Hypertonic Saline Resuscitation of Hemorrhagic Shock Increases In Vivo Neutrophil Interactions With Endothelium in the Blood-Brain Microcirculation

Wanfeng Gong, MD, Joshua A. Marks, MD, Paymon Sanati, Carrie Sims, MD, FACS, Babak Sarani, MD, FACS, Douglas H. Smith, MD, and Jose L. Pascual, MD, PhD, FRCPS(C)

Background: Resuscitation of hemorrhagic shock with isotonic crystalloids has been shown to activate polymorphonuclear neutrophils (PMNs). Although hypertonic saline (HTS) can reduce PMN activation and interactions with endothelial cells (EC) in systemic microvascular beds, no data exist demonstrating that the same occurs in the unique blood-brain barrier microcirculation. We hypothesized that resuscitation of hemorrhagic shock with HTS would blunt brain in vivo PMN-EC interactions.

Methods: Wistar rats (250–350 g) underwent craniotomy and placement of a window for live intravital viewing of pial vessels. Twenty animals were bled to a mean arterial pressure of 30 mm Hg to 35 mm Hg for 1 hour and resuscitated with shed blood and either 5% HTS (6 mL/kg) or Ringer’s lactate (RL) (2× shed blood volume). Circulating rhodamine-6G-labeled PMN in pial venules were captured by videomicroscopy at baseline (preshock), end of the shock period, after resuscitation, and every 15 minutes to 30 minutes for 2 hours. Hemodynamics and arterial gases were monitored. Off-line footage analysis allowed comparisons of PMN-EC interactions between groups.

Results: Animals in both groups developed significant metabolic acidosis (p < 0.01) after hemorrhage, but postresuscitation blood pressures were similar at all time points. Crystalloid resuscitation volumes were 10× greater in RL than HTS animals (p < 0.001). For all time points, we did not observe the expected reduction in PMN rolling and adhesion in HTS animals, instead demonstrated the PMN

Conclusions: In contradistinction to studies evaluating the systemic microcirculation, HTS may activate PMN-EC crosstalk in the blood-brain microcirculation. Further studies are needed to analyze whether this effect is due to the unique nature of the blood-brain interface.

Key Words: Intravital microscopy, Hypertonic saline, Ringer’s lactate, Hemorrhagic shock, Cranial window, Neutrophil, Endothelium.

(J Trauma. 2011;XX: 000–000)

Survivors of multiorgan trauma are often admitted to the intensive care unit for prolonged periods of time during which they are at risk for various complications. In particular, infections leading to local and systemic inflammation may lead to sepsis and organ dysfunction, which is associated with a high mortality.1 Despite recent advances in resuscitation and critical care, late mortality rates have remained largely unchanged.2 Intense research has concentrated on finding resuscitation fluids that not only reverse shock and restore end-organ flow but also may curtail subsequent systemic inflammation, organ dysfunction, and death. Indeed, standard shock resuscitation with normal saline (NS) or Ringer’s lactate (RL) may worsen inflammation, particularly that mediated by the polymorphonuclear neutrophil (PMN).3 Studies evaluating systemic capillary beds have characterized the PMN’s physiologic progression from microvascular to target tissue where it effectively destroys invading pathogens and removes debris of tissue injury. After margination to the vessel wall, the PMN interacts in a stepwise fashion with endothelial cells (EC) before crossing the vessel wall and migrating to the tissue site of action. In conditions of systemic inflammation, as can occur after resuscitated shock, this sequence may become disrupted and inappropriately augmented leading PMN activation to result in host tissue injury.

