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HIGH-MOLECULAR-WEIGHT HYALURONAN—A POTENTIAL ADJUVANT TO FLUID RESUSCITATION IN ABDOMINAL SEPSIS?

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ABSTRACT— While fluid resuscitation is fundamental in the treatment of sepsis-induced tissue hypoperfusion, a sustained positive fluid balance is associated with excess mortality. Hyaluronan, an endogenous glycosaminoglycan with high affinity to water, has not been tested previously as adjuvant to fluid resuscitation in sepsis. In a prospective, parallel-grouped, blinded model of porcine peritonitis sepsis, we randomized animals to intervention with adjuvant hyaluronan (add-on to standard therapy, n = 8) or 0.9% saline (n = 8). After the onset of hemodynamic instability, the animals received an initial bolus of 0.1% hyaluronan (1 mg/kg/10 min) or placebo (0.9% saline) followed by a continuous infusion of 0.1% hyaluronan (1 mg/kg/h) or saline during the experiment. We hypothesized that the administration of hyaluronan would reduce the volume of fluid administered (aiming at stroke volume variation <13%) and/or attenuate the inflammatory reaction. Total volumes of intravenous fluids infused were 17.5 ± 11 versus 19.0 ± 7 mL/kg/h in intervention and control groups, respectively (P = 0.442). Plasma IL-6 increased to 2,450 (1,420–6,890) pg/mL and 3,690 (1,410–11,960) pg/mL (18 hours of resuscitation) in the intervention and control groups (nonsignificant). The intervention counteracted the increase in proportion of fragmented hyaluronan associated with peritonitis sepsis (mean peak elution fraction [18 hours of resuscitation] intervention group: 16.8 ± 0.9 versus control group: 17.9 ± 0.6 [P = 0.031]). In conclusion, hyaluronan did not reduce the volume needed for fluid resuscitation or decrease the inflammatory reaction, even though it counterbalanced the peritonitis-induced shift toward increased proportion of fragmented hyaluronan.

KEYWORDS—Animal model; peritonitis; inflammation; fluid therapy; colloid

INTRODUCTION

Sepsis is associated with cardiovascular compromise due to absolute and relative hypovolemia, vasodilation, myocardial depression (1), and derangements of the microcirculation (2). While effective fluid resuscitation is essential to antagonize sepsis-induced tissue hypoperfusion (3), the optimal approach to fluid therapy has not been established (4).

A positive fluid balance is associated with higher mortality in septic patients (5). Crystalloids are the first-line fluids recommended for resuscitation in patients with sepsis and septic shock, whereas albumin can be added to the resuscitation strategy when considerable quantities of crystalloids are needed (3). Resuscitation with albumin may be associated with lower total volume administered as compared with crystalloids (6). However, its role as a resuscitation fluid in sepsis is not clear (7,8). The administration of other colloids is not recommended for volume resuscitation in sepsis/septic shock (3,7).

Hyaluronan (HA) is a polyanionic, linear glycosaminoglycan, composed of alternating β-D-glucuronate and *N*-acetyl-β-D-glucosamine (9,10). Because of its large molecular size and negative charges, HA has pronounced hydrophilic and colloid osmotic properties (11,12). The dominating forms of HA *in vivo* have molecular weights of greater than 1,000 kDa and are referred to as high-molecular-weight HA (HMW-HA) (13). High-molecular-weight HA is an important constituent of the endothelial glycocalyx layer (13) and is paramount in the maintenance of vascular integrity (14,15).

Inflammation leads to shedding of HA from the vascular endothelial layer (16). Degradation of HMW-HA is mediated by hyaluronidases (17), as well as by reactive oxygen (18) and nitrogen species (19). While intact HMW-HA exhibits anti-inflammatory properties (20), degraded, or low-molecular-weight HA (LMW-HA), has a proinflammatory effect (21). Hyaluronan in plasma has a high turnover rate with a half-life of 2–5 minutes and is removed primarily by the liver (9). Elevated levels of plasma HA correlate with more severe disease in critical illness, but studies have rendered conflicting results regarding any possible association with mortality (22–25).

Pretreatment with systemically administered HMW-HA in a rat model of sepsis and mechanical ventilation did not only improve macrocirculation but also reduced the inflammatory response in the lung as well as the degree of lung injury (26).

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Intravenous administration of HA in humans did not result in any serious adverse events (10). Systemically administered HMW-HA has not been studied in peritonitis-induced sepsis in larger animals or in humans.

We developed, to the best of our understanding, a clinically relevant intensive care, fecal peritonitis/sepsis model to test the hypothesis that administration of HMW-HA would reduce the volume of fluid administered during resuscitation and/or attenuate the hyper-inflammatory state associated with peritonitis-induced sepsis.

MATERIALS AND METHODS

Animals and ethic statements

The study (protocol: dx.doi.org/10.17504/protocols.io.bwt5peq6) was approved by the Animal Ethics Committee in Uppsala, Sweden (decision 5.8.18-01054/2017, DOUU 2019-014). The care of the animals was carried out in strict accordance with the National Institute of Health guide for the care and use of Laboratory animals (27), and all efforts were made to minimize suffering. After premedication and induction of anesthesia, all the animals received continuous intravenous analgesia and were under deep anesthesia. No animal was awake during any moment of the experiment. The study was performed at the Hedenstierna Laboratory, Uppsala University, Sweden.

Anesthesia and instrumentation

Sixteen pigs (*Sus scrofa domesticus*) of mixed Swedish, Hampshire, and Yorkshire breeds of both sexes (mean weight 29.4 ± 1.4 kg) were premedicated with Zoletil Forte (Virbac, Kolding, Denmark) (tiletamine and zolazepam) 6 mg/kg and Rompun (Elanco Denmark ApS, Ballerup, Denmark) (xylazine) 2.2 mg/kg i.m. After adequate sedation was established we placed the animals in a supine position and introduced a peripheral intravenous catheter in an ear vein. After a bolus of fentanyl (Braun, Danderyd, Sweden) of 5–10 μg/kg i.v., anesthesia was maintained with ketamine (Abcur, Helsingborg, Sweden) 30 mg/kg/h, midazolam (Accord-Healthcare, Solna, Sweden) 0.1–0.4 mg/kg/h, and fentanyl 4 μg/kg/h, in glucose 2.5% (Baxter, Kista, Sweden) during the whole experiment. After adequate depth of anesthesia was assured by absence of reaction to pain stimulus between the front hooves, rocuronium (Braun, Danderyd, Sweden) 2.5 mg/kg/h was added as muscle relaxant. Ringer's acetate (Baxter, Kista, Sweden) was infused i.v. at a rate of 30 mL/kg/h during the first hour and thereafter tapered down to 10 mL/kg/h until the induction of peritonitis.

