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Resuscitation of traumatic hemorrhagic shock patients with hypertonic saline - without dextran - inhibits neutrophil and endothelial cell activation

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Abstract

Background—Post-traumatic inflammation and excessive neutrophil activation cause multiple organ dysfunction syndrome (MODS), a major cause of death among hemorrhagic shock patients. Traditional resuscitation strategies may exacerbate inflammation and thus novel fluid treatments are needed to reduce these post-traumatic complications. Hypertonic resuscitation fluids inhibit inflammation and reduce MODS in animal models. Here we studied the anti-inflammatory efficacy of hypertonic fluids in a controlled clinical trial.

Methods—Trauma patients in hypovolemic shock were resuscitated in a pre-hospital setting with 250 ml of either 7.5% hypertonic saline (HS; n=9), 7.5% hypertonic saline + 6% dextran-70 (HSD; n=8), or 0.9% normal saline (NS; n=17). Blood samples were collected on hospital admission and 12 and 24 h post-resuscitation. Multi-color flow cytometry was used to quantify neutrophil expression of cell-surface activation/adhesion (CD11b, CD62L, CD64) and degranulation (CD63, CD66b, CD35) markers as well as oxidative burst activity. Circulating concentrations of soluble intercellular adhesion molecule (sICAM)-1, vascular cell adhesion molecule (sVCAM)-1, P-, E-selectins, myeloperoxidase (MPO), and matrix metallopeptidase (MMP)-9 were assessed with immunoassays.

Results—MODS, leukocytosis, and mortality were lower in the HS and HSD groups than in the NS group. However, these differences were not statistically significant. HS prevented priming and activation and neutrophil oxidative burst and CD11b and CD66b expression. HS also reduced circulating markers of neutrophil degranulation (MPO and MMP-9) and endothelial cell activation (sICAM-1, cVCAM-1, sE-selectin, and sP-selectin). HSD was less capable than HS of suppressing the upregulation of most of these activation markers.

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Conclusions—This study demonstrates that initial resuscitation with HS but neither NS nor HSD can attenuate post-traumatic neutrophil and endothelial cell activation in hemorrhagic shock patients. These data suggest that hypertonic resuscitation without dextran may inhibit post-traumatic inflammation. However, despite this effect, neither HS nor HSD reduced MODS in trauma patients with hemorrhagic shock.

Keywords

Hypertonic saline; resuscitation fluids; hemorrhage; neutrophils; adhesion molecules; inflammation; multi-organ failure; matrix metalloproteinase; myeloperoxidase; shock

Introduction

Despite significant advances in injury prevention, pre-hospital resuscitation strategies, damage control surgery and modern intensive care [1], trauma remains the fifth leading cause of death among all age groups in the United States and the major cause of death worldwide among persons under the age of 40 years [2]. Immediately after injury, the amount of blood loss is a main determinant of outcome. In later stages, posttraumatic hemorrhagic shock, initiated by massive tissue injury and ischemia/reperfusion, primes the innate immune system for an exaggerated systemic inflammatory response syndrome (SIRS) [3]. This process ultimately leads to multiple organ dysfunction syndrome (MODS), which is the leading cause of death among those patients who die in the intensive care unit [4]. Moreover, conventional resuscitation strategies often exacerbate the underlying cellular injury caused by hemorrhagic shock, and the type of fluid administered may play an important role in the development of secondary injury.

The underlying pathogenesis of these related syndromes involves a complex cascade of humoral and cellular activation events [5]. The mechanisms by which posttraumatic hemorrhagic shock triggers SIRS and MODS have been extensively studied but not fully understood. Excessive activation of neutrophils and endothelial cells results in upregulation of cell surface adhesion molecules, including selectins and integrins, promoting adherence of neutrophils to inflamed vascular endothelium. Resultant extravasation and sequestration of neutrophils into vital organs with subsequent degranulation and release of protease and other hydrolytic enzymes, liberation of reactive oxygen species, and the synthesis of inflammatory cytokines are crucial steps in the development of shock-mediated MODS [6]. In order to prevent these post-traumatic complications, various pharmacological approaches have been considered as possible strategies to reduce post-traumatic inflammation [6]. Among these treatments, hypertonic resuscitation has attracted particular interest because hypertonic fluids have been shown to reduce neutrophil activation and tissue injury in preclinical trials [7].

Treatment of neutrophils with hypertonic fluids activates intracellular signaling pathways that suppress cell activation processes through cAMP-mediated pathways [8, 9]. When administered *in vivo*, hypertonic resuscitation fluids have been shown to possess powerful anti-inflammatory properties that result in reduced secondary organ damage in various animal models of trauma [10], hemorrhagic shock, and sepsis [11–14]. These promising laboratory findings along with encouraging initial clinical trials have generated considerable interest in hypertonic resuscitation fluids as potential immunomodulatory treatments for trauma patients [15–17]. In addition to their pharmacological properties, small-volume hypertonic resuscitation strategies have the added logistic advantage of conserving fluid resources under far-forward battlefield conditions. Therefore, much effort has been devoted to study the efficacy of hypertonic fluids as alternative resuscitation fluids for combat casualty care. While initial studies demonstrated that hypertonic fluids have clear

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advantages over isotonic fluids, subsequent clinical trials have failed to convincingly demonstrate survival benefits and improvements of post-traumatic morbidity [17].

During this period, our laboratories have made progress in our understanding of the molecular mechanisms by which hypertonic fluids regulate neutrophil functions [9,12,18]. We found that hypertonic saline can inhibit neutrophil activation *in vitro* when clinically relevant doses are administered before or during cell stimulation. However, we also found that timing of hypertonic fluid treatment is critical to achieve this effect [19]. Hypertonic saline added to previously stimulated or primed neutrophils enhanced degranulation and increased the release of proteolytic enzymes including elastase, suggesting that delayed hypertonic resuscitation could aggravate neutrophil-mediated organ damage [8]. Indeed, a mouse hemorrhagic shock model has shown that administering hypertonic saline after the infusion of isotonic fluids can exacerbate lung tissue damage [19]. Furthermore, previous laboratory studies suggested that different resuscitation fluid formulations differentially modulate neutrophil activation and cellular injury profiles *ex vivo* [20]. However, there have been no human studies to date that have directly compared the immunomodulatory capacity of hypertonic saline alone versus hypertonic saline admixed with dextran for post-traumatic resuscitation. Thus, it has remained unclear whether undesirable effects of specific fluids and/or delayed resuscitation could have a negative impact on clinical outcome.

The current study was designed as an *a priori* sub-group analysis within a larger multi-center clinical trial, conducted in eleven trauma centers across North America to investigate the efficacy of pre-hospital resuscitation of trauma patients with two commonly used hypertonic resuscitation fluids: hypertonic saline (HS; 7.5% NaCl) and hypertonic saline with dextran (HSD; 7.5% NaCl + 6% dextran-70). The main focus of the parent trial was on determining the effects of these fluids on patient survival, along with post-traumatic measures of organ failure, infection, and sepsis. A detailed description of this trial can be found elsewhere [21]. In the present study, we evaluated neutrophil activation, adhesion, and degranulation markers along with other inflammatory mediators in a smaller cohort of patients admitted to two of the eleven trial centers. Our goal was to determine how pre-hospital treatment with hypertonic fluids affects the post-traumatic inflammatory response after hemorrhagic shock.