In the last two decades, hypertonic saline (HTS) has emerged as an alternative resuscitation fluid that is safe and efficacious. When compared with RL, HTS may be used in much smaller volumes resulting in rapid restoration of intravascular deficits.4 In addition, several studies have demonstrated that HTS possesses important immunomodulatory properties that may blunt overexuberant PMN responses to shock or resuscitation and decrease inflammation in the microcirculation. HTS has also been used for over a decade as a potent osmotherapeutic agent exerting a particular effect on injured brain tissue, drawing fluid intravascularly, reducing edema, and lowering intracranial pressure. Some investigators have postulated that this beneficial effect in swollen brain tissue might in part be due to counter-inflammatory properties of HTS on PMN function in the brain microcirculation.5,6

Although some in vitro and ex vivo studies have found reduced PMN surface receptor expression with HTS, it is intravital microscopy (IVM) that has offered direct real-time visualization of intravascular events and has demonstrated reduced EC-PMN in vivo in certain systemic vascular beds. However, it remains unclear if these changes also occur in the blood-brain barrier (BBB) microcirculation where immunocompetence is markedly different from that observed in the systemic circulation.7 We hypothesized that HTS resuscitation...
of hemorrhagic shock would result in decreased neutrophil-endothelial interactions in the BBB microcirculation and would follow similar patterns as those reported in systemic vascular beds.

MATERIALS AND METHODS

Animal Model

All studies were performed in accordance with the Institutional Animal Care and Use Committee of the University of Pennsylvania. Male Wistar rats (250–350 g) (Charles River Laboratories, Wilmington, MA) were acclimated for 3 days to 5 days on a 12-hour light/dark cycle. Anesthesia was induced with 4% isoflurane in oxygen, and animals were tracheally intubated with a 14-gauge angiocatheter and mechanically ventilated with 1.5% isoflurane/70% N₂O/30% O₂ using a small rodent ventilator (Harvard 683, Harvard Apparatus, Holliston, MA). The left femoral vein and artery were cannulated with polyethylene-50 tubing (BD Biosciences, Sparks, MD) for i.v. administration (resuscitation fluids, withdrawn blood, rhodamine-6G, and fentanyl), withdrawal of arterial blood gas samples, and for continuous systemic arterial pressure monitoring through a pressure transducer attached to a monitor (Omnicare 24 Monitor, Hewlett Packard, Palo Alto, CA). Blood pressure was monitored throughout the experiment. Body temperature was maintained at 37°C with a silicon heating pad.

Craniotomy

The craniotomy was performed as previously described. Briefly, the animal’s head was secured in a stereotactic frame (Stoelting, Wood Dale, IL) in the prone position to facilitate placement of a quartz cranial window. The scalp and underlying soft tissue over bilateral parietal lobes was dissected free and a 10 mm × 5 mm diameter oval shape craniotomy was performed on the left side of the midsagittal suture using a low-speed dental drill (0–8,000 rpm) (Henry Schein, Melville, NY) (Fig. 1). After the dura was carefully removed from the cerebral surface to expose the pia mater, a 10-mm diameter quartz window was glued to the skull using cyanoacrylate glue. After window placement, isoflurane was maintained with 70% N₂O/30% O₂ and an intravenous bolus of fentanyl was given to facilitate placement of a quartz cranial window. The scalp was closed with 5–0 suture using a low-speed dental drill (0 – 8,000 rpm) (Henry Schein, Melville, NY) (Fig. 1). After the dura was carefully removed from the cerebral surface to expose the pia mater, a 10-mm diameter quartz window was glued to the skull using cyanoacrylate glue. After window placement, isoflurane was discontinued, and an intravenous bolus of fentanyl was given (10 μg/kg) for 1 minute. Thereafter, anesthesia was maintained with 70% N₂O/30% O₂ and fentanyl (25 μg/kg/h IV).