The animals were under deep anesthesia during the whole experiment (up to 18 hours of sepsis after onset of circulatory instability), including euthanasia. Bolus doses of 100-mg ketamine i.v. were administered if signs of distress or reaction to pain stimulus were noted. In case an animal presented with refractory shock, it was euthanized just before circulatory collapse (rapidly decreasing systemic arterial pressure, bradycardia and a decrease in end-tidal CO_2).

The animals were tracheostomized and a tube with an internal diameter of 8 mm (Mallinckrodt Medical, Athlone, Ireland) was inserted in the trachea and connected to a ventilator (Servo I, Maquet, Solna, Sweden). Thereafter, volume controlled ventilation was maintained as follows: tidal volume 8 mL/kg, respiratory rate 25/min, inspiratory/expiratory time 1:2, inspired oxygen concentration (F_iO_2) 0.3, and positive end-expiratory pressure (PEEP) 8 cmH_2O. The settings of tidal volume, inspiratory/expiratory time, and PEEP were maintained constant throughout the protocol. Respiratory rate was adjusted aiming at arterial partial pressure of carbon dioxide ($PaCO_2$) less than 6.5 kPa, while FiO_2 was adjusted to keep arterial partial pressure of oxygen (PaO_2) greater than 10 kPa.

A triple-lumen central venous catheter for fluid infusions and a pulmonary artery catheter (Edwards Life-Science, Irvine CA) for measurement of pulmonary artery pressures and cardiac output (CO) were inserted *via* the right jugular vein. An arterial catheter for blood pressure measurement and blood sampling was inserted *via* the right carotid artery. A PiCCO (pulse index continuous CO) catheter (Pulsion, Munich, Germany) was inserted *via* the right femoral artery for estimation of stroke volume variation (SVV). Blood gas analyses were performed immediately after sampling and executed on an ABL 3 analyzer (Radiometer, Copenhagen, Denmark). Hemoglobin (hgb) and hgb oxygen saturation (SaO_2) were analyzed with a hemoximeter OSM 3 (Radiometer, Copenhagen, Denmark) calibrated for porcine hgb.

We performed a midline laparotomy and catheterized the bladder for urinary drainage. Transit-time flow probe (3 mm; Transonic Systems, Ithaca, New York) was applied around the renal artery. The flow probe was connected to a dual channel flow-meter (T 402; Transonic System, Inc, New York) and renal blood flow was recorded continuously. After identification of the caecum a small incision was made, feces was collected and the incision closed. After insertion of a large-bore intraperitoneal drain, the abdominal incision was closed.

Study protocol and protocolized resuscitation

Preparation of HMW-HA solution

Five grams of HMW-HA 1,560 kDa (Sodium hyaluronate Lot# 027362 HA15M-5; Lifecore Biomedical LCC, Chaska, MN) was dissolved in 500 mL 0.9% saline to yield a stock concentration of 1% (10 mg/mL). The solution of 1% HMW-HA 1,560 kDa was produced under sterile condition in laminar air flow and stored as 50 mL aliquots at −20°C before use. On the day of experiment, aliquots were thawed and the stock solution was diluted 1:10 in 0.9% saline, to yield 0.1% concentration.

Pilot study—safety profile and kinetics of HMW-HA injection

A pilot study was performed before the experimental peritonitis model to study safety of intravenous HMW-HA injection and simplified plasma kinetics. Animals were divided into two groups, pilot HA group (n = 3) and pilot control group (n = 2). The pilot HA group was injected with HMW-HA and the pilot control group with 0.9% saline. Each individual animal in the pilot HA group received a total of six consecutive injections with the 0.1% HMW-HA solution aiming to achieve plasma concentrations of 1,000, 5,000, 10,000, 30,000, 50,000, and 100,000 ng/mL by injecting 0.065, 0.325, 0.65, 1.95, 3.25, and 6.5 mL/kg, respectively. The pilot control group received six injections with equal amounts of 0.9% saline. Blood samples (EDTA) for HA analyses were taken at 3 and 45 minutes after each injection and vital parameters were recorded at T = 0 (baseline), 5, 10, 15, 25, 35, and 45 minutes.

The experimental time line for the main series is presented in Figure 1. Baseline measurements were performed after a period of at least 30 min of stabilization after preparation. Peritonitis was established with a peritoneal instillation of autologous feces (2 g/kg of body weight (28) in 200 mL warmed 5% glucose solution), after which the large-bore intraperitoneal drain was removed and the abdominal wall closed. The infusion of Ringer's acetate was discontinued at the time of the induction of fecal peritonitis.

To simulate an intensive care setting, the respiratory, circulatory, and metabolic maintenance treatments followed a predefined protocol to support vital parameters according to typical invasive monitoring and repeated measurements and sampling. Both intervention and control groups were subject to a protocolized resuscitation that was initiated at onset of circulatory instability (S0) with Ringer's acetate 10 mL/kg/h. The resuscitation protocol was equal to both groups and aimed at MAP > 60 mm Hg, guiding fluid and norepinephrine administration by changes in SVV and MAP, respectively. If MAP less than 60 mm Hg and SVV greater than 15% fluid was administered, in case of hypotension without increased SVV,

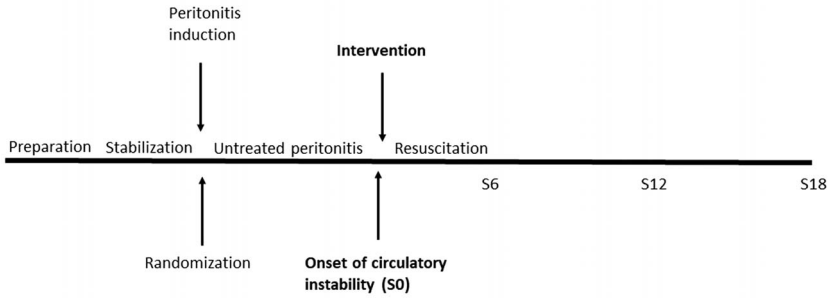

FIG. 1. **Experimental time line.** Preparation was followed by at least 30 minutes of stabilization. Thereafter peritonitis was induced. Intervention and resuscitation was initiated at the onset of circulatory instability (S0). S6, S12, and S18 refer to consecutive time points, respectively (hours after S0).

infusion of norepinephrine 5 mL/h (40 μg/mL) was started after a bolus of 0.5 mL (40 μg/mL) and increased stepwise. The fluid therapy consisted of boluses of Ringer's acetate of 150 mL, repeated until SVV was steady less than 15%. If MAP was stable greater than 60 mm Hg, infusion was first tapered down to 5 mL/kg/h, and if the animal continued to be stable and SVV maintained less than 13% the infusion was stopped.