Materials and Methods

Ethics Statement

This clinical study was conducted under the United States (US) regulations for Exception from Informed Consent for Emergency Research (21 CFR 50.24) and the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans in Emergency Health Situations (Article 2.8). The protocol was reviewed and approved by the US Food and Drug Administration and Canadian Institute for Health Research. The protocol was also approved by all participating Institutional Review Boards (US) and Research Ethics Boards (Canada), as previously described [21]. Blood samples were drawn from healthy volunteers (controls) after obtaining written informed consent in accordance with the principles of the Declaration of Helsinki and with the approval of the institutional review boards of the participating hospitals in Washington, Toronto, and Boston.

Clinical trail

Study Design and Setting—This prospective experimental study was conducted as an a priori subgroup analysis as part of a larger randomized, placebo-controlled, double-blinded trial under the Resuscitation Outcomes Consortium (ROC) — a multicenter clinical trials network designed to conduct interventional studies in the pre-hospital setting following life-threatening trauma [21].

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Study Participants—For the current study, we included 34 shock patients that included patients with a pre-hospital systolic blood pressure (SBP) of ≤70 mmHg and patients that had a SBP between 71 and 90 mmHg as well as a concomitant heart rate ≥108 beats per min. These enrollment criteria were chosen based on the rationale delineated previously [21]. Patients were excluded if they were <15 years of age, pregnant, or if they received intravenous fluid therapy in the field (>2,000 ml of isotonic crystalloid fluids, any colloids, or any blood products) prior to treatment with study fluids. In addition to study fluid (250 ml), the additional fluid volume administered en rout to the emergency department was <1,000 ml. Excessive delay in treatment was another reason for exclusion, i.e., if the time interval between call received for dispatch and study intervention was >4 h, patients were excluded. Other exclusion criteria were pre-hospital cardiopulmonary resuscitation, severe hypothermia (body core temperature <28°C), drowning or asphyxia due to hanging, burns >20% total body surface area, isolated penetrating head injury, inability to obtain intravenous access, or if a potential subject was known to be a prisoner. A group of twenty asymptomatic adult donors served as a healthy control group.

Interventions—As previously reported [21], the randomized, placebo-controlled, double-blinded three-arm trial compared the effects of a 250-ml bolus infusion of hypertonic saline *versus* hypertonic saline with dextran *versus* normal saline as the initial resuscitation fluids given to injured patients assessed to be in hemorrhagic shock. All study fluids used for this trial were purchased from BioPhausia Inc., Stockholm, Sweden. The company provided identical 250-ml infusion bags that contained either 7.5% NaCl combined with 6% dextran-70 (HSD; RescueFlow), 7.5% NaCl without dextran (HS), or 0.9% NaCl (normal saline, NS). These intravenous bags were distributed among the eleven different geographic regions participating in this clinical trial of the ROC. Paramedics administered the treatment solutions in a blinded fashion via intravenous access as the initial resuscitation fluid given within 4 h of the incident. Once the study fluid had been administered, additional fluids could be given as guided by local Emergency Medical Services protocols. Investigators agreed to follow established guidelines for management of common conditions in the critically ill trauma patient according to best practice guidelines as previously described [21]. Clinical data collected upon hospital admission included age, gender, mechanism of injury, and Injury Severity Score (ISS). The severity of illness was quantified using the Multiple Organ Dysfunction Score (MODS) at the time of admission to the intensive care unit (ICU). Additional clinical outcome parameters collected were the 28-day survival rate, fluid and blood transfusion requirements, physiologic parameters, and evidence of infections.

Blood samples—Two (Toronto and Seattle) of the eleven regional centers participating in the parent ROC trial and the Beth Israel Deaconess Medical Center in Boston collaborated in the substudy described in this report to assess cellular and soluble immuno-inflammatory responses in patients treated with HS, HSD, or NS. IRB approvals to exchange patient samples among the three institutions was obtained at each institution.

Serial samples of venous blood were collected into EDTA, sodium heparin and non-additive Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) at the time of admittance to the hospital emergency department (within 3 hours of resuscitation) and subsequently at 12-, and 24-h post-resuscitation. A single blood sample was obtained by venipuncture from each of 20 age-matched healthy volunteers. EDTA blood samples were used to assess routine clinical laboratory values, including plasma sodium concentrations and leukocyte differential counts. In addition, freshly drawn heparinized whole-blood was processed immediately to assess neutrophil cell-surface activation, adhesion and degranulation markers, and circulating concentrations of soluble endothelial adhesion molecules, MPO, and MMP-9 were subsequently determined.

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Neutrophil cell-surface receptors

Whole blood samples were used to analyze with flow cytometry the expression of specific surface molecules that indicate various states of neutrophil activation. Neutrophil adhesion was assessed with antibodies that recognize CD62L (L-selectin) and CD11b; while activated cells express increased levels of CD11b on the cell surface they shed CD62L from the cell surface. In addition to these adhesion markers, we also assessed markers of degranulation using antibodies that recognize CD35, CD66b, and CD63. These degranulation markers are present on the inner membranes of secretory vesicles (CD35), specific granules (CD66b), and azurophilic granules (CD63). Neutrophil degranulation results in the fusion of these vesicular membranes with the cell membrane and the expression of CD35, DC66b, and CD63 on the cell surface. Freshly collected whole blood samples (100-μl) were placed in 12×75-mm polystyrene Falcon tubes and incubated for 20 min at room temperature in the dark with saturating concentrations of CD66b-FITC, CD63-PE, CD14-PerCP, CD11b-APC, CD64-FITC, CD35-PE, CD62L-APC antibody conjugates (BD Biosciences, San José, CA). CD14 was assessed to facilitate separation of gated cell populations. Appropriate isotype-matched antibodies were used in separate tubes to control for autofluorescence and non-specific binding. After the incubation period, erythrocytes were removed by adding 2 ml of FACS™ Lysing Solution (BD Biosciences) for 10 min followed by centrifugation at 500×*g* for 5 min at room temperature. Cells were washed once with CellWASH™ (BD Biosciences) and resuspended in 400 μl of a 1% paraformaldehyde solution. Stained cell suspensions were acquired on a dual-laser FACSCalibur flow cytometer (BD Biosciences) calibrated for four-color analysis using CaliBRITE™ beads (BD Biosciences). For each sample, 10^3 neutrophils were acquired with CellQuest® software (BD Biosciences) using a live-gate setting to distinguish the neutrophil population from other cells and debris according to the CD14-PerCP fluorescence versus side-scatter (SSC) light characteristics. Data analysis was performed with FlowJo software v.8.7 (Tree Star Inc., Ashland, OR). Electronic analysis gates and quadrant markers were set to define positive and negative populations using fluorescence histogram data according to the non-specific staining of isotype-matched negative controls. Results were recorded as percentage (%)-positive cells and mean channel fluorescence intensity (MFI; in arbitrary units, a.u.). Absolute cell counts were obtained by multiplying the corresponding percentages of cells derived from FACS analysis by total leukocyte counts obtained with a hematology analyzer (Coulter Electronics, Hialeah, FL).