Pial IVM

The animal mounted on the stereotactic frame was transferred to an intravital microscope (ECLIPSE FN1, Nikon Instruments, Melville, NY) and the window was imaged with a 20× water immersion objective lens and a 10× eyepiece. A digital camera (QuantEM, Photometrics, Tucson, AZ) was used to project the images onto a computer monitor and digital analysis software (NIS-Elements Advanced Research Software, Nikon Instruments, Melville, NY) was used to display and capture footage. Recordings were stored for playback analysis in an external high-capacity hard drive (Seagate, Scotts Valley, CA). To visualize PMN-EC interactions occurring in the pial circulation under the cranial window, neutrophils were labeled with 0.3% rhodamine-6G (300 μg/mL in 0.9% saline), which was administered as an intravenous bolus (1 mL) followed by a continuous infusion at a rate of 1 mL/h. The rhodamine-6G-associated fluorescence was visualized by epi-illumination at 510 nm to 560 nm, using a 590-nm emission filter.

Hemorrhagic Shock Model

Hemorrhagic shock was induced by withdrawing blood from the femoral artery into a syringe previously flushed with 100 units of heparin until mean arterial pressure (MAP) reached 30 mm Hg to 35 mm Hg. Hypotension was maintained for 60 minutes by further blood withdrawals if MAP increased above 40 mm Hg or by reinfusion of withdrawn blood if MAP fell below 35 mm Hg. Withdrawn blood was maintained at 37°C in a warmed water bath. Animals were then resuscitated for 20 minutes with the above regimens. The timeline for the experimental procedure is illustrated in Figure 2.

Blood Gas Analyses

Arterial blood gas analysis was conducted at baseline (preshock, after a 10-minute stabilization period after the completion of surgery) and after shock to confirm an adequate shock state was reached. The blood was analyzed using an i-Stat blood analyzer (Abbott Laboratories, Abbott Park, IL) to obtain the following parameters: pH, bicarbonate (HCO₃), base excess, SaO₂, PaO₂, PaCO₂, and lactate.

Study Groups and Experimental Protocol

Twenty animals underwent hemorrhage and were resuscitated with shed blood and one of two crystalloid regimens: group 1—RL (2× shed volume, n = 13) and group 2—HTS (6 mL/kg of 5% HTS, n = 7). A 6 mL/kg of 5% solution, the HTS concentration available in our pharmacy, was chosen because it is equiosmolar to the 4 mL/kg of 7.5% HTS regimen that is used in the majority of animal and human HTS studies.
Evaluation of the Cerebral Microcirculation

Each animal’s live microcirculation was videorecorded at the following time intervals: Pre-S (preshock, after a 10-minute stabilization period at the completion of surgery); End-S (at the end of the shock period); immediately after resuscitation (t = 0); every 15 minutes for an hour (t = 15, 30, 45, 60); and every 30 minutes for the subsequent hour (t = 90, 120) (Fig. 2). Recordings with epifluorescence microscopy were captured in 30 s to 60 s intervals to avoid undue tissue exposure to fluorescence illumination. All animals were killed by isoflurane or fentanyl overdose at the completion of experiments. Two to three postcapillary venules (25–50 μm diameter, 100 μm length) were selected per animal, and the same venular section was observed during all analyzed time points.

After completion of all experiments, recordings were reviewed by a laboratory member offline. Rolling neutrophils were defined as the mean number of fluorescing cells crossing a 100-μm venular segment at a velocity less than that of erythrocytes and visibly interacting with the endothelial surface (rolling cells/100 μm/minute). Neutrophils were considered adherent to the venular endothelium if they remained stationary for at least 30 s. Preadherence was defined as the number of immobile neutrophils in the same 100 μm vessel section at the initiation of the 30-s period. Total neutrophil adherence was the sum of neutrophil adherence and preadherence.

Statistical Analysis

All data were presented as mean ± standard error of the mean unless otherwise stated. Differences between groups were compared using the paired Student’s t test if they were normally distributed otherwise the Mann-Whitney U test was used using SPSS software (SPSS, Chicago, IL). A two-tailed p value of <0.05 was considered statistically significant.