Experimental design

This was a prospective, parallel-grouped, blinded study with animals randomized (block randomization, sealed opaque envelope) after peritonitis induction into two treatment groups: intervention with HMW-HA (n = 8, both sex, 3 female and 5 male) or control group (n = 8, both sex, 4 female and 4 male). The researchers were blinded for the group allocation until a master file for the whole experiment was produced. After the onset of hemodynamic instability (MAP <60 mm Hg for >5 min), the intervention group received an initial bolus of 0.1% HMW-HA solution of 1 mg/kg over 10 minutes. The initial bolus was followed by a continuous infusion of the same concentration of 1 mg/kg/h during the rest of the experiment, aiming at a plasma HA concentration at 10,000–15,000 ng/mL. The control group received the same volume of vehicle (0.9% saline) both as initial bolus and continuous infusion. Immediately after the intervention was initiated, a protocolized resuscitation was started together with piperacillin/tazobactam 2 gram in 10 mL of 0.9% saline, given i.v. every 6 hours.

The primary outcome of the present study was fluid balance and secondary outcome was inflammatory reaction. The primary outcome of the pilot study was to study the safety profile of HMW-HA injection in the anesthetized animal, and the secondary outcome in the pilot study was to describe simplified plasma kinetics.

Analyses and physiologic parameters

We analyzed arterial blood gases at baseline, at the onset of circulatory instability and every hour for the after 18-hour duration of the experiment. At the same time points, hemodynamic parameters (systemic arterial and pulmonary arterial pressures, CO, heart rate), respiratory parameters (FiO_2, SaO_2, end-tidal CO_2, plateau pressure, dynamic and static compliance) and urine output were measured. We calculated modified shock index (MSI) as HR/MAP (29).

Every 3 hours, mixed venous blood gas analyses were performed, while plasma and urinary samples were collected for analysis every 6 hours. Stroke volume variation was monitored continuously to guide fluid resuscitation.

Cytokine and HA analyses

Porcine-specific sandwich ELISAs were used for the determination of TNF-α, IL-6, IL-8, and IL-10 in plasma (DY690B [TNF-α], DY686 [IL-6], DY535 [IL-8], and DY693B [IL-10]; R&D Systems, Minneapolis, MN). The ELISAs had total coefficient of variations of approximately 6%. Hyaluronan concentration was measured with a commercial ELISA kit (Hyaluronan DuoSet, DY3614; R&D Systems, Minneapolis, MN).

Analysis of plasma HA size distribution by chromatography

Hyaluronan plasma concentrations were measured and a predetermined amount of plasma was analyzed using gel filtration chromatography on a Sephacryl S-1000 (GE Healthcare Europe GmbH, Diegem, Belgium) glass column (length 26 cm, diameter 0.6 cm). Samples were eluted at 70 μL/min with 0.15 M sodium acetate, 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS; Sigma-Aldrich, Steinheim, Germany) and 30 fractions of 200 μL each were collected. Controlled size HA samples of 2,200, 600, and 140 kDa (HA; Merck Life Science BV, Darmstadt, Germany) were used as standards. The HA concentration was measured in each fraction using Hyaluronan DuoSet kit (DY3614; R&D Systems, Minneapolis, MN, DY3614) and expressed as a percentage of the total HA recovered. Fractions were defined as HMW (fractions 1–10: >2,200 kDa) versus LMW (fractions: 18–30 < 140 kDa) and LMW-HA/HMW-HA ratio was calculated. The mean peak elution fraction for each sample was calculated by multiplying the fraction number by its relative HA content and summing the results.

Tissue analyses *post mortem*

At the end of the experiment, the animals were euthanized with 100 mmol KCl i.v. under deep anesthesia, in accordance with the National Institute of Health guidelines (27). The skin of the animals was washed with soap, dried with paper, and sprayed with ethanol; thereafter, the chest wall and abdomen were opened. Tissue samples were collected from left lung (dorsal/basal), heart (left ventricle), liver, spleen, kidney, and small intestine. The samples were immersed in 10% buffered formalin immediately. A veterinary pathologist who was blinded for the group allocation evaluated the samples histologically, and inflammatory lesions were graded in a semiquantitative way: 4, very severe (numerous leukocytes in most parts of the section), 3, severe (numerous leukocytes in many parts of the section); 2, moderate (moderate numbers of leukocytes diffusely or focally distributed); 1, mild (low number of leukocytes diffusely or focally distributed); or 0, lesions were not observed.

Wet-to-dry ratio was measured in samples from the above mentioned tissues. Samples were weighed and dried in an oven, at 50° C, until the weight did not differ between two consecutive measurements.

Bacterial investigations

Every third hour, 0.5 mL arterial blood was collected from a sterile arterial catheter for quantitative blood cultures. Therefrom, a 100 μL was cultured on three separate cysteine lactose electrolyte deficient (CLED) agar plates, then cultured at 37°C overnight, and CFU quantified with viable count technique the following day. A colony forming unit on only one CLED plate from a time point was interpreted as a contamination; otherwise, the median of counted CFU per milliliter was calculated. More than 1 CFU/mL were considered a positive blood culture. Colonies were sent to specification to a MALDI Biotyper (tof-user@FLEX-PC).

Samples from the lung, spleen, and liver were also collected for tissue culture after spraying the organ (surface) with 99% ethanol. One gram of each tissue was placed in a sterile mortar and mashed in 3 mL saline 0.9%, from where 200 μL was cultured on CLED plates and quantified as described previously.

