Preservation of hepatic blood flow by direct peritoneal resuscitation improves survival and prevents hepatic inflammation following hemorrhagic shock

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Hurt RT, Matheson PJ, Smith JW, Zakaria ER, Shaheen SP, McClain CJ, Garrison RN. Preservation of hepatic blood flow by direct peritoneal resuscitation improves survival and prevents hepatic inflammation following hemorrhagic shock. Am J Physiol Gastrointest Liver Physiol 303: G1144–G1152, 2012. First published September 17, 2012; doi:10.1152/ajpgi.00278.2011.—Conventional resuscitation (CR) from hemorrhagic shock (HS) results in gut and liver hypoperfusion, organ and cellular edema, and vital organ injury. Adjunct direct peritoneal resuscitation (DPR) with dextran prevents gut vasoconstriction, hypoperfusion, and injury. We hypothesized that DPR might also improve hepatocellular edema, inflammation, and injury. Anesthetized male SD rats were assigned to groups (n = 8/group): 1) sham (no HS); 2) HS (40% MAP/60 min) + intravenous fluid conventional resuscitation [CR; shed blood + 2 vol saline (SAL)/30 min]; 3) HS + CR + DPR (30 ml ip 2.5% glucose dextran); or 4) HS + CR + SAL (30 ml ip saline). Histopathology showed lung and liver injury in HS + CR and HS + CR + SAL up to 24 h postresuscitation (post-RES) that was not in shams and which was prevented by adjunct DPR. Wet-to-dry weight ratios in HS + CR revealed organ edema formation that was prevented by adjunct DPR. HS + CR and HS + CR + SAL had 34% mortality by 24 h post-RES, which was absent with DPR (0%). Liver IFN-γ and IL-6 levels were elevated in CR compared with DPR or shams. TNF-α mRNA was upregulated in CR/sham and DPR/sham. IL-17 was downregulated in DPR/sham. CXCL10 mRNA was upregulated in CR/sham but downregulated in DPR/sham. Despite restored central hemodynamic performance after CR of HS, liver blood flow was compromised up to 24 h post-RES, and the addition of DPR restores and maintains liver perfusion at 24 h post-RES. DPR prevented liver injury, histological damage, and edema formation compared with CR alone. DPR provided a mitigating anti-inflammatory dampening of the systemic inflammatory response. In all, these effects likely account for improved survivorship in the DPR-treated group.

hemorrhagic shock; liver blood flow; liver injury

Despite advances in treatment and therapies, hemorrhagic shock (HS) remains a major cause of morbidity and mortality following trauma in the United States (18). The clinical role the liver plays during HS and in subsequent multiple organ failure (MOF) is unclear. The largest and most recent clinical study followed 1,962 trauma patients with injury severity score > 14 (ISS > 14) during a 3-year period (17). Of the patients who met the study’s inclusion criteria, 154 (7.9%) exhibited signs of liver dysfunction during their hospital course. In general, these patients with liver dysfunction were older and had higher injury severity scores and lower prehospital blood pressure (17). Patients who developed high serum levels of bilirubin, a serum marker of liver injury, had longer stays in the intensive care unit and higher mortality (16.2% vs. 2.5%) compared with patients with normal or slightly elevated bilirubin. While these observational studies suggest an association between HS and significant liver dysfunction, necrosis, and mortality, the mechanisms of this liver injury have not been well defined.

Experimental evidence suggests that despite return of central hemodynamic performance by aggressive intravenous fluid resuscitation, the gut and liver experience a progressive vasoconstriction and hypoperfusion after HS (5, 14, 21, 42). End organ tissue hypoperfusion has been linked to several mechanisms of postresuscitation (post-RES) injury: endothelial cell dysfunction, tissue hypoxia, compromised capillary filling and fluid exchange, deranged electrolyte handling, and altered release of cytokine mediators that exaggerate the systemic inflammatory response (40). These mechanisms of cellular and end organ tissue injury translate clinically into organ dysfunction, which can progress to MOF and death. While the role of the gut as a potential motor for MOF has been extensively studied and promoted, the role of the liver in post-RES tissue injury has not been well defined (5, 21). This paper will examine hepatic perfusion, function, and histopathology following resuscitated HS and will analyze the potential for a new resuscitation regimen termed direct peritoneal resuscitation (DPR) to ameliorate the HS/resuscitation-induced liver injury. We propose that DPR can be used in conjunction with conventional intravenous fluid resuscitation to prevent the hepatic pathophysiological consequences associated with post-RES injury.