RESULTS

Adequacy of Shock Model

All animals were hemorrhaged until their MAP reached 30 mm Hg to 35 mm Hg. In a subgroup of animals from both groups (n = 11), arterial blood gas samples were obtained and analyzed at baseline (preshock) and end of the shock period (postshock) to establish whether shock was metabolically discernible (Table 1). Preshock blood gases demonstrated a mild respiratory acidosis likely from preintubation hypoventilation. However, postshock animals demonstrated a severe lactic metabolic acidosis that was only partially compensated, consistent with severe hemorrhagic shock.

Resuscitation and MAP

Hemorrhage resulted in MAPs that were equal in both groups (HTS: 32.0 ± 1.5 vs. RL: 31.5 ± 1.6 mm Hg; p = NS) (Fig. 3). After fluid administration, neither resuscitation regimen resulted in MAP return to preshock levels. HTS resulted in a greater blood pressure rise immediately after resuscitation and for 90 minutes thereafter, but differences with RL animals were not significant. Both resuscitation regimens have been shown to adequately resuscitate this model of class III hemorrhage.11,12 The Shock Model was Considered Adequate as Hemorrhaged Animals Demonstrated a Significant Lactic Metabolic Acidosis Despite Compensatory Hypocapnia

<table>
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<th>pH</th>
<th>Pre-shock</th>
<th>Postshock</th>
<th>p</th>
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<tbody>
<tr>
<td>7.30 ± 0.09</td>
<td>7.10 ± 0.17</td>
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<td>pCO₂ (mm Hg)</td>
<td>51 ± 19</td>
<td>24 ± 6.7</td>
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<td>Lactate (mmol/L)</td>
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<td>8.4 ± 3.4</td>
<td>0.0086</td>
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</table>

Effect of Crystalloid on Neutrophil Interactions With Endothelium

Neutrophils were labeled with rhodamine-6G administered before imaging and were visible with epifluorescence (Fig. 4). Figure 5 illustrates the percent preshock neutrophil rolling levels occurring at each time point after resuscitation. Rolling levels in both groups were significantly lower at the end of the shock period when compared with every time point.
thereafter ($p = NS$). After resuscitation, RL animals displayed rolling levels that were consistently lower than HTS animals ($p = NS$). HTS animals always demonstrated rolling levels that were higher than baseline ($p = NS$).

Baseline (preshock) adhesion levels started significantly lower in HTS than RL animals ($0 \pm 0 vs. 1.09 \pm 0.4; p = 0.04$). Despite this, HTS consistently tended to have higher PMN adhesion throughout all posttreatment time points when accounting for baseline adhesion numbers (Fig. 6). Although no statistical differences were seen in postresuscitation PMN adhesion levels, RL animals demonstrated a late increasing trend in the number of adherent cells that approached, but did not surpass the level of adherence in HTS. Thus, the expected reduced PMN adherence with HTS administration was not seen, instead trending to increased postresuscitation adhesion. To summarize, trends in rolling and adhering PMNs favored fewer interactions after RL resuscitation, consistently, for all time points although this did not reach statistical significance.

**DISCUSSION**

In the last decade, significant investigational efforts have been dedicated to finding resuscitation strategies that could confer resistance to the development of subsequent complications. Standard resuscitation with isotonic crystalloids (NS, RL) remains the principal treatment of shock, but increasing data suggest that such resuscitation is, in itself, injurious and contributes to host inflammation by profoundly

![Figure 3](image-url) MAP. Initially, HTS animals tended to have a higher MAP after resuscitation ($p > 0.05$).

![Figure 4](image-url) Representative image of pial microcirculation observed at 200× magnification. Note the neutrophils interacting with the endothelium (white arrows).