Statistical analysis

To determine sample size, we performed a power analysis. We used data from a previous peritonitis protocol where the fluid balance of the control group (n = 8) was 14 ± 4 mL/kg/h. We chose to test the hypothesis that the difference in fluid balance would be 1.5 × SD between the two groups. Aiming at detecting a difference of 6 mL/kg/h between groups in fluid balance, a power of 0.8 and a significance level of less than 0.05 yielded a sample size of eight animals in each group. We tested data for normality by applying the Shapiro-Wilk test.

To describe each group separately from baseline to onset of circulatory instability (S0), we used the Student *t* test, whereas the one-way ANOVA was used to describe the groups separately throughout the experiment. The two-tailed Student *t* test, the Mann-Whitney *U* test, and the two-way ANOVA were used to compare the two groups, pending distribution of data. Multiple imputation was used to replace missing data because of early deaths.

The data are expressed as mean ± SD or median (interquartile range [IQR]) as appropriate. We conducted the statistical analyses using SPSS v. 27.0.0 software (SPSS, Inc, Chicago, IL). A *P* value less than 0.05 was considered to be statistically significant. Bonferroni correction was not used.

The results are presented as n = 8 per group at the baseline, at the onset of circulatory instability (S0), at 6 (S6) and 12 (S12) hours after onset of circulatory instability, as well as at the end (last observation, before imminent death, or at 18 hours [S18]). Comparisons between the groups over time are presented herein (two-way ANOVA) after performing multiple imputation (i.e., 5) of which *P* values are reported as an interval.

RESULTS

Pilot study—safety profile and kinetics of HA

All the animals in the pilot study (n = 5) survived the experiment with no apparent acute adverse reactions observed after HMW-HA injections. The actual measured increase in plasma HA concentrations after every injection was 73 ± 13.5% of the aimed concentration. HA and removal rate was concentration dependent until the last injection (see Table, Supplemental Digital Content 1, http://links.lww.com/SHK/B628, which demonstrates HA kinetics). Plasma HA concentrations did not return to baseline levels within 45 minutes, resulting in an accumulation effect on the total HA concentration. The pharmacokinetics of plasma HA followed a nonlinear pattern (Table 1).

Hyaluronan concentration in sepsis/peritonitis

Plasma HA concentrations were comparable in the two groups at baseline and at onset of circulatory instability. Plasma HA concentration (median [IQR]) increased to 12,280 (33,780), 9,060 (43,630), and 9,760 (49,020) ng/mL during infusion at 6, 12, and 18 hours of the experiment. Peritonitis/sepsis per se was associated with 3-fold increase of HA at 18 hours in the control group (from 54 ng/mL (30) at baseline to 172 (154) ng/mL at S18; Fig. 2).

TABLE 1. **Aim versus measured HA concentration increase after injection**

Injection HA, mg/kg	Aim (HA), ng/mL	ΔHA increase, ng/mL
0.065	1,000	570 ± 135
0.325	5,000	3,219 ± 3,102
0.65	10,000	7,175 ± 2,412
1.95	30,000	21,367 ± 2,782
3.25	50,000	48,067 ± 18,845
6.5	100,000	79,151 ± 8,836

Net increase of HA concentration after each injection with HA. Values are presented as mean ± SD.

The HA size distribution was comparable between the groups at baseline ($P = 0.994$). The distribution curve shifted to the right at the end of the protocol in both groups; this shift toward low-molecular-weight fractions was more pronounced in the control group (see Fig. 2, a and b), Supplemental Digital Content, http://links.lww.com/SHK/B629, which illustrate the right shift of HA molecular weight fractions from baseline [2a] to the end of the experiment [2b]). The right shift was confirmed by a statistically significant increase of mean peak elution fraction in the control group versus the intervention group from baseline to end of protocol (control group: from 15.2 ± 0.9 to 17.9 ± 0.6, vs. intervention group: from 15.8 ± 0.9 to 16.8 ± 0.9, two-way ANOVA, $P = 0.031$). Similarly the LMW-HA/HMW-HA ratio was comparable between groups at baseline ($P = 0.486$), and while peritonitis sepsis was associated with increased LMW-HA/HMW-HA ratio ($P = 0.004$), and the HA infusion maintained the ratio ($P = 0.154$) (Fig. 3).

Hemodynamics

Fourteen of the 16 animals survived the experiment until euthanasia (18 hours after onset of circulatory instability), while one animal died of refractory shock during the 18-hour observation period in both treatment (T = S8) and control groups (T = S14).

The intervention group presented with circulatory instability (from the baseline to onset of circulatory instability S0, defined as MAP <60 mm Hg > 5 min) within 3.8 ± 1.3 h and the control group within 3.8 ± 1.6 h ($P = 0.966$) from the induction of peritonitis.

The onset of circulatory instability was accompanied by an increase in HR, mean pulmonary arterial pressure, SVV, hgb, and temperature in both intervention and control groups (Tables 2, 3). Lactate increased in intervention group during the same period, but not in the control group (Table 3).

All hemodynamic parameters as well as arterial blood lactate changed comparably in the two groups as a function of time over the length of the resuscitation period (Tables 2, 3). Neither did the groups differ in regard to wedge pressure, nor central venous pressure as a function of time (Tables 2, 3).

Modified shock index was comparable at baseline and at the onset of circulatory instability in the two groups. Hyaluronan infusion was associated with lower MSI as compared with placebo at several time points during the resuscitation period (see Fig. 3, Supplemental Digital Content, http://links.lww.com/SHK/B630, which illustrate the evolution of MSI during the experiment).

Respiratory parameters

Onset of circulatory instability was accompanied by comparable decrease of SaO_2 and PaO_2/FiO_2 ratio from baseline in the two groups. There was a gradual decrease in both dynamic and static compliance in both intervention and control groups, respectively, throughout the protocol (Table 4). As respiratory rate was adjusted (intervention group: range 25–50 per minute) (control group: range 25–40 per minute) to maintain normocapnia, we observed that $PEEP_{TOT}$ was stable at 8 cm H_2O in the both groups throughout the experiment. A progressive increase in plateau pressure throughout the protocol was statistically significant in the control group ($P = 0.001$–0.004) but not in the intervention group ($P = 0.350$–0.594) throughout the resuscitation period.