Oxidative burst activity

The intracellular oxidative burst capacity (i.e., generation of superoxide and secondary reactive oxygen species) of whole blood neutrophils was measured with flow cytometry, using commercially available fluorometric assay kits (PHAGOBURSTR® kit, Orpegen Pharma, Heidelberg, Germany). This test provides a quantitative assessment of burst activity by measuring the percentage and fluorescence intensity (MFI, in the FL1 530-nm emission channel) of cells that oxidize the fluorogenic substrate dihydrorhodamine (DHR)-123 to green fluorescent rhodamine (Rho)-123 in the presence of hydrogen peroxide]. Briefly, three 100-μl aliquots of each heparinized whole blood sample were placed in separate test tubes and combined with 20 μl of either wash buffer (as an unstimulated control), N-formyl-methionyl-leucyl-phenylalanine (fMLP), or phorbol 12-myristate 13-acetate (PMA) at final concentrations of 5 and 8.1 μM, respectively. Simultaneously, 20 μl of DHR-123 substrate solution was added to each tube and then samples were incubated in a water bath at 37° for 20 min. In all cases, reactions were stopped by placing chilling tubes in an ice bath. Erythrocytes were lysed by addition of 2 ml of BurstTest Lysing Solution for 10 min. After centrifugation at 250 *g* for 5 min, the lysates were discarded and the remaining white cells resuspended and incubated with anti-CD14-APC at room temp for 15 min. The cells were then washed with 3 ml of Wash Solution and centrifuged at 250×*g*. Finally, 200 μl of and

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propidium iodide (PI) solution in PBS (200 μg/ml) was added to each sample on ice. Samples were analyzed using a FACSCanto (BD Biosciences) flow cytometer within 30 min. Neutrophil populations were gated using a CD14-APC/SSC-defined gate; 10,000 neutrophil events were recorded for each sample. Data were analyzed in FlowJo™ software v.8.7 (Tree Star) and the results are presented as the % Rho-123-positive neutrophils and as Rho-123 MFI expressed as a percentage of the untimulated control sample.

Soluble endothelial adhesion molecules, myeloperoxidase, and matrix metallopeptidase 9

Plasma and serum samples were isolated and stored at −80°C until analysis. Serum concentrations of soluble ICAM-1, VCAM-1, E-selectin, P-selectin were measured in duplicate with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (Quantikine®, R&D systems Inc., Minneapolis, MN) and read on an automated microplate photometer (EL340, BIO-TEK Instruments, Winooski, VT). Plasma levels of myeloperoxidase (MPO) and matrix metallopeptidase 9 (MMP-9) were measured using ultra-sensitive electrochemiluminescence-based immunoassay detection and analyzed on a Sector Imager 6000 system according to the manufacturer's instructions (Meso Scale Discovery, LLC, Gaithersburg, MD).

Statistical analyses

All data were expressed as mean ± SEM. Biomarker levels were treated as normally distributed continuous variables. For statistical analyses, Student's *t* test was used for continuous variables and χ^2 test was used for categorical predictor variables. The non-parametric Mann-Whitney *U* test was used for continuous variables that were not normally distributed. Serial comparisons (*time* × *treatment*) of biomarkers between treatment groups and control group were made using repeated measures ANOVA with *post-hoc* Bonferroni/Dunn testing. All analyses were two-tailed and p-values < 0.05 were considered statistically significant.

Results

Hypertonic resuscitation does not improve clinical outcome

The current report summarizes our findings with a small subgroup of trauma patients of the larger ROC trial [21]. In this subgroup of patients, we assessed a number of immunological parameters to determine how hypertonic fluids affect the inflammatory response to trauma. This subgroup included a total of 34 patients with hemorrhagic shock who were enrolled in the ROC study sites in Seattle and Toronto. Of the 34 patients, 9 received 250 ml HS, 8 received an equal volume of HSD, and 17 patients received 250 ml of NS (Table 1). There were no significant differences between these 3 treatment groups with regards to gender distribution, age, type of injury, and injury severity scores (ISS). The average multiple organ dysfunction score (MODS) of patients treated with NS was higher than those of the HS and HSD groups, but these differences were not statistically significant. However, patients treated with HS arrived in the emergency department with an average systolic blood pressure that was significantly higher than in the other treatment groups (Table 1). All of the patients treated with HSD or HS survived, while two patients in the NS group died within a week after hospitalization. Interestingly, while the average systolic blood pressures in the HS group was higher than in the HSD group, these differences could not be explained by corresponding sodium levels. As expected, the average plasma sodium concentrations in patients treated with HS and HSD were similar and significantly higher than in the NS group with sodium levels ranging in the lower bracket of normal values for healthy adult controls.

Leukocyte and neutrophil counts in all patients were significantly elevated compared to age-matched healthy controls (Fig. 1). While leukocyte and neutrophil counts generally

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decreased after admission, they remained significantly elevated above healthy control values throughout the 24-h observation period. Total leukocyte counts did not differ among patient groups. However, the initial neutrophil count in the HS group was significantly lower than in the NS group.

HS inhibits trauma-induced neutrophil activation

As a measure of neutrophil activation, we assessed the expression of adhesion molecules and degranulation markers on the cell surface of neutrophils in whole blood samples of trauma patients. Neutrophil CD11b expression in the NS group was significantly upregulated compared to values of healthy controls (Fig. 2A). CD11b expression in patients treated with HS was significantly lower compared to the NS group. In fact, throughout the observation period, values in the HS group did not differ from healthy controls. In the HSD group, only initial CD11b values at admission were lower than in the NS group. While neutrophil activation results in increased CD11b expression, L-selectin (CD62L) expression decreases because of a process termed L-selecting shedding. In the HSD and NS groups, but not the HS group, we found evidence of L-selectin shedding on admission with CD62L expression levels that were significantly lower than in healthy controls (Fig. 2B). Interestingly, in the patients treated with HSD, L selectin shedding was, most pronounced with CD62L expression values remaining significantly lower compared to the NS group (Table 2). Taken together with the CD11b expression data, these findings suggest a higher degree of neutrophil activation in the HSD group compared to the HS group.

HS inhibits trauma-induced neutrophil degranulation

A hallmark of the inflammatory response leading to host tissue damage after trauma is neutrophil degranulation, which is responsible for the release of proteolytic enzymes and other cytotoxic mediators such as myeloperoxidase (MPO) that cause secondary host tissue damage [22]. Neutrophils possess four discrete types of granules that contain specific sets of cytotoxic mediators; and these granules are released in a hierarchical fashion by the mobilization of secretory vesicles, the most easily mobilized granule type, followed by gelatinous (tertiary), specific (secondary), and then azurophilic (primary) granules [22]. While exocytosis of these granules causes the release of luminal proteins into the extracellular space, this process also results in the incorporation of granule membrane proteins into the cell surface, which can be assessed by flow cytometry. Using antibodies recognizing CD35, CD66b, and CD63, we assessed degranulation of secretory vesicles (CD35), specific granules (CD66b), and azurophilic granules (CD63), respectively. At admission to the emergency department, CD35 was upregulated in the NS but not in the HS or HSD groups, suggesting that hypertonic resuscitation in the field attenuates degranulation of secretory vesicles (Fig. 2C). However, over the course of the subsequent 24-h observation period, CD35 expression increased in all treatment groups to levels that were significantly higher than in healthy controls. This indicates that the initial inhibition of this degranulation process is relatively short-lived.

While both hypertonic fluids prevented initial CD35 expression, only HS reduced upregulation of CD66b, which suggests that HS may be more effective than HSD in preventing degranulation of specific granules (Fig. 2F). Neutrophil CD63 expression is a measure of exocytosis of elastase-containing azurophilic granules. We found that CD63 expression in the HS and HSD groups was initially lower than in the NS group and in healthy controls (Fig. 2D). One possible reason for this finding could be differential effects on cell adherence and numbers of circulating CD63 positive neutrophils. This notion is supported by the lower percentage of CD63 positive neutrophils in the HS and HSD groups compared to the NS and control groups (Table 2).