![Figure 5](image-url) Rolling neutrophils observed in the pial circulation and expressed as percent of levels observed before hemorrhage (mean % ± standard error of the mean). Absolute baseline (Pre-S) rolling levels did not differ between groups (HTS: 111 ± 21.6 vs. RL: 164 ± 74.9, $p = 0.40$). End-S (end of shock period) rolling levels were lower than Pre-S (preshock) in both groups, but this was only significant in HTS animals ($*p = 0.01$). End-S rolling levels in both groups were lower than levels at each time point thereafter ($\hat{O}p < 0.05$).
activating the innate immune system. In particular, PMN activation in the microcirculatory milieu and its exaggerated interaction with endothelium has been implicated as a key element in host progression to systemic inflammation.

Although PMNs are essential in destroying invading organisms and cellular debris, their release of toxic oxygen free radicals and proteinases may become overexuberant and cause host organ injury. In physiologic conditions, PMN transmigration into tissues follows an orderly sequence of steps, initiated by PMN margination and selectin-mediated rolling along the vessel wall. These initial weak interactions permit strong adhesion of surface PMN β2-integrins (CD11b) to endothelial receptors of the immunoglobulin superfamily (intercellular adhesion molecules [ICAM]-1 and ICAM-2). Through diapedesis, PMNs then pass to the interstitial space, to the source of tissue injury or infection where they release a powerful arsenal of oxygen free radicals and proteinases that destroy invading pathogens through adjacent degranulation and phagocytosis. With systemic inflammation, this process may become indiscriminate and self-perpetuating causing inadvertent release of these toxic substances and resulting in host tissue destruction. Indeed, disruption of this orderly sequence has been implicated in host progression to the systemic inflammatory response syndrome, and multiple organ dysfunction after sepsis, burns, multiple injuries, and resuscitated hemorrhagic shock.

HTS has been offered as an alternative fluid by several human trials that have shown how it rapidly draws cellular and parenchymal water into the vasculature reducing tissue edema and increasing effective circulating volume, HTS (3–21%), alongside mannitol, has been used extensively as an osmotherapeutic agent to reduce intracranial pressure in a variety of neurologic emergencies including stroke, subarachnoid hemorrhage, and traumatic brain injury. In addition, increasing evidence suggests that HTS reduces systemic inflammation by blunting neutrophil activation.

Some animal studies have shown HTS to reduce PMN L-selectin and CD11b and EC ICAM-1 in hemorrhagic shock and hepatic ischemia or reperfusion. In models evaluating the microcirculation directly using live IVM, HTS decreased PMN-EC interactions in models of sepsis, burns, and ischemia or reperfusion. In hemorrhagic shock, although no IVM study has evaluated interactions in brain, diminished PMN-EC interactions with HTS resuscitation have been reported in liver and cremaster. Our group has also demonstrated HTS to blunt PMN-EC interactions in an IVM mouse cremaster model subjected to hemorrhagic shock alone or combined with infection. Little is known regarding the effect of HTS on the brain pial microcirculation after hemorrhagic shock. Pial windows in models of sepsis and brain injury have shown reductions in EC-PMN interactions with HTS. In disagreement with these studies, HTS animals in this study consistently demonstrated trends of greater rolling and adhering PMNs after resuscitation than in RL counterparts, and this despite starting with lower baseline adhesion.

Yet other studies have also demonstrated that HTS may worsen PMN activation and tissue injury in certain conditions. Patrick et al found enhanced elastase release in lipid primed neutrophils exposed to hypertonicity and postulated that HTS could aggravate tissue damage by augmenting neutrophil's cytotoxic mediator release. In a rodent burn study, administration of HTS at 0 or 4 hours after burns augmented intestinal mucosal lipid peroxidation, neutrophil sequestration, intestinal permeability, and villi sloughing compared with animals receiving isotonic saline. The same authors subsequently used another model evaluating these effects in lung and also showed that HTS increased pulmonary PMN sequestration, permeability, and inflammatory cell infiltration while augmenting PMN oxidative burst. More specifically in the brain, several authors have reported the ability of hypertonic fluids to disrupt the BBB and have used this property to promote passage of circulating elements into brain tissue. Using hypertonic mannitol, for example, one group used the resultant increased BBB permeability to promote passage of circulating monocytes into brain tissue. Another group used the same hypertonic fluid to promote the transvascular passage of chemotherapeutic agents into brain tissue of animals with cerebral tumors. Together, these data suggest that hypertonicity may promote PMN-mediated brain microvascular permeability.