Fluid balance, norepinephrine dosage, kidney function, and electrolytes

The total volumes of fluid administered during the experiment were 17.5 ± 11 versus 19.0 ± 7 mL/kg/h in intervention and control groups, respectively ($P = 0.442$). Weight gain was 12.5 ± 3.1 kg in the intervention and 14.0 ± 2.3 kg in the control group ($P = 0.328$). The average norepinephrine dosage was 1.2 ± 1.6 and

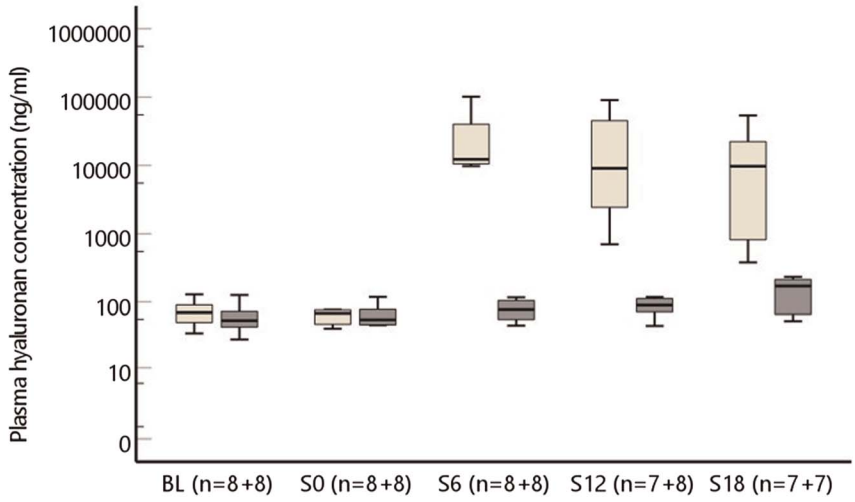

FIG. 2. **Plasma HA concentrations in intervention (white) and control groups (gray) at baseline, onset of circulatory instability (S0), 6 (S6), 12 (S12), and 18 hours (S18) after onset of circulatory instability**. HA. hyaluronan.

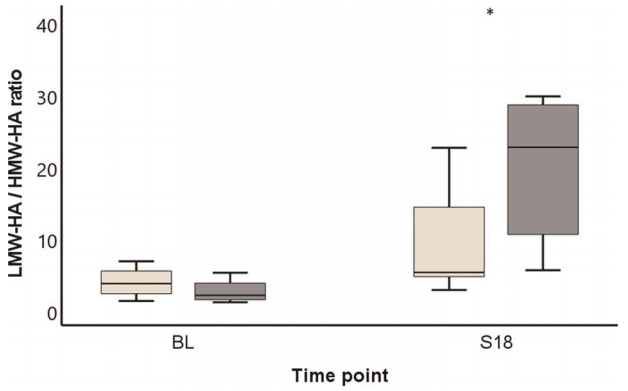

FIG. 3. **Low-molecular-weight HA/high-molecular-weight HA ratio at BL and at the end of the experiment (S18).** White represent intervention group and gray control group (*) $P < 0.05$ (Mann-Whitney U test, $P = 0.015$). BL, baseline.

1.0 ± 0.8 μg/kg/min in the intervention and the control groups, respectively ($P = 0.721$).

Urine production decreased from baseline to onset of circulatory instability from 4.1 ± 4 to 0.5 ± 0.4 mL/kg/h ($P = 0.045$) and from 3.0 ± 2 to 0.5 ± 0.4 mL/kg/h ($P = 0.015$) in the intervention and control groups, respectively. Hourly diuresis was comparable in the two groups throughout the resuscitation period, in average 2.1 ± 1.3 mL/kg/h in the intervention and 1.7 ± 0.9 mL/kg/h in the control group ($P = 0.442$).

Renal arterial blood flow decreased from baseline to onset of circulatory instability in both intervention and control group: from 132 ± 79 to 61 ± 46 mL ($P = 0.001$) versus from 138 ± 63 to 72 ± 34 mL ($P = 0.002$). Blood flow changed comparably in the two groups as a function of time ($P = 0.873–0.976$) throughout the protocol.

Plasma creatinine increased from baseline 77 ± 17 to 100 ± 19 μmol/L at onset of circulatory instability in intervention group and from 72 ± 12 to 92 ± 14 μmoL/L in control group, with comparably increasing plasma concentrations throughout the resuscitation period in both groups, respectively, as a function of time. Plasma urea, whole blood Na, whole blood K, base excess and HCO_3^- followed a similar pattern over time in the two groups (Tables 3, 5).

Finally, there were no differences between groups in urine creatinine, urea, sodium, or potassium concentrations between groups as a function of time (Table 5).

Cytokines

The concentration of IL-6, IL-8, IL-10, and TNF-α in plasma increased from baseline to onset of circulatory instability (S0) in both intervention and control groups. The dynamics in cytokine concentrations in plasma were comparable in the two groups throughout the experiment (Table 6).

Wet-to-dry ratio

Wet-to-dry ratios at the end of the experiment were comparable in the two groups (see Table, Supplemental Digital Content 4, http://links.lww.com/SHK/B631, which demonstrates wet-to-dry ratios of *post mortem* samples).

Blood and tissue cultures

Blood cultures

All animals, except one in the intervention group, had positive blood cultures at some time point during the experiment. Number of colony forming units per milliliter ($P = 0.682$) were comparable in the two groups throughout the experiment. Positive cultures were of mixed etiology, with a dominance (>90%) of Escherichia coli.

Tissue cultures

All animals had viable bacteria in at least one organ (lung, liver, spleen). All three tissue cultures had viable bacteria in five animals in intervention group and in four animals in the control group. The two groups did not differ in colony forming units per gram in either of the tested organs.