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In addition to the degranulation markers described above, we also assessed the expression of CD64 (Fcγ receptor I) on the cell surface of neutrophils. CD64 is a sensitive marker of cell activation and bacterial infection [23]. Similar to our findings with CD66b, treatment with HS but not with HSD attenuated the initial upregulation of CD64 seen on admission of trauma patients (Fig. 2E). However, as with CD35 expression, these differences were transient and CD64 expression increased over time in all treatment groups and reached levels that were significantly higher than in controls within 12 h after admission. This trend was paralleled by overall higher percentages of CD64 positive neutrophils in all patients after admission to the hospital (Table 2).

Taken together these findings indicate that HS and, apparently to a lesser extent, HSD treatment can inhibit trauma-induced neutrophil activation, suggesting that hypertonic fluid resuscitation is indeed able to attenuate specific aspects of the inflammatory processes involved in neutrophil activation.

HS abolishes neutrophil priming after trauma

In order to assess in more detail how the different resuscitation fluids affect neutrophil activation, we measured oxidative burst of neutrophils in whole blood samples freshly collected from trauma patients (Fig. 3). Heparinized whole blood samples were incubated with or without fMLP or PMA and oxidative burst was assessed using flow cytometry. In the NS group but also the HSD group fMLP-induced oxidative burst was significantly higher than in the HS group reaching values ~4-times higher than in healthy controls. By contrast, oxidative burst in the HS group was not different from that of healthy controls and remained at these levels throughout the entire 24-h observation period. Stimulation of control cells with PMA induced an oxidative burst response that was about 9-times higher than the response elicited by fMLP. This response was further increased by trauma resulting in ~6 fold higher levels in the NS and HSD groups. However, despite the powerful oxidative burst response triggered by PMA, neutrophils of patients in the HS group showed a clearly lower response compared to the HSD and NS groups. Taken together these data demonstrate that trauma results in profound neutrophil priming that exacerbates oxidative burst in response to subsequent stimuli such as fMLP or PMA. Importantly, we found that HS but not HSD is able to attenuated neutrophil priming.

Evaluation of the proportion of rhodamine (Rho)-123 positive neutrophils revealed marked differences between the three treatment groups (Table 3). On admission, which best reflects the initial response to trauma and resuscitation, we found a significantly smaller percentage of Rho-123 positive neutrophils in the HS group compared to the HSD and NS groups. This was true for unstimulated cells as well as for cells stimulated with fMLP, indicting that HS reduces cell activation as well as priming by trauma.

HS treatment reduces MPO and MMP-9 release

The findings described above indicated that HS treatment reduces neutrophil priming in trauma patients. In order to study the effect of HS resuscitation on neutrophil activation in more detail, we assessed concentrations of cytotoxic mediators released from neutrophils in plasma samples of trauma patients treated with HS, HSD, or NS. Myeloperoxidase (MPO) and matrix metallopeptidase (MMP)-9, also known as type IV collagenase or gelatinase B are neutrophil proteases that are released from the azurophilic and gelatinous (tertiary) granules, respectively [24]. MPO and MMP-9 concentrations in the plasma of trauma patients were significantly higher than in healthy controls (Fig. 4). In the HS and HSD groups, the initial MMP-9 levels at admission were significantly lower than in the NS group, suggesting that both hypertonic resuscitation fluids can reduce the activation of neutrophils in response to trauma. This was not observed in the case of MPO, where plasma levels at

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admission did not differ among the three treatment groups. In the HS group and, to a lesser extent, in the HSD group, MPO and MMP-9 concentrations were below admission values 12 h after resuscitation. At this time point, MMP-9 values in the HS group were significantly lower than in the HSD group. These findings suggest that the suppressive effect of HS on MMP-9 release remains for at least 12 h after resuscitation. At the 24-h time point, MMP-9 levels increased again and did not differ among the three treatment groups, while MPO concentrations at this time point were lower in the HS group than in the HSD group.

HS treatment reduces endothelial cell activation and damage

The results described above suggest that HS treatment reduces neutrophil activation. Neutrophil activation and the sequestration of activated cells to host organs are critical steps in the progression of MODS. Neutrophils are sequestered from the circulation by adhesion molecules expressed on endothelial cells. Along with L-selectin, E- and P-selectins regulate initial neutrophil rolling on the endothelial surface, while ICAM-1 and VCAM-1 induce firm adhesion. These molecules are released from the endothelium in response to inflammation and can thus serve as biomarkers of endothelial cell activation and/or damage [25]. We found that trauma patients had significantly elevated levels of circulating sICAM-1, sVCAM-1, sE-selectin, and sP-selectin levels over the 24-h observation period after admission (Fig. 5). On admission, however, only sP-selectin levels were significantly elevated compared to the concentrations found in healthy controls. While all patients showed initially elevated sP-selectin levels on admission, these levels decreased in the HS group and reached normal values within 12 h after admission. In contrast to sP-selectin, the concentrations of the other three biomarkers increased over time after admission, suggesting progressive endothelial activation and dysfunction. Interestingly, in the HS group but, these increases were attenuated compared to the other two treatment groups. In fact, in the HS group, sICAM-1, sVCAM-1, and sE-selectin levels remained at normal control levels throughout the 24-h observation period. In the HSD group, however, plasma sICAM-1 and sVCAM-1 concentrations at the 24-h time point were significantly higher than in the HS and NS groups, suggesting more severe endothelial cell damage.

Discussion

The optimal dose, timing, and composition of intravenous fluids for resuscitation of traumatic hemorrhagic shock patients remain controversial. Previous experimental and clinical studies have shown that small-volume hypertonic/hyperoncotic resuscitation fluids are effective intravascular volume expanders and attenuate post-traumatic inflammatory cascades, as compared to conventional isotonic crystalloids. To our knowledge, this is the first report to show that pre-hospital treatment with HS versus HSD differentially modulates neutrophil and endothelial cell functions. We demonstrate that HS is more effective than HSD in reducing post-traumatic alterations in cellular and soluble neutrophil and endothelial cell activation marker profiles.

Hypertonic fluids have been shown to suppress neutrophil responses *in vitro* and to ameliorate neutrophil-mediated host tissue damage in animal models of shock [10–14]. However, despite the generally encouraging results of the many laboratory studies that had been performed with hypertonic fluids, clinical trials have yielded relatively unimpressive results [17,26]. Based on previous work, we hypothesized that the reason for the disappointing results of clinical trials may have been due to pro-inflammatory side effects caused by delayed administration of hypertonic fluids. This hypothesis was based on findings that hypertonic saline increases degranulation of neutrophils that were stimulated with fMLP before being exposed to hypertonicity [8]. Moreover, it was shown that the protective effect of hypertonic resuscitation in a mouse model of shock was lost or reversed when hypertonic saline was administered after the infusion of isotonic fluids [27]. Based on

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the assumption that delayed treatment could have a negative impact on the clinical efficacy of hypertonic resuscitation, we designed a clinical trial protocol that would reduce the likelihood of such undesired side effects, namely by administering hypertonic fluids in a pre-hospital setting before any other treatments.