MATERIALS AND METHODS

Studies were approved by the Institutional Animal Care and Use Committee and Biohazard Safety Committee at the Louisville Veterans Affairs Medical Center. Rats were housed in an American Association for the Accreditation of Laboratory Animal Care (AAALAC)-approved facility. Male Sprague-Dawley rats (200–220 g; Harlan, Indianapolis, IN) were acclimated on a 12:12-h light-dark cycle for 1–2 wk prior to use and were fed standard rat chow (20 g/day) and water ad libitum. Weights were recorded daily to ensure positive weight gain.

Surgical anesthesia was induced by pentobarbital injection (50 mg/kg ip), and supplements (12.5 mg/kg sc) were given as needed. Normal saline (2 ml sc) was given to maintain homeostasis, and body temperature (37.0 ± 1.0°C) was held constant by feedback controller. After the loss of blink and withdrawal reflexes, a tracheotomy was performed (PE-240 tubing; Becton Dickinson, Parsippany, NJ), and rats spontaneously breathed room air. Cannulae (PE-50) were inserted for monitoring blood pressure (left femoral artery), galactose infusion...
(left femoral vein), blood withdrawal (right femoral artery), and resuscitation (right femoral vein). After equilibration (45–60 min), mean arterial pressure (MAP) and heart rate (HR) were continuously monitored and recorded every 15 min (Digi-Med Signal Analyzers, Louisville, KY). Baseline MAP, HR, and serum galactose values were monitored until values remained stable (<10% variation). Survival surgery for the 24-h post-RES group was performed in a dedicated space under aseptic conditions and sterile techniques.

Animal model of HS/RES. HS to 40% of baseline MAP was achieved by bleed withdrawal (1 ml/min) into a heparin-rinsed syringe (10 IU). HS was continued for 60 min by manual withdrawal or infusion of blood as needed. Conventional RES (CR) was return of shed blood over 5 min followed by infusion of warmed saline (2× vol of shed blood/25 min). Adjunct DPR was given at the time of CR by intraperitoneal injection of prewarmed peritoneal dialysis solution (30 ml 2.5% Delflex; Fresenius, Ogden, UT). This contains 2.5% dextrose, 5.67 g/l sodium chloride, 3.92 g/l sodium lactate, 0.257 g/l calcium chloride, and 0.152 g/l magnesium chloride at a pH of 5.5 and osmolarity of 398 mosmol/l. Volume-matched controls were given prewarmed normal saline (30 ml ip) at the time of CR. Rats were randomized by coin toss prior to anesthesia to these groups (n = 8/group): 1) sham (no HS), 2) HS+CR, 3) HS+CR+DPR, or 4) HS+CR+SAL (30 ml ip saline). The experimental protocol was followed for 4-h or 24-h post-RES for a total of eight groups (Fig. 1).

Galactose clearance for estimation of effective hepatic blood flow (EHBF). The galactose clearance method to assess effective hepatic blood flow (EHBF) has been well described (9). The assumptions inherent in this technique are that systemic galactose is solely metabolized and cleared from the plasma by the liver, and thus, the steady-state galactose clearance accurately reflects liver blood flow. Briefly, a steady-state galactose concentration (GCSS) was obtained within 30–40 min after bolus of galactose (2.6 mg·1 ml⁻¹·5 min⁻¹) followed by constant galactose infusion (I; 13 mg·ml⁻¹·h⁻¹). The steady-state galactose concentration (GCSS) was obtained within 30–40 min after bolus of galactose (2.6 mg·1 ml⁻¹·5 min⁻¹) followed by constant galactose infusion (I; 13 mg·ml⁻¹·h⁻¹). EHBf was determined in triplicate every 30 min by EHBf/GCSS.

Histopathology, blood chemistry analysis, serum ELISA, and edema estimation. At the completion of the protocol, an arterial blood complete diagnostic panel provided liver enzyme alanine aminotransferase (ALT), bilirubin, total protein, blood urea nitrogen, creatinine, sodium, potassium, calcium, chloride, and glucose (VS2 Analyzer; Abaxis, Union City, CA). Wet-to-dry (WD) weight ratios were performed in ileum, liver, and lung samples. Serum cytokine and high mobility group box protein-1 (HMBG1) protein levels were measured according to manufacturers’ instructions (IL-6, IFN-γ, and IL-10; R&D Systems, Minneapolis, MN and HMGB1; IBL International, Toronto, Canada). Hematoxylin and eosin-stained liver samples were evaluated for liver injury by pathologist blinded to experimental protocol and groups. Specimens with no and minimal injury were scored 0, focal necrosis scored 1, centrilobular necrosis (zone 3) scored 2, submassive necrosis (zone 2 and 3) scored 3, and massive necrosis scored 4. Each sample was examined first on low power and then high power (magnification, ×400). Ten high-power fields were examined per slide and the scores were averaged for each sample.

