The Effect of Erythropoietin on Microcirculation Perfusion and Tissue Bioenergetics of the Small Intestine in a Hemorrhagic Shock and Resuscitation Rat Model

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Background: Erythropoietin (EPO) can exert acute hemodynamic and anti-inflammatory effects in addition to erythropoiesis. We tested the hypothesis that EPO given at resuscitation with saline will improve capillary perfusion and tissue oxygenation in the gut using a hemorrhagic shock model.

Methods: Sprague-Dawley rats were bled 30 mL/kg to maintain a mean arterial blood pressure of 40 mm Hg for 50 minutes and then randomized to one of four resuscitation groups (n = 6 per group): blood, blood + recombinant human EPO (rHuEPO), saline, and saline + rHuEPO. Intravenous rHuEPO (1,000 U/kg) was given at the start of resuscitation. Intravital microscopy was used to measure perfused capillary density, flow motion of red blood cell (RBC), and tissue NADH fluorescence 60 minutes after resuscitation. Venous oxygenation saturation (Svo2) was also measured in a second experiment.

Results: In the blood ± rHuEPO resuscitation group, the perfused capillary density, RBC flow motion scores, and NADH fluorescence returned to near normal values. The saline + rHuEPO group compared with the saline group demonstrated an increased RBC flow motion score (2.32 vs. 1.60; p < 0.01); however, the perfused capillary density was not significantly increased (23.03 Cap/mm vs. 21.61 Cap/mm; p = 0.40). The saline + rHuEPO group also demonstrated statistically significant lower NADH fluorescence than the saline group after shock following resuscitation (110% ± 3.64% vs. 122% ± 4.26%; p < 0.05) suggesting decreased tissue dysoxia. The Svo2 in the saline + rHuEPO group was higher when compared with the saline group (45% vs. 38% by continuous oximetry; 38% vs. 29% by co-oximetry; p < 0.05).

Conclusion: Our results suggest that the addition of rHuEPO at the time of saline resuscitation may have beneficial effects in hemorrhagic shock by improving tissue perfusion and decreasing dysoxia in the gut. 

Key Words: Recombinant human erythropoietin, Microcirculation, Intravital microscopy, NADH fluorescence, Venous oxygen saturation.

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(EPO), which can mediate tissue protection, is distributed in a wide variety of tissues including the cardiovascular, neurologic, renal, and gastrointestinal system where both physiologic and pharmacological doses of EPO have been shown to exert complex actions that can promote the maintenance of homeostasis of the organism under stressors such as oxidation-induced ischemic-reperfusion injury. We have recently demonstrated in a murine sepsis model that recombinant human EPO (rHuEPO) can improve tissue bioenergetics by improving both tissue microcirculation and mitochondrial function that was sustained for at least 6 hours after treatment.

In this study, we hypothesize that the administration of 1,000 U/kg of rHuEPO in a hemorrhagic shock model will improve small intestine microcirculation and tissue bioenergetics and thus ameliorate metabolic dysfunction caused by ischemic-reperfusion injury.

MATERIALS AND METHODS

The study protocol was approved by the University of Western Ontario Council on Animal Care and the animals were managed according to the guidelines set forth by the institutional Council on Animal Care. Rats were acclimatized to the laboratory for 1 week and with access to rat chow and water ad libitum.

Surgical Preparation

The studies were performed on Sprague-Dawley rats (200–280 g). The animals were anesthetized with intraperitoneal injections of sodium pentobarbital (55 mg/kg; Sigma, Oakville, ON, Canada) and the animal’s temperature was monitored and maintained at 36°C to 37.5°C by a heating pad and lamp throughout the experiment. The right carotid artery and jugular vein were cannulated with PE-50 polyethylene tubing (Becton-Dickinson Company, Sparks, MD). The mean arterial blood pressure (MAP) and heart rate (HR) were measured by the arterial line with a pressure transducer (78353A; Hewlett Packard, Mississauga, ON, Canada) and the jugular vein was used for the administration of drugs, blood, or saline during resuscitation. After cannulation, a laparotomy was performed to exteriorize the distal segment of the ileum. The terminal ileum was placed onto moistened gauze and irrigated liberally with warmed saline. Then, the rat was transferred to the intravital microscope stage and kept in a left lateral recumbent position on the thermostatic platform. The exteriorized ileum was then placed gently on a Plexiglass stage in a bath of warmed saline, and the longitudinal muscular layer from the serosal side was selected for visualization and covered with Saran wrap throughout the entire examination period to avoid desiccation. After a stabilization period of 30 minutes to 45 minutes on the microscope platform, a set of baseline parameters were recorded before induction of hemorrhagic shock. The animals tolerated the anesthetic agent and surgical procedures without the need for mechanical ventilation.

Experimental Design

Heparin (Leo Pharma, Ajax, ON, Canada) 150 U/kg in 0.5 mL volume of saline was given intravenously (i.v.) to the animals before blood withdrawal and induction of shock. Blood was withdrawn (30 mL/kg) with a 10 mL-syringe containing 0.5 mL heparin-saline (7 U/mL blood) by a continuous syringe pump for 10 minutes to simulate acute blood volume loss. The bled animal’s MAP was maintained at 40 mm Hg during the shock period (SP) by either further withdrawal or reinfusion of blood. The blood was stored at room temperature for 50 minutes before use in the blood-resuscitated animal groups. At the end of the 60-minute SP, the animals were randomly allocated to the following resuscitation groups (n = 6 per group) in experimental series 1 to assess the microcirculation and NADH fluorescence of the distal rat ileum: (a) blood resuscitation in which the shed blood was reinfused within 5 minutes; (b) saline resuscitation in which a volume of normal saline equal to three times the volume of shed blood was infused within 15 minutes as per Advanced Trauma Life Support 3 to 1 rule for fluid and blood resuscitation; (c) blood resuscitation + rHuEPO injection (1,000 U/kg) given i.v. at the start of shed blood reinfusion; and (d) saline resuscitation + rHuEPO injection (1,000 U/kg) given i.v. at the start of saline resuscitation.