A subsequent multicenter trial conducted by the ROC was based on this concept and has allowed us to test the hypothesis that pre-hospital treatment with HS or HSD improves the clinical efficacy of hypertonic fluid resuscitation. However, unfortunately this trial had to be suspended prematurely on the basis of futility and potential safety concerns [21]. The Data Safety Monitoring Board noted that mortality was higher in the HS and HSD groups compared to the NS group. This was the case in the post randomization subgroup of patients who did not receive any blood products in the first twenty-four hours. Detailed data analysis indicated that this was due to an earlier mortality in the HS and HSD groups, but that there were no differences in overall 28-day mortality between the treatment arms [21]. Despite the early termination of the parent clinical trial, we were able to carry out the immunological studies presented in this report using blood samples of patients who had been enrolled in the Toronto and Seattle study sites. However, the resulting small sample size represents a limitation of our current study.

Nevertheless, our findings suggest that hypertonic resuscitation can indeed reduce the activation and priming of neutrophils. These results, thus, largely recapitulate previous findings of preclinical laboratory studies. However, unexpectedly our study also revealed marked differences between the HS and HSD treatment groups. This is somewhat surprising, given that the mechanisms by which hypertonic fluids influence immune cells are thought to depend primarily on osmotic effects, which were not markedly different between the HS and HSD groups. Exposure of immune cells to hypertonic fluids has been shown to result in rapid cell shrinkage that is accompanied by the release of cellular ATP; extracellular ATP is converted to adenosine and adenosine suppresses neutrophils by autocrine mechanisms that involve A2a adenosine receptors on the cell surface [9]. Since both HS and HSD increased plasma sodium concentrations to comparable levels (Table 1), it is unlikely that differences in the osmotic effects triggered by these fluids could explain the divergent responses of neutrophils to these resuscitation fluids. Yet, we found remarkable differences between the HSD and HS groups with regard to several post-traumatic inflammatory responses we studied.

One possible explanation for these differences could be related to the synthetic colloid dextran 70, which is the main distinction between HSD and HS. Dextran 70 may elicit an inflammatory response that is negligible when administered in small quantities to healthy subjects, but that might be amplified in severely traumatized patients by the immunomodulatory effects of hypertonic saline. Although the effects of dextran 70 on the induction of acute anaphylactoid and inflammatory reactions have been examined previously [28], specific inflammatory responses to dextran 70 after traumatic and hemorrhagic shock have not been well studied. However, some previous evidence supports the notion that dextran can increase oxidative burst of neutrophils *in vitro* and the transcription of inflammatory cytokines and chemokines, such as tumor necrosis factor-α and interleukin-8 [29,30]. Dextrans are polysaccharides produced by lactic-acid bacteria such as *Leuconostoc mesenteroides*. Special purification methods are required to isolate dextran from culture media and to eliminate bacterial byproducts in order to obtain refined dextran preparations suitable for clinical applications. While it may not be feasible to quantitatively eliminate all trace contaminants and remaining culture byproducts are not thought to cause dextran reactions, such byproducts could play at least a contributory role to mild inflammatory responses that have been observed on rare occasions [30]. Previous *in vitro* studies have shown that hypertonic saline at clinically relevant doses can enhance T

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cell responses by autocrine feedback mechanisms that involve ATP release and P2 receptor activation [31–33]. Therefore, it is possible that dextran in combination with hypertonic saline could exacerbate otherwise mild subclinical inflammatory responses, which could explain why HSD was less effective than HS in suppressing some of the inflammatory markers we studied.

However, despite the differences between HSD and HS we found in our substudy, the overall results of the parent ROC trial indicate that there are no measureable differences in clinical outcome between patients who received HSD or HS. This suggests that the increased inflammatory markers we found in the HSD group may not affect the overall clinical outcome in trauma patients. This notion is also supported by the fact that the HSD and HS groups in our current substudy did not differ with regard to average MODS scores (Table 1).

Over the last few years, we have found that osmotic cell shrinkage in response to HS induces the release of intracellular ATP from many different cell types in addition to neutrophils. Because all these different cell types express their own cell type-specific subsets of the nineteen known ATP and adenosine receptors as well as members of the ecto-enzyme families that metabolize released ATP, the responses of different cell types to HS likely vary greatly. Extracellular ATP and autocrine signaling via these purinergic receptors are being increasingly recognized as a general motif in immune cell activation [34]. Yet, little information is currently available about how HS-induced ATP release affects immune cells other than neutrophils and $CD4^+$ T cells that we have studied in more detail in our previous work. Moreover, the response of the endothelium to HS is poorly understood but likely to have a major role in regulating neutrophil adhesion and sequestration to host organs. Thus, it is possible that still unknown purinergic signaling events triggered by trauma and HS could be reasons for the lack of clinical efficacy of HS resuscitation. As an example, we recently found that trauma causes a dramatic increase in A3 adenosine receptor expression by neutrophils [35]. A3 receptors have a co-stimulatory role in neutrophil activation by counteracting the anti-inflammatory effect of A2a receptors [18]. Hence, increased A3 receptor expression in trauma patients may diminish the protective effects of HS [36].

In summary, our current study shows that pre-hospital resuscitation with HS, but not HSD, suppresses markers of neutrophil and endothelial cell activation/damage as compared with isotonic fluid. This study establishes, for the first time in trauma patients, that different formulations of hypertonic resuscitation fluids differentially regulate various cellular and molecular inflammatory pathways. Notwithstanding the lack of clinical utility observed in the parent ROC multi-center trial [21], the specific immunomodulatory effects observed in the patient cohorts studied here warrant further investigation of hypertonic resuscitation as a means to dampen excessive post-traumatic inflammatory responses. However, the design of effective hypertonic resuscitation strategies will require a detailed knowledge of how hypertonic fluids influence purinergic signaling mechanisms and immune cell functions.

Acknowledgments

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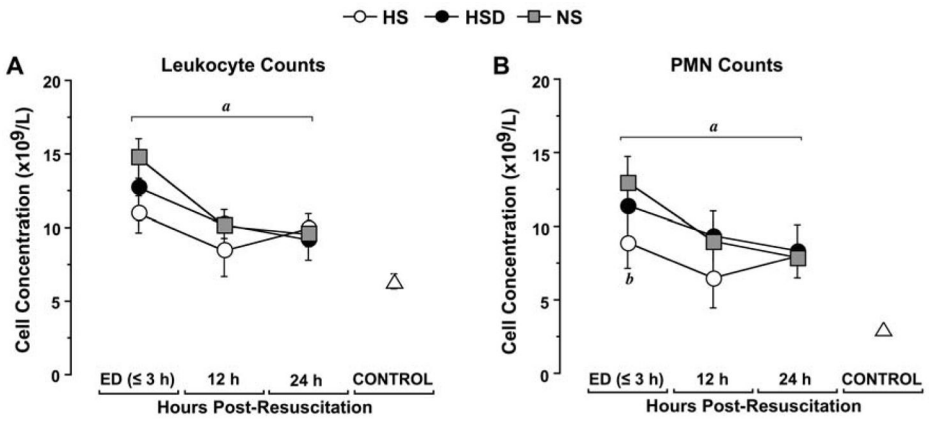

Fig. 1. Leukocyte and neutrophil counts were significantly elevated in all trauma patients

Peripheral blood samples were collected in EDTA vacutainers from healthy controls (n=20) and trauma patients resuscitated with NS (n=17), HSD (n=8), or HS (n=9) at the time of emergency department admission (≤3 h post-resuscitation) and 12 and 24 h post-resuscitation. Total leukocyte counts (**A**) and neutrophil (**B**; PMN) differential counts were determined with a Beckman Coulter Hematology Analyzer. Statistics: [a]$P < 0.05$ vs. age-matched healthy controls; [b]$P < 0.05$ vs. time-matched NS-treated patients; [c]$P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.