Although progression of PMN-EC interactions has been well characterized in systemic capillary beds, little is known on how these interactions occur at the BBB. Indeed, the level of immune surveillance in brain capillary beds under normal conditions is a fraction of that observed in other capillary beds. The blood-brain microvascular endothelial barrier is known to be distinct to that of systemic capillary bed in different ways. Specialized EC in brain microcirculation possess unique tight junctions that are present in fewer numbers but which may alter PMN-EC crosstalk and change EC responses to HTS exposure. In addition, PMN migration
may not only occur through these junctions (between EC) but also involve transcellular diapedesis involving the formation of pores through the EC itself.39

Indeed, other authors have shown that HTS may have differential effects on PMNs located in different anatomic sites. One group demonstrated that compared with RL, HTS resuscitation had no effect in peritoneal PMN sequestration while significantly reducing neutrophil lung accumulation.40 Our study demonstrated that hemorrhagic shock resuscitated with HTS tended consistently to increase neutrophil rolling more than RL. Although these changes were not statistically different between groups, the greater number of rolling and adhering cells in HTS animals when compared with RL counterparts was pervasive throughout all time points after resuscitation. Furthermore, although significant acidosis was seen with shock in both groups, blood pressure tended to be higher in HTS resuscitated animals further lending support to an independent inflammatory effect of HTS. This result is opposite to what our group and others demonstrated in cremaster and other systemic capillary beds. This divergence could be explained by unique BBP PMN-EC crosstalk, affected differently by HTS resuscitation—an important mechanistic question that remains unanswered.

This study has important limitations. Five percent HTS was used and not 7.5%, which is much more common in published models. However, we ensured doses used were equiosmolar and salt load was the same as published models. The number of animals in each group is small and greater numbers may have led to statistically significant differences. In addition, a group undergoing shock but no resuscitation could have been added as control but instead, we used each animal as its own control by comparing postresuscitation time points to preshock. Our hemorrhagic shock model could also be criticized for also administering shed blood, potentially diluting the effect of each individual crystalloid. Yet, this model was clinically relevant as it represents class III hemorrhagic shock where the clinical standard of care always requires administration of blood in addition to crystalloid. In addition, although the RL regimen used has been confirmed by others to be adequate,11,16,41 certain groups20,42 administer 3× (instead of 2×) the shed blood volume in RL, suggesting that our RL animals were potentially underresuscitated. However, if this premise is true, it would further strengthen the findings of increased PMN-EC interactions with HTS despite this proposed underresuscitation in RL animals. Although it was unfortunately not performed, arterial blood gas analysis of animals after resuscitation with each regimen would have been useful to clarify resuscitation adequacy of each regimen. Although not significant, HTS animals tended to have a slightly greater volume of blood resuscitation. Thus, the finding of more “activated” PMN may have been a reflection of worse shock in HTS animals. Yet, when looking at degree of acidosis which was no different in the two groups after hemorrhage and more importantly, blood pressure which tended to be higher in HTS animals, we would venture that animals in both group reached a similar state of shock. Clearly, a completely random method to assign resuscitation fluid for each experiment would have been optimal.

CONCLUSIONS

We have thus shown in a model of rat hemorrhagic shock observed with in vivo brain microscopy that HTS resuscitation tends to consistently increase neutrophil rolling and adhesion to endothelium. Although this result does not concur with studies evaluating HTS in other vascular beds and under different injurious stimuli, it may reflect a unique effect of HTS on BBB PMN-EC interactions in the setting of hemorrhagic shock. Further studies will be needed to better elucidate the mechanism behind these in vivo effects.

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