RNA purification from liver tissue. In the 4-h post-RES groups, liver sections (~3 mm cubes) were placed in RNAlater (Ambion Division of Applied Biosystems, Austin, TX) and stored at −20°C for 24 h and then at −80°C until RNA purification (<90 days). Purification of total RNA was performed according to manufacturer’s instructions (RNeasy Mini Kits; Qiagen Sciences, Germantown, MD). RNA purity was calculated by absorbance ratio (A260/A280) (44). All samples from the present study used in the RT²-PCR assays had A260/A280 ratios of between 1.9 and 2.1. Following RNA quantification, samples were frozen and stored in a −80°C freezer until RT²-PCR was performed. The storage time was <2 mo for all samples.

Real-time PCR. RNA samples were converted to cDNA using RT according to the manufacturer’s instructions (OneStep First Strand Kit; Qiagen Sciences), which also eliminated genomic DNA contamination. SA Biosciences RT²-PCR SuperArrays’ 96-well plates with cytokine, chemokine, and receptor primers (cat. no. PARN-011; SuperArray Bioscience, Frederick, MD) were used in a Chromo 4 Analyzer (MJ Research, Waltham, MA). Each plate was run on the three-step cycle program of the Chromo 4 Analyzer, and gene expression analysis from the PCR arrays was run on Qiagen spreadsheets. Changes in gene expression were deemed significant at ≥2-fold up- or downregulation. Genomic DNA, RT, and positive PCR controls were all within normal limits according to the manufacturer’s instructions.

Statistical analysis. Data are expressed as mean ± SE, and differences between groups were determined by one-way or two-way ANOVA. The null hypothesis was rejected at P < 0.05. When differences were found using ANOVA, one of the following post hoc tests was performed: Tukey-Kramer’s honestly significant different test, Bonferroni’s test, or repeated-measures ANOVA and Dunnett’s test.

Fig. 1. Experimental time line for the 4-h and 24-h protocols. We utilized a pressure hemorrhage (HEM) of withdrawal or infusion of blood (via femoral artery) to maintain a level of 40% of baseline mean arterial blood pressure (MA) for 60 min. A 200-g Sprague-Dawley rat requires roughly 7 ml of total blood withdrawal to obtain this HEM profile. Resuscitation (RES) was return of shed blood over 5 min and then intravenous infusion of 2 equal volumes of normal saline over 25 min. Direct peritoneal resuscitation (DPR) of 30 ml of prewarmed (37°C) 2.5% Delflex solution (Fresenius, Ogden, UT) was given via intraperitoneal injection at the time of shed blood resuscitation. The hemorrhagic shock + conventional resuscitation + saline (HS+CR+SAL) volume group was given 30 ml of prewarmed normal saline via intraperitoneal injection at the time of shed blood return. BL, baseline; LBF, liver blood flow. ***LBF time points.

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Table 1. Summary of baseline values

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<th>4 h</th>
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<tr>
<td></td>
<td>Sham</td>
<td>HS + CR</td>
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<tr>
<td>Body weight, g</td>
<td>209.1 ± 1.7</td>
<td>209.1 ± 1.4</td>
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<tr>
<td>MAP, mmHg</td>
<td>114.5 ± 1.7</td>
<td>118.0 ± 1.8</td>
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<tr>
<td>Heart rate, beats/min</td>
<td>369.7 ± 6.5</td>
<td>392.5 ± 6.4</td>
</tr>
<tr>
<td>Liver blood flow, ml·min⁻¹·g⁻¹</td>
<td>8.3 ± 0.8</td>
<td>8.8 ± 0.5</td>
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<tr>
<td>Hemorrhage volume, ml</td>
<td>0</td>
<td>7.0 ± 0.2</td>
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Summary of baseline values for body weight, blood pressure, heart rate, liver blood flow, and hemorrhage volume required to maintain 40% of baseline mean arterial blood pressure during the hemorrhage (HEM) period. HS, hemorrhagic shock; CR, conventional resuscitation; DPR, direct peritoneal resuscitation; SAL, saline. There were no differences between groups in baseline values (not significant, *P > 0.05 by ANOVA).

