Command of the PLA. Written informed consent was obtained from all patients or their representatives.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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