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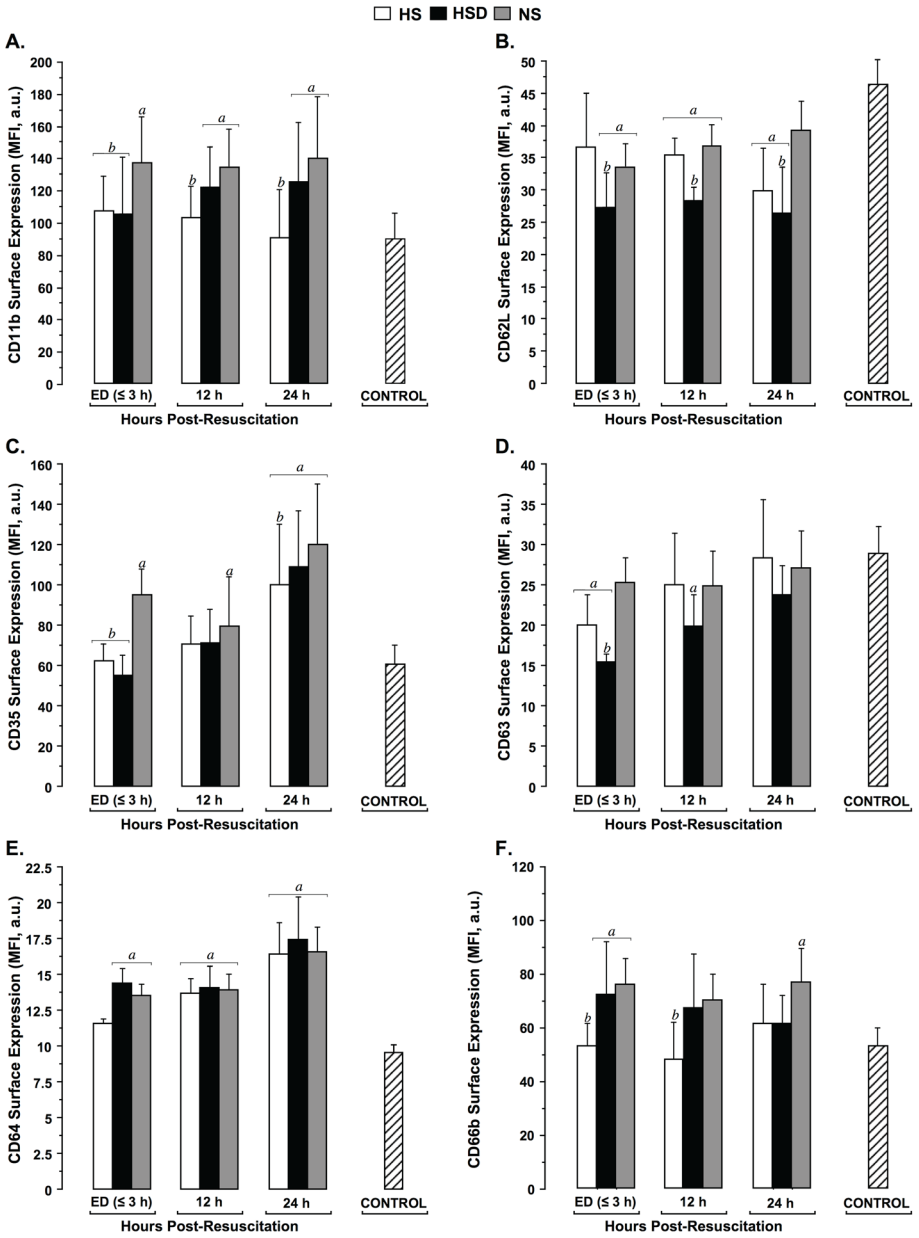

Fig. 2. Neutrophil cell-surface activation, adhesion, and degranulation markers

Cell-surface expression of CD11b (**A**), CD62L (**B**), CD35 (**C**), CD63 (**D**), CD64 (**E**), and CD66b (**F**) were assessed by multiparameter flow cytometry using freshly drawn heparinized whole blood. Results were expressed as mean fluorescence intensity (MFI ± SEM) in arbitrary units (a.u.). The corresponding percentage of receptor-positive cells is shown in Table 2. Blood was sampled serially from patients resuscitated with normal saline (NS; n=17), hypertonic saline-dextran (HSD; n=8), or hypertonic saline (HS; n=9) upon hospital admission (ED ≤3 h) and 12 and 24 h after resuscitation. Blood samples from age and gender matched healthy volunteers served as control (n=20). Statistical analyses: $^{a}P < 0.05$ vs. age-matched healthy controls; $^{b}P < 0.05$ vs. time-matched NS-treated patients; $^{c}P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.

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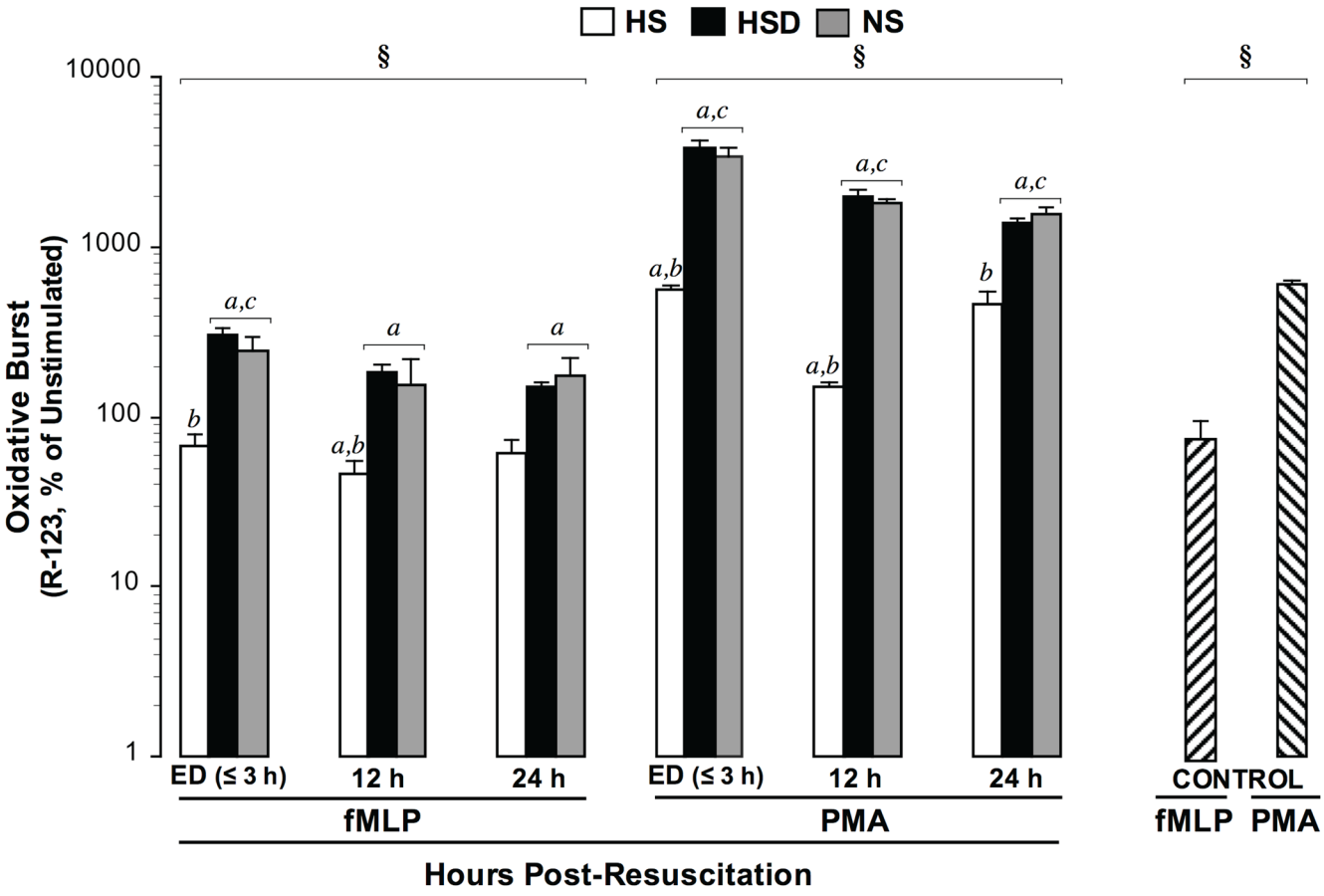