RESULTS

Table 1 shows baseline values for body weight, MAP, HR, EHBf and hemorrhage volume, which were not different between groups at 4- or 24-h post-RES. MAP, HR, and EHBf responses are shown in Figs. 2 and 3 for 4- or 24-h post-RES, respectively. Resuscitation restored MAP in all three hemorrhage groups (HS + CR, HS + CR + SAL, and HS + CR + DPR). During hypovolemic shock, EHBf (ml·min⁻¹·100 g body wt⁻¹) decreased about 50% compared with baseline values or sham (7.8 ± 0.7); HS + CR (3.4 ± 0.2, *P < 0.05), HS + CR + SAL (3.4 ± 0.2, *P < 0.05), and HS + CR + DPR (3.4 ± 0.2, *P < 0.05). All RES protocols restored EHBf to baseline levels initially, but in HS + CR and HS + CR + SAL, there was a progressive decline in EHBf over the 4-h post-RES period, and adjunct DPR normalized EHBf compared with sham levels (Fig. 2). At 24-h post-RES, EHBf was decreased compared with sham (6.5 ± 0.3) in the HS + CR (4.6 ± 0.2, *P < 0.05) and HS + CR + SAL (4.3 ± 0.3, *P < 0.05) groups, but adjunct DPR normalized EHBf in the HS + CR + DPR group (6.1 ± 0.5) (Fig. 3, bottom).

High-mobility group box 1 (HMGB1), a marker of hepatocyte cell death, was increased compared with sham in the HS + CR group at 4- and 24-h post-RES (Fig. 4). Adjunct DPR prevented this elevation in HMGB1 at both time points compared with HS + CR. These data suggest that improved EHBf abrogates the degree of liver injury during resuscitated HS. This finding was supported by the pathologist scoring of liver hematoxylin and eosin samples from these groups. At 24-h post-RES, histological scoring of the sham liver specimens revealed no/minimal injury (100%). Two of eight (25%) liver specimens from the HS + CR group had centrilobular or submassive necrosis, and the remainder had focal necrosis (75%). Similarly, the HS + CR + SAL group had two of eight (25%) livers scored as submassive necrosis, one of eight (12.5%) as massive necrosis, and the remainder (62.5%) graded as focal necrosis. No animal from the CR or SAL groups was scored as no/minimal injury. Adjunct DPR decreased microscopic liver injury compared with the other HS groups at 24-h post-RES. No HS + CR + DPR liver samples were scored as centrilobular, submassive, or massive necrosis (0%), six of eight (75%) had focal necrosis and two (25%) had no/minimal injury. These findings correlated with the impaired hepatocellular function in HS + CR and HS + CR + SAL as assessed by serum liver enzyme levels (ALT) at 4- and 24-h post-RES (Table 2). Resuscitated HS (HS + CR and HS + CR + SAL) significantly elevated ALT at 4-h (79.6 ± 9.6 and 78.9 ± 10.5, *P < 0.05) and 24-h (134.1 ± 63.8 and 84.9 ± 20.4, *P < 0.05) post-RES compared with sham at 4-h (42.4 ± 4.7) and 24-h (38.1 ± 1.5), respectively. Adjunct DPR prevented liver injury as evidenced by normalized serum ALT levels at 4-h (48.4 ± 6.0, not significant) and 24-h (40.9 ± 1.7, not significant) post-RES, which were not different than shams.

Tissue edema (total tissue water) was evaluated by WD weight ratios at 4- and 24-h post-RES (Fig. 5). In the liver, resuscitated HS (HS + CR and HS + CR + SAL) significantly increased WD weight ratios at 4- and 24-h post-RES compared with sham, and the addition of DPR (HS + CR + DPR) pre-

Fig. 2. Top and middle: central hemodynamic variables for the 4-h postresuscitation (PR) groups. MAP dropped during hemorrhage and remained at ~40% of baseline for the hypovolemic period. MAP returned to baseline following resuscitation. Bottom: effective hepatic blood flow (EHBf) for the 4-h PR groups. As in the 2-h PR groups, the end of the hypovolemic period EHBf was decreased to ~60% of baseline in all shock groups. EHBf returned to baseline immediately postresuscitation (post-RES). In CR and CR + SAL groups, EHBf was significantly lower than baseline. CR + DPR, and sham beginning at 60 min PR and persisting up to 4 h post-RES. IP SAL, intraperitoneal saline; DPR, direct peritoneal resuscitation.
DPR animals survived the full length of the protocol (8/8), suggesting a mortality benefit with adjunct DPR following resuscitated HS.

