Fluid resuscitation with hemoglobin-vesicle solution does not increase hypoxia or inflammatory responses in moderate hemorrhagic shock

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ABSTRACT
The aim of the present study was to compare the hypoxic and inflammatory effects of transfusing hemoglobin-vesicles (HbV) or lactated Ringer’s (LR) solution on several organs in a hemorrhagic shock model. Hemorrhagic shock was induced in 48 anesthetized rats by withdrawing 28 mL/kg blood. The animals were resuscitated by replacing the blood with an equal volume of HbV solution or three times the volume of LR solution. The heart, lung, liver, kidney and spleen were extracted at different time points following resuscitation, and mRNA expression levels of hypoxia-induced factor 1-alpha (HIF-1α) and tumor necrosis factor-alpha (TNF-α) were determined. Blood lactate concentrations in the HbV group rapidly returned to baseline levels, whereas elevated lactate concentrations in the LR group were prolonged. There were no significant differences between the two resuscitation groups in terms of HIF-1α and TNF-α expression in the organs examined. HIF-1α and TNF-α expression in the lungs was significantly greater than in other organs. Our results suggest that resuscitation from hemorrhagic shock with HbV did not increase hypoxic or inflammatory effects in major organs, compared with resuscitation using LR solution, despite prolonged elevation of blood lactate.

Hemorrhagic shock is caused by hypovolemia and a loss of blood components, and it is usually corrected by infusion of crystalloids and colloids. Decreased blood flow and/or reduction in hemoglobin (Hb) during hemorrhagic shock can, however, lead to tissue hypoxia and critical anemia, which requires red blood cell (RBC) transfusion. During emergency care or perioperative periods in which RBCs are not available, RBC substitutes, such as those derived from Hb, are used (5, 8, 18, 28).

Hb-based oxygen carriers (HBOCs) are a valuable resource in prehospital care, large-scale disasters, and remote hospitals, in which stored blood is either not available or is rapidly depleted. The safety and efficacy of HBOCs can be evaluated in terms of hemodynamics (28), systemic and regional vasoconstriction (10, 11), tissue oxygenation, immunomodulation (13), and post-injury multiple organ failure (MOF) (20). The standard approach to restoring oxygen delivery in hemorrhagic shock has been crystalloid administration to expand intravascular volume, followed by stored RBCs for critical anemia. However, the initial transfusion therapy after hemorrhagic shock may have adverse immunoinflammatory effects that increase the risk of MOF (20, 27, 32). Hb vesicles (HbV) are artificial oxygen carriers (24, 25, 28, 31). They consist of phospholipid vesicles (liposomes) that encapsulate purified human Hb with polyethylene glycol chains at the surface. The aim
of the present study was to evaluate the use of HbV during resuscitation following hemorrhagic shock and to determine its oxygenation and proinflammation effects on multiple organs.

MATERIALS AND METHODS

Animal preparation. This study was approved by the Ethics Committee for Animal Experiments at Nippon Medical School, Japan. A total of 48 male Sprague-Dawley rats, aged 10 to 13 weeks weighing 308 ± 43 g (mean ± standard deviation (SD)), were anesthetized with 2–4% sevoflurane. Heated blankets were used to maintain core body temperature at 37°C. Lactated Ringer’s solution (LR) was infused at a rate of 1 mL/kg/h via the tail vein, until baseline blood pressure measurements were obtained.

Following laparotomy, a 24G Teflon catheter was inserted into the inferior vena cava, and the common iliac artery was catheterized to allow mean arterial blood pressure (MAP) measurement and blood withdrawal for inducing hemorrhagic shock. Arterial pressure and central venous pressure (CVP) were measured with a pressure transducer (TP-300T; Nihon Koden, Tokyo, Japan) for 2 h following fluid resuscitation. The transducer was connected to a computer and electronic signals were configured to represent pressure changes by analysis software (MacLab/s; ADInstruments Japan, Nagoya, Japan).