Cultures of lung tissue had viable bacteria in five animals in intervention group (median, 50; IQR, 475) and in six animals in the

TABLE 2. **Hemodynamic parameters**

	Group	Baseline (n = 8 + 8)	S0 (n = 8 + 8)	S6 (n = 8 + 8)	S12 (n = 7 + 8)	S18 (n = 7 + 7)	*P* range
MAP, mm Hg	HA	87 ± 13	55 ± 3	63 ± 8	67 ± 5	68 ± 6	0.059–0.187
	Control	69 ± 7	57 ± 1	65 ± 3	63 ± 4	63 ± 9	
HR, bpm	HA	84 ± 15	141 ± 44*	130 ± 28	116 ± 26	118 ± 29	0.961–0.988
	Control	83 ± 19	160 ± 34*	149 ± 13	136 ± 27	127 ± 19	
MPAP, mm Hg	HA	16 ± 2	19 ± 3*	22 ± 7	20 ± 3	22 ± 3	0.632–0.788
	Control	17 ± 1	20 ± 4*	21 ± 3	24 ± 4	23 ± 5	
Wedge, mm Hg	HA	9 ± 2	7 ± 1	9 ± 2	9 ± 2	9 ± 2	0.936
	Control	8 ± 2	6 ± 1	8 ± 1	9 ± 2	10 ± 3	
SVV, %	HA	10 ± 5	15 ± 3*	15 ± 3	11 ± 2	12 ± 4	0.448–0.701
	Control	7 ± 2	17 ± 5*	18 ± 5	15 ± 4	16 ± 4	
CO, L/min	HA	3.1 ± 0.8	2.5 ± 0.7	3.1 ± 0.8	2.9 ± 0.7	2.9 ± 0.7	0.722–0.916
	Control	3.0 ± 0.8	2.1 ± 0.4	3.2 ± 0.5	3.2 ± 0.5	3.6 ± 1.2	
CVP, mm Hg	HA	8 ± 3	7 ± 3	9 ± 3	9 ± 4	10 ± 5	1.000
	Control	7 ± 2	8 ± 4	8 ± 2	10 ± 3	11 ± 4	
T, °C	HA	39.0 ± 0.9	40.5 ± 1.0*	40.4 ± 0.6	40.3 ± 0.7	40.0 ± 0.7	1.000
	Control	38.2 ± 0.8	39.9 ± 0.8*	39.8 ± 0.6	39.9 ± 0.5	39.6 ± 0.7	

Onset of circulatory instability (S0), 6 hours of circulatory instability (S6), 12 hours of circulatory instability (S12), and 18 hours of circulatory instability at the end of the protocol (S18). Values are presented as mean ± SD. Significance level as range after performing multiple imputation (*P* range).

*Baseline versus S0, $P < 0.05$, *P* values from paired *t* test.

CVP, central venous pressure; HR, heart rate, MPAP, mean pulmonary arterial pressure, T, temperature; Wedge, wedge pressure.

TABLE 3. **Arterial blood gas analysis**

	Group	BL (n = 8 + 8)	S0 (n = 8 + 8)	S6 (n = 8 + 8)	S12 (n = 7 + 8)	S18 (n = 7 + 7)	*P* range
pH	HA	7.45 ± 0.05	7.36 ± 0.07	7.38 ± 0.07	7.38 ± 0.08	7.36 ± 0.12	0.931–0.990
	Control	7.44 ± 0.02	7.35 ± 0.04	7.37 ± 0.05	7.35 ± 0.10	7.26 ± 0.17	
Hgb, g/L	HA	96 ± 7	135 ± 14*	114 ± 9	112 ± 6	111 ± 6	0.389–0.527
	Control	93 ± 6	139 ± 12*	122 ± 6	113 ± 7	107 ± 4	
Lactate, mmol/L	HA	2.6 ± 0.8	3.4 ± 1.1*	2.2 ± 1.8	1.3 ± 0.4	1.3 ± 0.6	0.254–0.500
	Control	2.2 ± 0.5	2.5 ± 0.6	2.0 ± 0.5	2.1 ± 1.4	2.4 ± 2.8	
BE, mmol/L	HA	3.1 ± 3.2	–0.2 ± 4.1	–0.7 ± 3.2	–0.9 ± 4.3	–1.5 ± 6.2	0.953–0.992
	Control	3.9 ± 1.7	–0.1 ± 2.5	–0.4 ± 2.8	–2.7 ± 3.9	–5.1 ± 8.9	
HCO_3^-, mmol/L	HA	27.3 ± 2.9	24.0 ± 3.6	23.7 ± 2.9	23.7 ± 3.7	23.2 ± 5.2	0.955–0.987
	Control	27.9 ± 1.5	23.9 ± 2.1	23.9 ± 2.5	22.2 ± 3.6	20.1 ± 7.2	
Na, mmol/L	HA	135 ± 2	131 ± 3	129 ± 3	126 ± 2	125 ± 3	0.985–0.999
	Control	135 ± 1	131 ± 2	129 ± 2	126 ± 1	125 ± 2	
K, mmol/L	HA	3.9 ± 0.3	4.9 ± 0.8	5.5 ± 0.6	5.2 ± 0.5	5.0 ± 0.8	0.878–0.980
	Control	3.8 ± 0.4	5.0 ± 0.7	5.5 ± 0.5	5.6 ± 0.4	5.5 ± 0.9	

Onset of circulatory instability (S0), S6, S12, and S18 refer to consecutive time points, respectively (hours after S0). Values reported as mean ± SD. Significance level as range after performing multiple imputation (*P* range).
*Baseline versus S0, $P < 0.05$, *P* value from paired *t* test.
BE, base excess.

control group (median, 200; IQR, 4400) ($P = 0.382$). Liver tissue cultures had viable bacteria in six animals in intervention group (median, 254; IQR, 405) and in five animals in control group (median, 236; IQR, 1,682) ($P = 1$). Tissue cultures of spleen had viable bacteria in all animals in intervention group (median, 5,174; IQR, 15,692) and in all but one animal in the control group (median, 1,813; IQR, 8,107) ($P = 0.195$).

Histology

Lung samples showed acute inflammatory lesions in samples from four animals of the intervention group and seven animals of the control group, the lesions varied in intensity between individual animals. Lung lesions were comparable in intervention (median, 1; IQR, 2) and control groups (median, 2; IQR, 2) ($P = 0.234$) (see Fig. 5, a and b, Supplemental Digital Content, http://links.lww.com/SHK/B632, which illustrate lung lesions from one animal in intervention [5a] and control group [5b]).