Liver IFN-γ levels at 4-h post-RES were significantly higher in HS + CR animals compared with sham (Fig. 7, middle), and adjunct DPR prevented this increase in IFN-γ. Liver IL-6 levels at 4-h post-RES were significantly higher in HS + CR animals compared with sham and DPR prevented the increase in IL-6 in the HS + CR + DPR group (Fig. 7, left). Liver IL-10 levels were not different between the groups at 4-h post-RES (Fig. 7, right). In all mRNA studies (cytokine, C-C chemokine, and C-X-C chemokine panels), the sham, HS + CR, and HS + CR + DPR groups were examined. The HS + CR and HS + CR + DPR levels were standardized to the sham levels. Resuscitated HS downregulated mRNA expression of IFN-γ, IL-15, IL-1f5, and IL-4 in both HS + CR and HS + CR + DPR. Resuscitated HS upregulated mRNA expression of IL-1β, IL-1r1, IL1r2, and IL-8rβ in both HS + CR and HS + CR + DPR. However, IL-10ra, IL-2rg, and IL-6ra mRNA levels were downregulated in HS + CR, but DPR prevented these changes. The addition of DPR downregulated IL-17 mRNA but upregulated mRNA expression of IL-13ar1 compared with HS + CR. Also, the addition of DPR prevented the increase of TNF-α mRNA that occurred in HS + CR alone. Other cytokine mRNAs were measured including: IL-10, IL-11, IL-13, IL-16, IL-18, IL-1α, IL-2rb, IL-3, TGF-β, and IL-6st, but there were no significant differences between HS + CR/sham and HS + CR + DPR/sham, which was defined a priori as ± 2-fold mRNA induction (Fig. 8).

Hepatic C-C chemokine mRNA induction (up- or downregulation) was assessed at 4-h post-RES (Fig. 9). Hepatic CCL19 and CCL25 mRNA levels were downregulated in HS + CR but not in HS + CR + DPR. CCL2 (i.e., monocyte chemoattractant protein 1, MCP-1) was upregulated in both HS + CR and HS + CR + DPR but was 2.5-fold greater in HS + CR (5.75) vs. HS + CR + DPR (3.23). CCL20 and CCL22 mRNA expression was downregulated in both HS + CR and HS + CR + DPR. CCL3 prevented this change. In the lung, resuscitated HS groups (HS + CR and HS + CR + SAL) had significantly increased WD weight ratios at 4- and 24-h post-RES compared with sham, and adjunct DPR prevented the increased lung WD weight ratios. Similarly, in the ileum, the WD weight ratios were increased in the HS + CR and HS + CR + SAL groups compared with sham at the 4-h post-RES time point, and DPR prevented the increase in WD weight ratios in resuscitated HS. At 24-h post-RES, the level of edema formation in the ileum was not different between sham, HS + CR, and HS + CR + SAL, but was diminished in the DPR treatment group. The WD weight ratios at 4-h post-RES in abdominal skeletal muscle were again significantly increased in HS + CR and HS + CR + SAL groups compared with sham and normalized by the addition of DPR to the treatment strategy. Abdominal muscle WD weight ratios were not different between groups at 24-h post-RES.

Survivorship at 24-h post-RES (Fig. 6) showed 100% survival in the sham (8/8). Resuscitated HS (HS + CR and HS + CR + SAL) increased mortality compared with sham. At 4- and 5-h post-RES, one (1/11) HS + CR animal expired at each time point (2/11 total). At 24-h another animal (total of 3/11) expired for a final 24-h survival of 73% (8/11). At 5-h post-RES, one (1/9) HS + CR + SAL animal expired, and at 24-h one additional animal expired for a final 24-h survival of 78% (7/9). All HS + CR + DPR animals survived the full length of the protocol.
Table 2. Complete metabolic panel

<table>
<thead>
<tr>
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<th>4 h</th>
<th>24 h</th>
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<tr>
<td></td>
<td>Sham</td>
<td>HS + CR</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>1.6 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Total protein, g/dl</td>
<td>3.5 ± 0.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Globulins, g/dl</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Alkaline Phosphatase, U/l</td>
<td>131.3 ± 9.2</td>
<td>141.9 ± 6.0</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>42.4 ± 4.7</td>
<td>79.6 ± 9.6*</td>
</tr>
<tr>
<td>Amylase, U/l</td>
<td>607.1 ± 24.5</td>
<td>628.4 ± 19.4</td>
</tr>
<tr>
<td>Total bilirubin, mg/dl</td>
<td>0.2 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>11.0 ± 1.0</td>
<td>13.0 ± 1.5</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.3 ± 0.0</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Na⁺ (mmol/l)</td>
<td>145.1 ± 1.1</td>
<td>143.3 ± 0.7</td>
</tr>
<tr>
<td>K⁺ (mmol/l)</td>
<td>4.0 ± 0.2</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>Ca²⁺, mg/dl</td>
<td>6.9 ± 0.3</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>Phos³⁻, mg/dl</td>
<td>5.3 ± 0.4</td>
<td>5.6 ± 0.1</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>129.9 ± 8.5</td>
<td>132.5 ± 4.3</td>
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</table>