Experimental procedure. Fifteen minutes after the preparation was complete, hemorrhagic shock was induced by withdrawing 28 mL/kg blood over 20 min, and maintaining the state for 15 min without fluid resuscitation. Animals were then randomly assigned to one of eight groups (n = 6 per group) based on treatment and time of sacrifice. Animals were resuscitated by infusing HbV solution (Oxygenix Co. Ltd., Tokyo, Japan, [Hb] = 10 g/dL) at the same volume as LR or by infusing three times the volume of LR. Each group was described according to the method of fluid resuscitation and the time of intentional sacrifice from the fluid resuscitation (e.g., the group, which includes the animals resuscitated using HbV solution and sacrificed 2 h after the resuscitation, was described as HbV-2H). Arterial blood (0.2 mL) was sampled before hemorrhagic shock (baseline), after hemorrhagic shock (T1), and 1 h (T2) and 2 h (T3) after fluid resuscitation. An ABL 700 (Radiometer A/S, Copenhagen, Denmark) was used to measure Hb concentration, hematocrit, blood lactate concentration and pO2. MAP and CVP were recorded before and after blood withdrawal and 1 h and 2 h after fluid resuscitation.

RNA extraction and RT-PCR. Following a 2 h observation period, the heart, lung, liver, kidney, and spleen of animals in the 3XLR-2H and HbV-2H groups were removed. The same organs were removed from the remaining rats 24, 72, and 168 h after resuscitation under sevoflurane anesthesia. Organs were placed in liquid nitrogen and stored at −80°C pending RNA extraction. RNA isolation, quantification, and RT-PCR were performed according to established methods (6, 26).

Briefly, total RNA was extracted from each tissue sample using the chaotrophic Trizol method followed by Isogen-chloroform extraction and isopropanol precipitation. Residual genomic DNA was eliminated with DNase I (Takara Shuzo, Otsu, Japan). One microgram of each total RNA sample was reverse transcribed at 37°C for 1 h in a 20 μL solution with mouse Moloney leukemia virus reverse transcriptase and hexanucleotide random primers (Takara Shuzo). RNA was quantified by measuring absorbance at 260 nm, and each sample was diluted to 0.4 μg/μL.

PCR primers and TaqMan fluorogenic probes were designed using the Primer Express software program (Applied Biosystems, Foster City, CA) and had the following sequences: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward 5’-G AAAGTGAAGGGTACGTGTC-3’, reverse 5’-GGTGTGATGGGATTTC-3’, and probe FAM-CAAGCTTCCGTTTCTCAGCC-Tamra. TNF-α: forward 5’-G CACTGTCTACCCAAGTACCT-3’, reverse 5’-GATGAGAGGGAGCCCATTTG-3’, and probe FAM-ACCACGCTTCTCTGCTT-Tamra. HIF-1α: forward 5’-ACACCTCTACCCAAGTACCT-3’, reverse 5’-TGTCGACTGTAATCTC-3’, probe FAM-ACCACGCTAAGGACT-Tamra. GAPDH was used as the housekeeping gene.

Quantitative PCR was carried out in a 50 μL solution containing 20 ng cDNA, 25 μL TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA), 900 nM forward and reverse primers, 200 nM TaqMan probe and deionized water. PCR conditions were 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The 6-FAM-labeled TaqMan probe was cleaved during amplification to generate a fluorescent signal that was measured using an ABI PRISM 5700 Sequence Detector (Applied Biosystems). Samples and calibration curve samples were run in triplicate. Values were interpolated automatically from the standard curve. A similar system utilizing a separate
GAPDH probe and primer set (TaqMan GAPDH control reagent kit; Applied Biosystems) was designed and run for GAPDH along with each sample to correct for total nucleic acid content. Relative amounts of mRNA were calculated by the comparative critical threshold (CT) method (Applied Biosystems).

**Statistical analysis.** Data are expressed as mean ± SD. Statistical analyses were performed with Statview® version 5.0 for Macintosh software (Abacus Concepts Inc., Berkley, CA). Differences in MAP, CVP, Hb concentration, blood lactate concentration and gene expression between resuscitation groups and time after resuscitation were analyzed with two-factor factorial ANOVA and the Tukey-Kramer test at the 95% confidence level. Within group differences were analyzed with one-factor ANOVA and the Tukey-Kramer test for comparison with each baseline value. p-values < 0.05 were considered statistically significant.

