Direct Peritoneal Resuscitation Alters Hepatic miRNA Expression after Hemorrhagic Shock

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BACKGROUND: MicroRNAs (miRNAs) are small segments of noncoding RNA that regulate gene expression and protein function, and therefore are key regulators of cellular processes including those of the inflammatory cascade after hemorrhagic shock (HS). We have previously shown that direct peritoneal resuscitation (DPR), as an adjunct to traditional IV fluid resuscitation, improves visceral blood flow and reduces pro-inflammatory cytokines released during HS. The effects of DPR on hepatic miRNA (miR) expression patterns after resuscitated HS are not known.

STUDY DESIGN: Male Sprague-Dawley rats were divided into 3 groups: sham (no HS); conventional resuscitation (CR; HS, then resuscitated with shed blood and 2 volumes of saline); and DPR (CR plus 30 mL peritoneal dialysis solution). Animals were sacrificed at 4 hours, and miRNAs were measured using reverse transcription polymerase chain reaction.

RESULTS: Use of DPR downregulated 68 of 92 hepatic miRNAs compared with only 2 of 92 upregulated when compared with CR alone, $p < 0.01$). Specifically, miR-9-5p, miR-122-5p, and miR-146, which regulate NFκB, were downregulated 4.1-, 3.4-, and 0.86-fold, respectively; miR-29a and miR-126 were upregulated 0.88- and 3.7-fold when DPR was compared with CR.

CONCLUSIONS: Adding DPR downregulated most hepatic miRNAs compared with CR alone. Some miRNAs were affected more significantly, suggesting that although this clinical intervention causes a near-global downregulation of hepatic miRNA, it still targets specific inflammatory pathways. Use of DPR for resuscitation of patients in HS may reduce hepatic inflammation to improve patient outcomes after hemorrhage. (J Am Coll Surg 2016;223:68–75. © 2016 Published by Elsevier Inc. on behalf of the American College of Surgeons.)

MicroRNAs (miRNAs) are small, noncoding pieces of RNA that regulate gene expression. Approximately 2,000 human miRNAs have been described, and these are thought to regulate more than half of all messenger RNAs (mRNAs).[1] They are found in numerous gene pathways and are associated with a broad array of human pathology, ranging from hypertension and cardiac remodeling,[1,2] to the immune response,[3] to cancer progression and metastasis.[4] The initial miRNA transcript is processed in the nucleus by the Drosha-DGCR8 complex to form pre-miRNA, after which it is transported to the cytoplasm and cut by Dicer to produce mature miRNA.[5] These mature miRNAs then bind to complementary mRNAs, promoting mRNA degradation and downregulating production of their respective proteins.[3] These miRNAs have also been implicated in toll-like receptor signaling and the body's stress response.[6]

Due to this association with the body's inflammatory stress response, several studies have begun to elucidate the effects of trauma on miRNA expression. Zhang and colleagues[7] demonstrated that changes in miRNA expression correlate with susceptibility to sepsis in blunt trauma patients; Truettner and associates[8] showed that specific

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Abbreviations and Acronyms

CCL	= CC motif ligand
CR	= conventional resuscitation
DPR	= direct peritoneal resuscitation
HS	= hemorrhagic shock
IL	= interleukin
miRNA and miR	= microRNA
mRNA	= messenger RNA
PCR	= polymerase chain reaction

miRNAs are increased after traumatic brain injury and can be targeted in neuroprotective strategies. Furthermore, a study from the University of Missouri examined many miRNAs in trauma patients with hemorrhagic shock and saw numerous changes in miRNA expression compared with that in controls, particularly in those related to expression of toll-like receptor 3 and toll-like receptor 4.[9]

Our lab has been studying a form of resuscitation known as direct peritoneal resuscitation (DPR), which has been shown to reduce the inflammation associated with trauma resuscitation. Use of DPR involves infusion of hyperosmotic fluid into the abdomen in addition to conventional intravenous resuscitation. This hyperosmolarity causes dilation of the visceral arterioles,[10,11] improves blood flow to the visceral and thoracic organs,[12,13] and reduces tissue edema and necrosis.[14,15] The addition of DPR is also associated with a reduction in serum inflammatory cytokines and other inflammatory mediators in the setting of hemorrhagic shock or acute brain death.[14,16,17] Given that DPR has a direct effect on the hepatic vasculature, we believed that examination of miRNA within the liver would be an appropriate way to begin to determine the cellular mechanism behind how DPR works. We hypothesized that use of DPR would be associated with downregulation of pro-inflammatory miRNAs.

METHODS

All protocols were approved by the Robley Rex Veterans Affairs Medical Center Institutional Animal Care and Use Committee. Male Sprague-Dawley rats between 200 and 225 grams were housed for 2 weeks before use. Anesthesia was induced with 50 mg/kg intraperitoneal pentobarbital and supplemented with additional doses of 25 to 50 mg/kg intraperitoneal pentobarbital as needed. Body temperature was maintained at 37°C using a rectal probe and heating pad. Anesthesia was confirmed with loss of reflexes. A tracheostomy (PE-240 polyethylene tubing) was placed and animals were allowed to spontaneously breathe room air. The right carotid artery was cannulated (PE-50 polyethylene tubing) and used to measure blood pressure and heart rate. The right jugular vein was cannulated and used for an infusion of galactose (2.6 mg/mL bolus followed by continuous 13 mg/mL/hour). The left femoral artery was cannulated and used for blood draws, and the left femoral vein was cannulated and used for infusion of intravenous fluids and blood.

Rats were randomly assigned to 3 groups of 8: sham (no hemorrhage); hemorrhagic shock (HS) + conventional resuscitation (CR); and HS + CR + DPR. Hemorrhage was achieved using blood withdrawal, and rats were hemorrhaged to 40% of baseline mean arterial pressure for 60 minutes before resuscitation. Conventional resuscitation included infusion of shed blood over 5 minutes, followed by 2 equal volumes of normal saline over 25 minutes. Peritoneal resuscitation consisted of a single injection of 30 mL 2.5% Delflex peritoneal dialysis solution (Fresenius USA).

Rats were sacrificed at 4 hours after resuscitation, after which the organs were removed and cut into pieces. Tissues used for cytokine analysis and polymerase chain reaction (PCR) were flash frozen in liquid nitrogen and stored at −80°C. The specific liver samples used for this study were chosen randomly, and were not standardized to any specific lobe of the liver. For protein extraction, tissues were placed in Qiagen RLT buffer and lysed at 50 Hz for 15 minutes using the Qiagen Tissuelyser II. To measure mRNA levels, RNA was extracted from the lung tissue using the Qiagen QiaCube using the "Purification of DNA and RNA from large samples of animal tissues or cells" protocol.[18] This was then converted to complementary DNA and then mRNA using the Qiagen RT^2 First Strand Kit following the manufacturer's instructions.[19] Real-time PCR for mRNA and miRNA were both carried out in the MJ Research RTC-200 PCR machine, which performs 40 cycles of amplification with a melting curve calculated after each cycle. Results were quantified using Qiagen's complementary software, which measures fold change between groups. Protein levels were then assessed with quantitative ELISA using the Luminex MagPlex and 23-plex rat cytokine kit. Statistical analysis was performed using SigmaPlot (Systat Software Inc) using a 2-way ANOVA and Tukey-Kramer Honestly Significant Difference post-hoc test. Significance was set at $p \leq 0.05$.

RESULTS

Table 1 shows selected hepatic cytokine protein values for all animal groups. After hemorrhage and conventional resuscitation, there was a broadly increased level of pro-inflammatory cytokines compared with that in sham animals. The DPR groups showed significantly reduced