Summary of complete metabolic panel findings at 4 h and 24 h postresuscitation in the sham, HS + CR, HS + CR + SAL, and HS + CR + DPR groups. Included in the panel were serum levels of albumin, total protein, globulins, alkaline phosphatase, alanine transaminase (ALT), amylase, total bilirubin, blood urea nitrogen (BUN), creatinine, sodium (Na⁺), potassium (K⁺), calcium (Ca²⁺), phosphate (Phos³⁻), and glucose. Of note is the finding of increased levels of ALT in the HS + CR groups compared to sham levels, which were corrected by the infusion of DPR solution into the peritoneal space during resuscitation. *P < 0.05 vs. sham, †P < 0.05 vs. HS + CR, ‡P < 0.05 vs. HS + CR + SAL.

and CCL4 mRNA levels (i.e., macrophage inflammatory protein MIP-1α and MIP-1β) were upregulated in both HS + CR and HS + CR + DPR. For CCL3 and CCL4, HS + CR was higher with onefold greater upregulation in CCL4 (MIP-2) compared with HS + CR + DPR. CCR7 mRNA expression was downregulated with adjunct DPR compared with HS + CR alone and CCR9 and CCR10 mRNA levels were downregulated in HS + CR but not HS + CR + DPR. Hepatic mRNA levels for other C-C chemokines and receptors were measured including; CXCL11, CXCL12, CCL17, CCL21h, CCL24, CCL5, CCL6, CCL7, CCL9, CCR1, CCR2, CCR5, and CCR6 and no differences were found.

Liver C-X-C chemokine mRNA induction (up- or down-regulation) at 4 h post-RES (Fig. 10). Hepatic mRNA expression for CXCL1 and CXCL2 was upregulated in both HS + CR and HS + CR + DPR. Liver CXCL10 mRNA levels were upregulated in HS + CR but downregulated in HS + CR + DPR. CXCL12 mRNA expression was downregulated in both HS + CR and HS + CR + DPR. Other C-X-C chemokine and receptor mRNA levels were measured including; CCL6, CXC6, CXCL9, CXCL11, CXC3 CXC5, CXC3C1, and CX3C11, but there were no significant differences detected at 4 h post-RES.

DISCUSSION

We report here that adjunct DPR provides benefits in resuscitated HS in liver blood flow, serum cytokine levels, liver cytokine and chemokine expression, liver injury, and survival. Conventional RES from HS impairs EHBF compared with sham, and adjunct DPR restores EHBF to sham and baseline levels.

Fig. 5. Wet-to-dry tissue weight ratios at 4 h post-RES suggests edema formation in the lung, liver, ileum, and abdominal muscle (Abd Musc) in the HS + CR and HS + CR + SAL groups (top). Edema formation was prevented by the addition of DPR to the resuscitation treatment (HS + CR + DPR), which resulted in sham levels throughout tissue types. There were no differences observed in the spleen. Wet-to-dry weight ratios at 24 h post-RES (bottom) again show edema formation in the liver and lung with conventional resuscitation alone or with intraperitoneal saline, while the addition of DPR prevented fluid shifts in the liver, gut, and lung.

Fig. 6. Survivorship curves showing the %surviving up to 24 h post-RES. Sham survival was 100% at 24 h, HS + CR produced a significant mortality rate (27%) compared with sham at 24 h post-RES and HS + CR + IP SAL also resulted in significant mortality (22%). However, the addition of DPR restored survival to 100% after resuscitation HS at 24 h post-RES (0% mortality).
These data suggest that DPR prevents three major pathophysiological events associated with HS and CR: 1) persistent liver hypoperfusion and vasoconstriction; 2) organ fluid sequestration; and 3) a liver-derived inflammatory response. Normalized EHBF might account for the improved survival and decreased inflammatory response observed in this and prior DPR studies, given the essential role of the liver in the hemorrhage-induced SIRS response (16, 46).

Liver injury and dysfunction drive the progression of MOF following resuscitated HS. Hepatocellular and sinusoidal endothelial cell dysfunction occur early during hemorrhage (HEM) and persist despite adequate fluid RES (3, 35, 39). End-organ damage is initiated and driven by a cascade of events involving multiple mediators and pathways (i.e., proinflammatory cytokines, reactive oxygen radicals, lipoxigenase derivatives, complement, intracellular Ca\(^{++}\) signaling, and hypoxia). A central unifying event in these processes might be compromised hepatic nutritive blood flow. The mechanisms of altered EHBF in this model could involve both vasoconstriction of the presinusoidal vessels (including the intestinal circulation) and direct sinusoidal constriction via altered control of local mediators of vascular tone.