Fig. 3. Neutrophil oxidative burst activity

Neutrophil oxidative burst activity was measured by assessing intracellular rhodamine (Rho)123 mean fluorescence intensity (MFI ± SEM) in arbitrary units (a.u.) of neutrophils in whole-blood samples from healthy controls (n=20) and trauma patients resuscitated with NS (n=17), HSD (n=8), or HS (n=9) at the time of emergency department admission (≤3 h post-resuscitation) and 12 and 24 h after resuscitation. Samples were incubated at 37°C with N-formyl-methionine- leucine-phenylalanine (fMLP, 5 μM) as a weak stimulus or with phorbol 12-myristate 13-acetate (PMA, 8.1 μM) as a strong stimulus. Rho-123 MFI values were expressed as a percentage of unstimulated control samples. Statistical analyses: $^{\S}P < 0.05$ vs. unstimulated control values; $^{a}P < 0.05$ vs. age-matched healthy controls; $^{b}P < 0.05$ vs. time-matched NS-treated patients; $^{c}P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.

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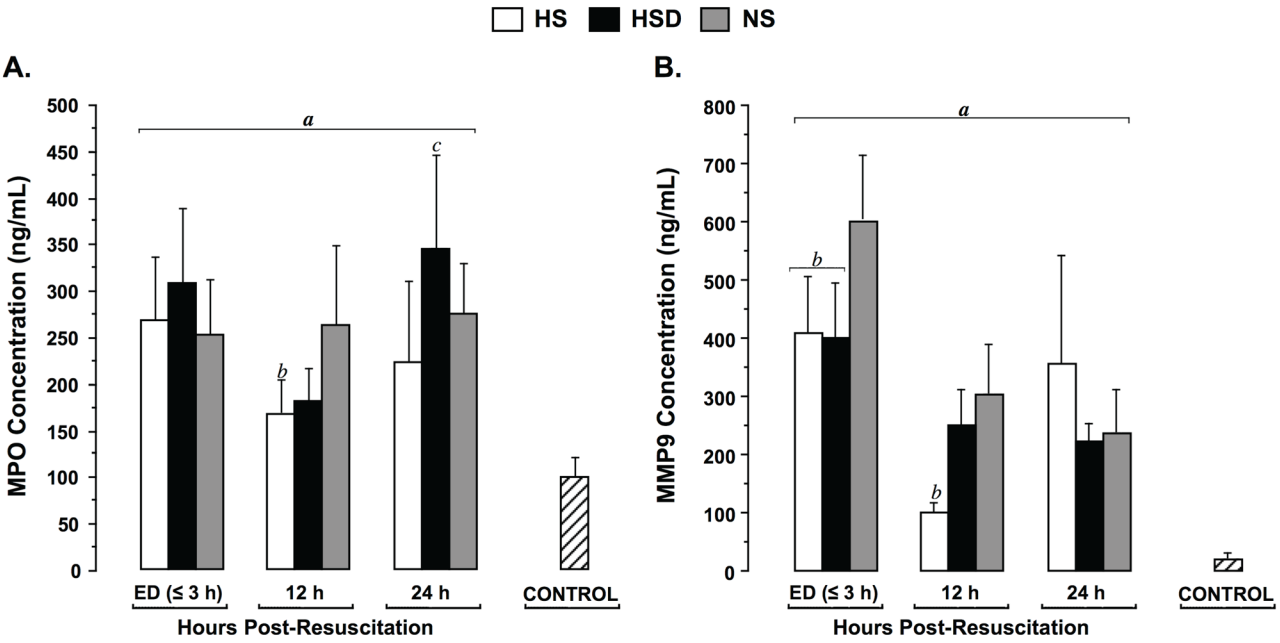

Fig. 4. Circulating myeloperoxidase and matrix metallopeptidase 9 levels

Plasma concentrations of MPO and MMP-9 were determined in healthy controls (n=20) and trauma patients resuscitated with NS (n=17), HSD (n=8), or HS (n=9) at the time of emergency department admission (≤3 h post-resuscitation) and 12 and 24 h after resuscitation, using electrochemiluminescence-based immunoassay detection methods. Statistical analyses: data are shown as mean ± standard error mean (SEM); [a]$P < 0.05$ vs. age-matched healthy controls; [b]$P < 0.05$ vs. time-matched NS-treated patients; [c]$P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.