**RESULTS**

All rats tolerated hemorrhagic shock, and received fluid resuscitation period and survived until the time of sacrifice. MAP and CVP at baseline were similar in the 3XLR and HbV groups (Table 1). MAP was significantly reduced by hemorrhagic shock and returned to baseline values by fluid resuscitation in both groups. Hemorrhagic shock reduced CVP and fluid resuscitation increased CVP, but not significantly. MAP was decreased in 3XLR group 2 h after resuscitation. Arterial blood lactate concentrations at baseline did not differ significantly between the 3XLR and HbV groups. Hemorrhagic shock increased arterial blood lactate concentration. Fluid resuscitation using HbV solution reduced the lactate level, but lactate concentrations in the 3XLR group remained elevated compared to baseline. Hb concentration and hematocrit at baseline were similar in both groups. After the fluid resuscitation, the Hb concentration and hematocrit in the HbV group were significantly higher than in the 3XLR group.

Expression of HIF-1α and TNF-α mRNA at various time points after fluid resuscitation is shown in Figs 1 and 2. There were no significant differences in gene expression in any organ between the 3XLR and HbV groups. However, HIF-1α expression in the lung was significantly higher than in the heart, liver, and kidney in both 3XLR and HbV groups (Fig. 1). HIF-1α mRNA expressions in the heart were significantly lower than in the lung and spleen. HIF-1α mRNA expression peaked 24 h after fluid resuscitation and then decreased in the most organs. In contrast, TNF-α mRNA gradually increased during the 168 h following resuscitation (Fig. 2). TNF-α expression in the lung was significantly higher than in the other organs examined.

The following table shows hemodynamics and arterial blood values:

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Time point</th>
<th>Baseline</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean arterial blood pressure (mmHg)</td>
<td>3XLR</td>
<td>83.3 ± 9.2</td>
<td>32.5 ± 4.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>80.0 ± 13.7</td>
<td>56.0 ± 20.4&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HbV</td>
<td>80.5 ± 13.7</td>
<td>30.2 ± 3.1&lt;sup&gt;1&lt;/sup&gt;</td>
<td>87.8 ± 14.8</td>
<td>68.7 ± 5.3</td>
</tr>
<tr>
<td>Central venous pressure (mmHg)</td>
<td>3XLR</td>
<td>4.0 ± 0.6</td>
<td>3.3 ± 1.9</td>
<td>4.5 ± 1.4</td>
<td>3.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>HbV</td>
<td>4.2 ± 1.2</td>
<td>4.0 ± 0.9</td>
<td>5.2 ± 2.1</td>
<td>4.7 ± 2.0</td>
</tr>
<tr>
<td>Hemoglobin concentration (g/dL)</td>
<td>3XLR</td>
<td>11.1 ± 2.2</td>
<td>8.5 ± 2.4</td>
<td>7.4 ± 0.7&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.0 ± 1.3&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HbV*</td>
<td>13.2 ± 1.5</td>
<td>9.5 ± 3.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>13.5 ± 0.6</td>
<td>13.0 ± 2.1</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>3XLR</td>
<td>34.2 ± 6.7</td>
<td>26.4 ± 7.1</td>
<td>23.0 ± 2.2&lt;sup&gt;1&lt;/sup&gt;</td>
<td>24.9 ± 3.7&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>HbV*</td>
<td>40.4 ± 4.6</td>
<td>29.4 ± 9.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>41.6 ± 2.0</td>
<td>40.1 ± 6.3</td>
</tr>
<tr>
<td>Blood Lactate concentration (mmol/L)</td>
<td>3XLR</td>
<td>2.1 ± 0.9</td>
<td>6.7 ± 2.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6.3 ± 2.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4.6 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>HbV</td>
<td>3.2 ± 1.1</td>
<td>5.8 ± 1.0&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2.6 ± 0.5</td>
<td>2.6 ± 0.3</td>
</tr>
</tbody>
</table>

T1, immediately after fluid resuscitation; T2, 1 h after resuscitation; T3, 2 h after resuscitation
<sup>1</sup> significantly different than baseline (p < 0.05)
*significantly different than LR group (p < 0.05)
DISCUSSION

Critical acute anemia during emergency care and the perioperative period is usually treated with RBC transfusion. However, this requires time-consuming blood typing and cross matching tests, and the preservation time of blood products is limited. Stored RBCs cannot be supplied during prehospital care, and costs are incurred for their transport to remote hospitals. Moreover, there is increasing evidence that RBC transfusions are associated with adverse effects on the immune response to injury and illness (27). Transfusion of more than six units of RBCs within the first 12 h after injury is an independent risk factor for MOF (20), and aged packed RBC transfusion further increases the risk of postinjury MOF (32). HBOCs may attenuate adverse immuno-inflammatory effects induced by allogenic RBC transfusion and ultimately reduce the incidence of postinjury acute respiratory distress syndrome (ARDS) and MOF (19).