Post-RES intestinal hypoperfusion in spite of corrected intravascular volume deficit accounts for impaired nutrient hepatic flow (3, 9, 32) and contributes to mortality in patients after resuscitated HS. Progressive vasoconstriction of small intestinal inflow and premucosal arterioles, which persists despite restoration of hemodynamic status, has been well documented (32). However, the large number of HEM/RES models used to study this phenomenon has complicated data interpretation. Our laboratory has utilized the same blood pressure dependent model for the past 20 yr to investigate this phenomenon in the microcirculation of the small intestine.

Reversing small intestinal vasoconstriction to prevent post-RES hypoperfusion has been achieved using agents including heparan sulfate, heparin, and pentoxifylline (10, 41, 43). We have focused on DPR to reverse the post-RES vasoconstriction in the ileum. Topical exposure of DPR in the ileum at the time of CR prevented impaired ACh dilator capacity in inflow (A1) and premucosal (proximal and distal A3) arterioles (48). In these studies, inflow (A1) blood flow, which was decreased in HS+CR alone, was normalized by the addition of DPR to levels greater than sham or baseline levels for the group (45). Subsequent studies have suggested a role of NO modulation by DPR as one possible mechanism of vasodilation (47).

The liver blood flow response following resuscitated HS reported here mirrors that of the ileum in vivo microcirculation studies. Presinusoidal portal venous vessels lack intrinsic autoregulation of blood flow through vasodilator and vasoconstrictor mechanisms (3, 19). This lack of portal autoregulation and the similarity of intestinal and hepatic blood flow responses suggests that intestinal portal outflow is a determining factor in EHBF. We propose that one mechanism of improved EHBF during resuscitated HS is improved intestinal blood flow by DPR-mediated vasodilation. However, other factors might contribute since DPR significantly elevated ileal A1 arteriolar blood flow to supra-baseline and supra-sham levels at 2-h post-RES, while only maintaining EHBF at baseline levels.

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**Fig. 7.** Liver cytokine levels for IFN-\(\gamma\), IL-6, and IL-10 at 4-h post-RES showing significantly increased levels in HS+CR or HS+CR+SAL compared with sham. Adjunctive DPR returned cytokine protein expression to sham levels at 4-h post-RES.

**Fig. 8.** Hepatic mRNA expression from SuperArray analysis of cytokines at 4-h post-RES. Significant gene expression induction was considered to be \(\pm\) 2.0-fold induction when standardized to sham levels. Cytokines that had opposite direction (up- vs. downregulation) mRNA induction in HS+CR/sham compared with HS+CR+DPR/sham were IL-1\(\alpha\), IL-6\(\alpha\), IL-10, IL-16, and TNFrsf1b. \(*P < 0.05\) upregulation compared with sham, \(\dagger P < 0.05\) downregulation compared with sham levels.
HMGB1 following HS can be detected in lung, liver, and serum as early as 1 h post-RES (8). Once released into the circulation, HMGB1 activates macrophages, including Kupffer cells, through interaction with toll-like receptor 4 (TLR-4). Once TLR-4s are activated, Kupffer cells produce large amounts of cytokines and chemokines including TNF-α, INF-γ, IL-6, IL-8, MCP-1, IL-17, and CXCL-10. Our previous data demonstrated elevated liver enzymes following HS, and we found elevated serum HMGB1 in this study (8, 21, 30). Adjunct DPR prevented hepatocyte cell death (HMB1) and improved liver histopathology scores and serum liver enzymes compared with HS + CR alone. Decreased HMGB1 release in DPR-treated groups at 4- and 24-h post-RES suggests preservation of hepatic cells, decreased generation of ROS and maintenance of gut integrity thus decreasing TLR-4 stimulated release of proinflammatory cytokines. Liver INF-γ levels were significantly higher early at 2-h post-RES in CR compared with sham and DPR. Liver TNF-α was upregulated in CR but not in DPR, suggesting that DPR prevents early release and upregulation of these two important proinflammatory cytokines.