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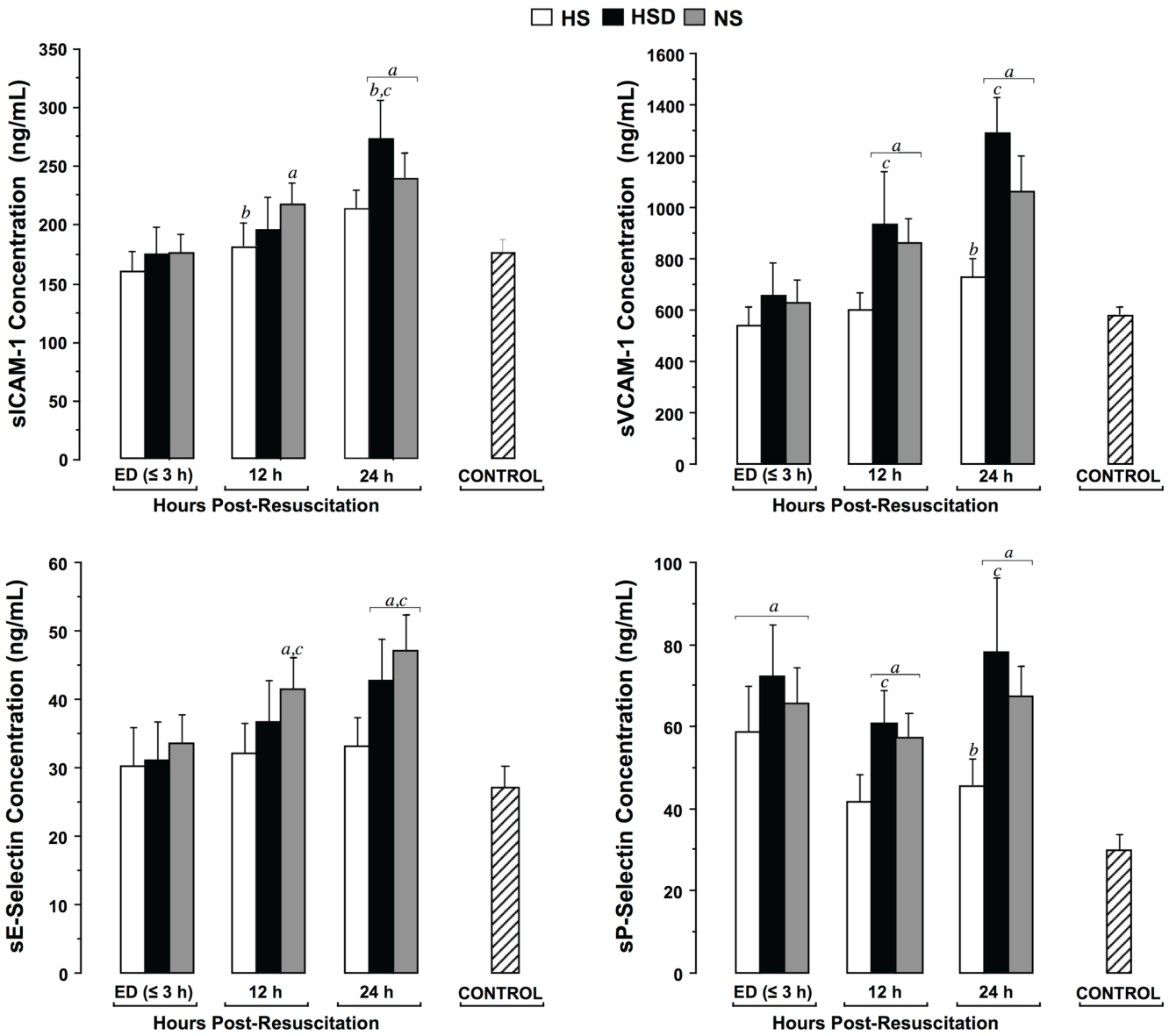

Fig. 5. Soluble endothelial adhesion molecules

Serum concentrations of soluble (s)ICAM-1 (**A**), sVCAM-1 (**B**), sE-selectin (**C**), and sP-selectin (**D**) were determined in healthy controls (n=20) and trauma patients resuscitated with NS (n=17), HSD (n=8), or HS (n=9) at the time of emergency department admission (≤3 h post-resuscitation) and 12 and 24 h after resuscitation. Statistics: data are shown as mean ± standard error mean (SEM); [a]$P < 0.05$ vs. age-matched healthy controls (n = 20); [b]$P < 0.05$ vs. time-matched NS-treated patients; [c]$P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.

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Table 1

Patient demographics and clinical characteristics of patients treated with the three different resuscitation strategies

Patients were treated in the field with HS, HSD, or NS and the initial clinical data shown were assessed on hospital admission.

Parameters	All patients	Resuscitation Treatment		
		HS	HSD	NS
Demographics				
Sample size	34	9	8	17
Sex, *n* (% male)	25 (73.5)	8 (88.9)	5 (62.5)	12 (70.6)
Age, yrs (SD) [a]	43 ±20	44 ±23	43 ±18	42 ±21
Clinical Characteristics				
Admission SBP, mean (SD), mmHg	123 ±29	141 ±31[b]	126 ±25	112 ±27
Admission plasma Na^+ (mM)	142±1	148±1[a,b]	148±1[a,b]	137±2
Type of injury, n (%)				
Blunt	29 (85.3)	8 (88.9)	7 (87.5)	14 (82.4)
Penetrating	5 (14.7)	1 (11.1)	1 (12.5)	3 (17.6)
ISS, mean (SD) [b]	22 ±12	24 ±17	21 ±9	21 ±11
Worst MODS score, mean (SD) [b]	5.7 ±5.9	4.9 ±3.9	4.8 ±5.3	6.6 ±7.2

Abbreviations: HS, hypertonic saline; HSD, hypertonic saline plus dextran; NS, normal saline; ISS, Injury Severity Score; MODS, Multiple Organ Dysfunction Score.

Statistics: Results are expressed as mean ± standard deviation (SD);

[a] $P < 0.05$ vs. age-matched healthy controls (n = 20);

[b] $P < 0.05$ vs. time-matched NS-treated patients; Mann-Whitney *U*-test.

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Table 2

Percentages of neutrophils positive for cell surface adhesion and activation markers.

	Cont.	HS			HSD			NS		
Markers		**ED≤3 h**	**12 h**	**24 h**	**ED≤3 h**	**12 h**	**24 h**	**ED≤3 h**	**12 h**	**24 h**
CD11b	98±1	98±1	99	99	99	98	94±5	99	99	99
CD62L	93±2	75±11[a]	95±1	68±18[a,b]	85±5	86±6	47±27[a,b,c]	87±4	87±7	86±6
CD35	97±1	93±1	92±1	83±16	91±4	95±4	98±1	94±1	95±1	98
CD66b	99±1	99	100	100	99	99	99	99	99	99
CD63	91±4	82±6	90±6	94±3	80±5	90±5	89±5	93±3	92±3	93±3
CD64	23±5	34±5	65±12[a]	68±14[a]	39±12[a]	43±22[a]	49±18[a]	45±9[a]	47±9[a]	62±8[a]

Data are shown as mean ± SEM of the percentage of surface marker-positive neutrophils as determined by flow cytometry.

Abbreviations: HS, hypertonic saline; HSD, hypertonic saline plus dextran; NS, normal saline; ED≤3 h, sample drawn ≤3 h after admission to the emergency department.

Statistics:

[a] $P < 0.05$ vs. age-matched healthy controls;

[b] $P < 0.05$ vs. time-matched NS-treated patients;

[c] $P < 0.05$ vs. time-matched HS-treated patients, by ANOVA

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Table 3

Oxidative burst activity expressed as percentage of rhodamine-123 positive neutrophils.

	Controls	Resuscitation Treatment								
		HS			HSD			NS		
		ED	12 h	24 h	ED	12 h	24 h	ED	12 h	24 h
none	69±11	54±10	51±7[a]	52±10	79±13 [a,c]	59±18	53.±20	91±5 [a,c]	78±4 [a,c]	58±28
fMLP	76±7	65±10	60±7[a]	70±5	91±18 [a,c]	87±3[a,c]	89±10 [a,c]	91±4 [a,c]	81±10 [c]	84±9 [a,c]
PMA	99±1	100	100	100	99	100	100	96±3	98±2	100

Data are percent DHR-positive neutrophils and values are expressed as mean ± SEM.

Abbreviations: HS, hypertonic saline; HSD, hypertonic saline plus dextran; NS, normal saline; fMLP, formyl-Met-Leu-Phe; PMA, phorbol 12-myristate 13-acetate. ED, time of admission to the emergency department (≤3 h).

[a] $P < 0.05$ vs. age-matched healthy controls;

[b] $P < 0.05$ vs. time-matched NS-treated patients;

[c] $P < 0.05$ vs. time-matched HS-treated patients, by ANOVA.