No significant lesions were visualized in heart tissue in either group. All but one animal in each group had acute focal/multifocal degeneration in the liver and coagulative necrosis of hepatocytes. Liver lesions were comparable between groups: intervention group (median, 3; IQR, 2) and control groups (median, 1; IQR, 1) ($P = 0.065$). In intestinal samples, the epithelial lining was generally preserved, but the gut mucosa in all pigs was infiltrated with mixed leucocytes to a varying degree. There was no difference between intervention (median, 2; IQR, 1) and control groups (median, 2; IQR, 2) ($P = 0.328$) as to inflammatory lesions of the intestine (see Fig. 6, a and b, Supplemental Digital Content, http://links.lww.com/SHK/B633, which illustrate histology of intestine, from one animal in intervention [6a] and control groups [6b]).

Lesions in kidney samples were rare (intervention: median, 0; IQR, 1; control group: median, 0; IQR, 1) and equally distributed between groups ($P = 0.959$). Three animals in the intervention group (median, 0; IQR, 3) and three animals in the control group (median, 0; IQR, 3) exhibited inflammatory lesions of the spleen, manifested as acute purulent inflammation of the splenic capsule. The capsular inflammation generally extended to the parenchyma

TABLE 4. **Respiratory parameters**

	Group	Baseline (n = 8 + 8)	S0 (n = 8 + 8)	S6 (n = 8 + 8)	S12 (n = 7 + 8)	S18 (n = 7 + 7)	*P* range
Pa_{O_2}/Fi_{O_2} ratio	HA	57 ± 4	47 ± 8*	44 ± 12	45 ± 6	40 ± 8	0.540–0.822
	Control	59 ± 4	50 ± 4*	48 ± 6	40 ± 13	35 ± 14	
Compliance dynamic, mL/cmH_2O)	HA	22 ± 4	21 ± 3	16 ± 5	15 ± 3	12 ± 3†	0.908–0.975)
	Control	24 ± 4	23 ± 3	19 ± 5	14 ± 6	13 ± 5†	
Compliance static, mL/cmH_2O	HA	25 ± 5	23 ± 4	19 ± 6	17 ± 4	14 ± 3†	0.951–0.992
	Control	28 ± 5	26 ± 3	21 ± 5	16 ± 6	15 ± 5†	
Plateau pressure, cmH_2O	HA	19 ± 4	20 ± 2	24 ± 7	25 ± 4	26 ± 5	0.942–1.000
	Control	18 ± 2	19 ± 1	22 ± 4	28 ± 10	29 ± 11†	
$PEEP_{TOT}$, cmH_2O	HA	8 ± 0	8 ± 0	8 ± 0	8 ± 0	8 ± 0	0.847–0.965
	Control	8 ± 0	8 ± 0	8 ± 0	8 ± 0	8 ± 2	
Pa_{CO_2}, kPa	HA	5.3 ± 0.5	5.9 ± 0.5	5.5 ± 0.6	5.5 ± 0.4	5.5 ± 0.4	0.584–0.972
	Control	5.5 ± 0.3	6.2 ± 0.6	5.7 ± 0.3	5.8 ± 0.3	6.0 ± 0.2	
Sa_{O_2}, %	HA	96 ± 1	93 ± 3*	91 ± 8	94 ± 1	93 ± 3	0.236–0.597
	Control	96 ± 0	94 ± 1*	94 ± 2	92 ± 4	88 ± 10	

Ratio of arterial partial pressure of oxygen to inspired oxygen fraction (Pa_{O_2}/Fi_{O_2} ratio), PEEP, Pa_{CO_2}, and Sa_{O_2}. Values are presented as mean ± SD. Significance level as range after performing multiple imputation (*P* range).
*Baseline versus S0, $P < 0.05$, *P* value from paired *t* test.
†The decrease (in dynamic and static compliance) was statistically significant in both groups, respectively, throughout the experiment ($P < 0.05$, one-way ANOVA), the increase in plateau pressure was statistically significant in the control group, but not in the intervention group ($P < 0.05$, one-way ANOVA).

TABLE 5. **Creatinine and urea in plasma and urine markers**

	Group	Baseline (n = 8 + 8)	S0 (n = 8 + 8)	S6 (n = 8 + 8)	S12 (n = 7 + 8)	S18 (n = 7 + 7)	*P*
Creatinine in plasma, μmol/L	HA	77 ± 17	100 ± 19*	111 ± 18	122 ± 28	145 ± 61†	0.980
	Control	72 ± 12	92 ± 14*	100 ± 17	123 ± 23	138 ± 36†	
Urea in plasma, mmol/L	HA	4 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	0.951
	Control	4 ± 1	5 ± 1	5 ± 1	5 ± 1	5 ± 1	
Creatinine urine, μmol/L	HA	10 ± 4	13 ± 3	7 ± 4	8 ± 4	8 ± 4	0.826
	Control	8 ± 5	14 ± 4	7 ± 3	9 ± 4	7 ± 4	
Urea urine, mmol/L	HA	270 ± 120	280 ± 70	150 ± 80	160 ± 60	150 ± 80	0.294
	Control	290 ± 150	370 ± 80	150 ± 50	140 ± 50	110 ± 50	
U K, mmol/L	HA	100 ± 50	70 ± 60	40 ± 10	60 ± 20	40 ± 20	0.490
	Control	70 ± 50	60 ± 50	70 ± 30	60 ± 20	40 ± 20	
U Na, mmol/L	HA	70 ± 50	40 ± 20	60 ± 20	40 ± 10	40 ± 20	0.735
	Control	60 ± 10	<20‡	40 ± 20	40 ± 20	30 ± 0	

Onset of circulatory instability (S0), S6, S12, and S18 refer to consecutive time points, respectively (hours after S0). Values are presented as mean ± SD. Significance level as range after performing multiple imputation (*P* range).
*Baseline versus S0, $P < 0.05$, *P* value from paired *t* test.
†Dynamics described within each group respectively, $P < 0.05$, *P* value from one-way ANOVA.
‡U-Na: at S0 U-Na was <20 mmoL/L in all animals in the control group.
U Na, sodium in urine; U K, potassium in urine.

in subcapsular areas. There was no difference between groups ($P = 1$) regarding inflammatory lesions of the spleen.