Early hepatic leukocyte sequestration following CR or reperfusion is predominately lymphocytes (1) attracted to the inflamed liver sinusoids by chemokines derived from resident lymphocytes and Kupffer cells following the generation of ROS and subsequent hepatic cell death (1). In the present study, HS + CR CCL2 (MCP-1) mRNA expression was increased 5.75-fold compared with 3.2-fold in the HS + CR + DPR (2-fold difference). CCL2 (MCP-1) is a potent chemotactic agent for monocytes and T-lymphocytes (11, 12), and CCL2 (MCP-1) levels were elevated 1–4 h following reperfusion or resuscitation in the heart, lung, kidneys, and serum (13, 15, 20). CCL2 (MCP-1) is the major chemoattractant for early monocyte and lymphocytes following reperfusion or RES (11, 12). Our findings suggest that DPR decreases early lymphocyte and monocyte sequestration and chemoattraction through decreased CCL2 (MCP-1) release in the liver 4-h post-RES.

IL-17 levels correlate with the severity of acute hepatic injury in patients with hepatitis B, alcoholic hepatitis, primary liver cirrhosis, and with the severity of injury following liver transplantation (14). Early hepatic leukocyte sequestration following CR or reperfusion is predominately lymphocytes (1) attracted to the inflamed liver sinusoids by chemokines derived from resident lymphocytes and Kupffer cells following the generation of ROS and subsequent hepatic cell death (1). In the present study, HS + CR CCL2 (MCP-1) mRNA expression was increased 5.75-fold compared with 3.2-fold in the HS + CR + DPR (2-fold difference). CCL2 (MCP-1) is a potent chemotactic agent for monocytes and T-lymphocytes (11, 12), and CCL2 (MCP-1) levels were elevated 1–4 h following reperfusion or resuscitation in the heart, lung, kidneys, and serum (13, 15, 20). CCL2 (MCP-1) is the major chemoattractant for early monocyte and lymphocytes following reperfusion or RES (11, 12). Our findings suggest that DPR decreases early lymphocyte and monocyte sequestration and chemoattraction through decreased CCL2 (MCP-1) release in the liver 4-h post-RES.

Thus, DPR appears to maintain hepatic nutrient blood flow partially through mechanisms that preserve gut perfusion and subsequent portal flow.

Other potential mechanisms of DPR-improved EHBF might involve nitric oxide and endothelin-1 dependent sinusoidal endothelial regulatory pathways (33). At 24-h post-RES, endothelial cell nitric oxide synthase expression in the liver is upregulated in DPR animals compared with HS + CR. That study suggests that neutrophil adhesion and inflammatory response can alter sinusoidal perfusion following HS. Additionally, sinusoidal endothelial cell edema following CR could lead to anatomical sinusoidal compression and subsequent hypoperfusion.

Ischemia and hypoxia that occur during severe HS cause significant tissue and organ damage. Even short periods of hypoxia can reduce ATP production, which can lead to failure of the Na⁺/K⁺ pump (22, 24, 38). This failure produces hepatocellular swelling and stress-induced unfolding of endoplasmic reticulum proteins, which might initiate hepatocyte apoptosis (6, 23). Hepatic cell death and necrosis release HMGB1 into the systemic circulation (4, 8, 30). Release of HMGB1 following HS can be detected in lung, liver, and
biliary cirrhosis, ischemia/reperfusion injury, and acute liver rejection (7, 27, 28, 31, 50), perhaps by late phase recruitment of neutrophils driven by early release of IL-17 from lymphocytes. This stimulates release of MIP1α, MIP-1β, and MIP-2 (CCL4) from Kupffer cells (29). In our study, DPR downregulated IL-17 and MIP-1β mRNA expression compared with CR alone. Decreased IL-17 expression might lessen C-X-C production in Kupffer cells, epithelial cells, fibroblasts, and endothelial cells (2, 25, 26, 34, 36). CXCL1 mRNA levels were upregulated in both DPR and CR, suggesting that DPR does not mitigate this response. Similar to ischemia/reperfusion (I/R) injury studies (37, 49), CXCL10 was upregulated in CR but downregulated by DPR, while there were no differences in CXCL9 and CXCL11 expression. In one hepatic I/R study, induction of CXCL10 was rapid (1-h post-RES) and specific (no upregulation of CXCL9 or CXCL11). CXCL10 knockout mice were protected from I/R injury with decreased liver enzyme levels and less severe hepatic damage on histopathology. Thus, a mechanism of hepatic protection offered by DPR appears to be prevention of CXCL10 upregulation.

We report the first thorough examination of the effects of adjunct DPR on the liver following resuscitated HS. DPR improved mortality and prevented hepatic hypoperfusion, edema formation, liver injury, and the proinflammatory response. DPR appears to be an effective and safe adjunctive therapy for trauma patients at risk for significant HS complications. In trauma patients, DPR might lessen inflammation and MOF to improve patient outcomes and mortality. In patients undergoing liver resection or transplantation, DPR may also prove beneficial to mitigate liver injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

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