In the present study, organ hypoxia and proinflammatory reactions were revealed by measuring HIF-1α and TNF-α mRNA expression after fluid resuscitation with crystalloid LR or HbV solutions. Despite a rapid recovery from elevated blood lactate...
concentrations in the HbV group, no significant group differences were found in the expression of HIF-1α or TNF-α in intrathoracic and splanchnic organs. A reduction in hemorrhagic volume is sufficient to depress MAP and increase blood lactate concentrations, leading to a 40–50% loss of circulating volume (24, 25, 31). Infection and bacterial translocation in the gut, which commonly occur after injury, and coincidental hemorrhagic shock, can also prolong the increased HIF-1α response (14). Although fluid resuscitation for hemorrhagic shock using HbV solutions does not always modulate HIF-1α expression in the liver and kidney, an unmodulated HIF-1α response is beneficial in cases of anemia, as erythropoietin production remains unchanged, while vascular tone is adjusted (9). Differences in HIF-1α expression between organs can provide tolerance against hemorrhagic shock. Centralization of circulating blood and tolerance of acute isovolemic anemia (16) provide protection against moderate hemorrhage.

Tissue hypoperfusion and vasoconstriction followed by acute hemorrhagic shock can lead to tissue hypoxia. Acute hypoxia stimulates the expression of HIF-1α and p38 mitogen-activated protein kinase (MAPK), particularly in the lung, which are linked to the proliferation of pulmonary artery fibroblasts and remodeling (30). Pulmonary and systemic vasoconstriction and low peripheral perfusion are associated with use of a modified Hb tetrametric solution (11, 22), which causes scavenging of nitric oxide (NO) and enhanced endothelin release (10). NO scavenging enhances hypoxic pulmonary vasoconstriction (1), worsens pulmonary hypertension and reduces cardiac output after hemorrhagic shock. In the present study, HIF-1α expression, a marker of hypoxia, did not increase following HbV resuscitation compared to LR resuscitation. This suggests that HbV does not increase NO scavenging or impede microcirculation based on an endothelial cell disorder (23). A definitive difference is seen with the use of HBOCs that induce vasoconstriction (11). For instance, blood lactate concentration decreased following resuscitation with HbV.

Injury, hemorrhagic shock and fluid resuscitation can produce an inflammatory response such as postinjury ARDS, an acute inflammation of the lungs (17). Our results suggest that inflammation of the lungs, as measured by TNF-α and HIF-1α expression, far exceeded that of other organs. LR resuscitation was shown to produce a smaller hemorrhagic shock effect in the lung than the equivalent volume of normal saline (29). It is, therefore, of clinical interest that in the present study there were no significant differences between HbV and LR resuscitation groups in the extent of inflammation in the lung or other organs.

The key cellular mediators in the pathogenesis of proinflammatory effects are neutrophil polymorphonuclear leucocytes (PMN) (3, 4) and endothelial cells (15). PMNs are primed by plasma from stored RBCs; and the older the RBCs, the greater the priming effect (21). To reduce the induction of cytokines following RBC transfusion, PMNs can undergo prestorage leukoreduction treatment (2, 7, 12). However this does not eliminate all inflammatory reactions. The use of RBC substitutes may overcome the problems of inflammatory reactions. However, in future studies it will be important to demonstrate that, in critical situations, fewer inflammatory reactions are induced by artificial oxygen carriers than by RBCs.

In conclusion, we demonstrated that fluid resuscitation with HbV solution for moderate hemorrhagic shock did not influence the expression of HIF-1α and TNF-α mRNA in the heart, lung, kidney, liver and spleen compared with fluid resuscitation using LR solution, despite blood lactate concentrations changing after fluid resuscitation. Inflammation of the lung was significantly greater than that of other organs after hemorrhagic shock and fluid resuscitation, but the extent of inflammation did not differ according to the type of fluid resuscitation.

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REFERENCES