DISCUSSION

While fluid administration is indispensable in the resuscitation phase of sepsis and early fluid administration is associated with a better outcome (3), fluid overload is associated with increased mortality (5). Intravenous colloids oppose the transcapillary fluid flux (30) and are more effective volume expanders than crystalloids. However, this advantage in sparing volume of total fluid administered during resuscitation is not associated with better outcome in critical illness (6).

In the current study, we tested an intervention with systemically administered HMW-HA as adjuvant in sepsis resuscitation. Plasma concentrations of HA were comparable in the intervention and control groups at baseline and at onset of circulatory instability. Peritonitis/sepsis was associated with 3-fold increase of HA in the control group during the resuscitation period. The intervention group showed a greater increase of plasma HA due to the infusion of exogenous HA, and concentrations were considerably higher in the intervention group than values previously reported in sepsis (15,23–25).

The main finding of the present study was that contrary to our hypothesis, HMW-HA infusion did not decrease the total volume of fluid resuscitation in the early phase of peritonitis-induced sepsis. Weight gain, urine production, tissue wet-to-dry ratios, and histology in the intervention and control groups were comparable. Furthermore, isolated hemodynamic parameters were comparable over the length of the experiment. However, a *post hoc* analysis of MSI (29) suggested that administration of HMW-HA was associated with less hemodynamic instability, possibly as a consequence of the colloid osmotic properties of the molecule. This finding may support the notion that HA exerts a protective effect in critical illness, as suggested previously (24).

Neither the histological analysis, bacterial cultures, nor the cytokine response in plasma revealed a difference in inflammation between the two groups in our study. Liu et al (26) studied the anti-inflammatory effect of HMW-HA in a rat model of sepsis and mechanical ventilation. While the inflammatory response and the degree of lung injury were reduced, macrocirculatory parameters did not improve (26); however, this protective effect has not been confirmed in larger animals. In humans, intravenous administration of HA in doses as high as 12 mg/kg did not result in any serious adverse events, nor did it increase plasma concentrations of fragmented HA (10).

The effect of HA on inflammation is dichotomous and depends on molecular weight. High-molecular-weight HA is anti-inflammatory and immunosuppressive (20). It reduces the

TABLE 6. **Cytokines in plasma**

Cytokine	Group	Baseline (n = 8 + 8)	S0 (n = 8 + 8)	S6 (n = 8 + 8)	S12 (n = 8 + 8)	S18 (n = 7 + 8)	*P* range
IL-6, pg/mL	HA	150 (–10 to 470)	5,650 (3,070 to 6,540)*	4,030 (2,670 to 6,570)	4,000 (1,730 to 8,380)	2,450 (1,420 to 6,890)	0.614–0.859
	Control	270 (130 to 460)	5,340 (3,460 to 6,870)*	3,700 (2,050 to 6,950)	3,910 (2,300 to 8,330)	3,690 (1,410 to 11,960)	
TNF-α, pg/mL	HA	150 (100 to 310)	240 (140 to 420) *	170 (90 to 320)	160 (130 to 190)	160 (140 to 250)	0.932–0.957
	Control	170 (90 to 380)	230 (140 to 390)*	140 (100 to 250)	160 (120 to 230)	150 (110 to 240)	
IL-10, pg/mL	HA	240 (–20 to 760)	820 (450 to 1,350)*	860 (690 to 1,480)	660 (430 to 1,140)	500 (360 to 770)	0.748–0.796
	Control	330 (220 to 570)	610 (490 to 820)*	720 (570 to 1,180)	600 (540 to 910)	540 (380 to 790)	
IL-8, pg/mL	HA	60 (50 to 80)	130 (90 to 150)*	120 (80 to 190)	90 (50 to 190)	80 (40 to 170)	0.694–0.741
	Control	60 (50 to 90)	110 (80 to 150)*	110 (70 to 130)	80 (60 to 110)	60 (40 to 130)	

Onset of circulatory instability (S0), S6, S12, and S18 refer to consecutive time points, respectively (hours after S0). One animal died before finishing the protocol in both groups, last sample before imminent death is included in the analyses of the time point after death, that is, S12 for the animal that died in group 1 (204 at S8) and S18 for the animal that died in group 2 (207 at S14), before finishing the protocol. Values are presented as median (95% CI).
*Baseline versus S0, $P < 0.05$, *P* value from paired *t* test.

proinflammatory cytokine response in plasma (31) and promotes resolution of infection (32). Fractions of HA, or LMW-HA, on the other hand, are a strong signal of tissue damage (33) and induce a proinflammatory response through several pathways (21,31,34) with production of proinflammatory cytokines and enhanced secretion of nitric oxide (32). In the current study, peritonitis sepsis in the control group was associated with a significant fragmentation of circulating HA (as assessed by the increased ratio of LMW-HA/HMW-HA ratio measured in plasma), which may reflect the shedding of glycocalyx into the circulation. Although the infusion of HMW-HA was able to counteract this increase as the ratio remained close to baseline values, this infusion was unable to reduce the state of hyperinflammation associated with the early phase of peritonitis-induced sepsis.

Our study has several limitations. Most importantly, it is an animal model, which implies possible species differences in host response, both with regard to infectious insult and to intervention, as compared with humans. It is also a study of limited size and small differences between groups might not have been detected, even more so because the peritonitis model used presents a heterogeneous panorama of disease severity. Furthermore, there are open questions about optimal concentration, timing, and mode of administration of an HMW-HA solution. Administration of rapid crystalloid infusions increases plasma concentration of HA and might disrupt the glycocalyx (35). Thus, the fact that we started an infusion of HMW-HA at onset of circulatory instability alongside crystalloid resuscitation (infusion and boluses as needed) might have interfered with the intervention. Limiting fluid resuscitation in the intervention group to nothing but HMW-HA solutions could be an alternative approach to study the potential benefit of HMW-HA solutions in sepsis resuscitation.

CONCLUSIONS

The current study does not support the hypothesis that HMW-HA reduces the volume of fluid administered during resuscitation and/or attenuates the hyperinflammatory state associated with peritonitis-induced sepsis. However, systemically administered HMW-HA is associated with maintained LMW-HA/HMW-HA ratio, as compared with the increased LMW-HA/HMW-HA associated with peritonitis sepsis. Although the present study does not support the role of HMW-HA in sepsis resuscitation, further studies are warranted mainly because of small sample size and lethal model of sepsis.

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