The same procedure was followed in experimental series 2 to measure venous oxygen saturation (Svo2) at the junction of the hepatic vein with the inferior vena cava in the following groups resuscitated with blood (n = 3); saline (n = 6); saline + rHuEPO (n = 6). A dose of 1,000 U/kg was chosen based on the literature review where doses from 1,000 U/kg to 5,000 U/kg of rHuEPO have demonstrated detectable cytoprotective effects. The animals were killed at the end of the 60-minute resuscitation period (RP).

Intravital Microscopy, Capillary Perfusion, Flow Motion Score, and NADH Fluorescence

The microcirculatory perfusion of the ileum was visualized using intravital microscopy, as previously described. Recordings of the microcirculatory flow in the longitudinal muscle layer were taken at baseline, end of the 60-minute SP, and end of the 60-minute RP. At each time point, a minimum of three separate fields were recorded to determine capillary perfusion for a period of 30 seconds. The perfused capillary count in each field was defined as the number of capillaries in which RBCs crossed a test line drawn perpendicularly to the direction of longitudinal muscle fibers. For each field, counts were obtained along three equally spaced lines drawn on the video monitor and then averaged and expressed as the number of perfused capillaries per millimeter (Cap/mm).

The RBC flow motion in the capillaries at each time point was also classified by a perfusion heterogeneity score described by Szabo et al.: score 0 (no flow); score 1 (sluggish—on/off perfusion); score 2 (low continuous flow); and score 3 (normal flow). An arithmetic average of values in a minimum of three separate fields was then used to determine the tissue capillary perfusion during the observation period.

To visualize the NADH fluorescence within the selected field, an epifluorescence optical unit was used as described. The intensified charge-coupled device camera was adjusted to produce a linear relationship between the intensity of NADH fluorescence using a series of different concentrations (7.8–1,000 μmol/L).
of β-NADH solutions (Sigma, Oakville, ON). The microscope setup was able to reliably measure fluorescence intensity for the different concentrations of β-NADH, generating a linear standard curve with a high correlation coefficient ($r^2 = 0.98$). Before each experiment, a 125-μmol/L concentration of β-NADH solution was used for calibration.

**Venous Oxygen Saturation Measurement**

Using the same animal preparation described earlier, the left femoral vein was cannulated with a 4F double-lumen oximetry catheter (Model 015HF4; Edwards Lifesciences LLC, Irvine, CA). The catheter has an optical module connector for $S\text{v}_o2$ monitoring and a distal lumen hub for blood sample withdrawal. The signal was processed and displayed by a Vigilance monitoring system (Edwards Lifesciences LLC). Before insertion, the catheter was calibrated according to the manufacturer’s instructions. An insertion length of ~8.3 cm was determined in our laboratory to place the tip at the hepatic vein juncture within the inferior vena cava, a region where a significant drop in the oxygen saturation ($O_2\text{ Sat}$) is observed as the catheter is advanced into position. The catheter’s position at the junction was confirmed with post-experiment laparotomies.

At baseline, 0.2 mL of blood samples were withdrawn slowly into a heparinized syringe and analyzed immediately using a bench co-oximeter (GEM OPL Co-Oximeter; Instrumentation Laboratories, Lexington, MA) for $O_2$ Sat and Hb concentrations. Slow withdrawal reduced the risk of admixture of venous blood from caval vessel tributaries (e.g., renal) and elevation of the $O_2\text{ Sat}$. For each experiment, an in vivo calibration of the monitor system was performed by using baseline Hb and $O_2\text{ Sat}$ values measured by the co-oximeter. At the end of hemorrhagic shock and RPs, venous blood samples were drawn and analyzed by the co-oximeter and correlated with the real-time $S\text{v}_o2$ values.

**Laboratory Assays**

Arterial blood samples of 0.1 mL were taken at each point to determine arterial lactate and Hb levels. The lactate level was measured with YSI 2300 Stat Plus lactate/glucose analyzer (Yellow Springs Instruments, Yellow Springs, OH), and the quantitative determination of Hb in arterial blood was performed with a Hemoglobin Reagent Set (Pointe Scientific, Carton, MI).

**Statistical Analysis**

The data were analyzed using the SPSS statistical software and all values were presented as mean ± SEM. Comparisons between groups were performed with one-way analysis of variance followed by Bonferroni’s post-hoc test for multiple comparisons. Within-group comparisons over time were made using a repeated-measures analysis of variance, with post-hoc paired $t$ tests to detect specific differences. A $p$ value <0.05 was considered to be statistically significant.

**RESULTS**

### Mean Arterial Pressure, Heart Rate, and Hb Levels

Animals were bled for 10 minutes to induce hemorrhagic shock with an initial MAP of 30 mm Hg that generally increased spontaneously to 40 mm Hg within 5 minutes (Fig. 1). The MAP of the shocked animals was maintained at 40 mm Hg throughout the 50-minute SP. In this study, we observed an overall mortality rate of 17% due to anesthesia accidents or excessive bleeding resulting in MAPs <40 mm Hg. During the 60-minute RP, the MAP of the blood and blood + rHuEPO groups returned to near-baseline values. In contrast, the MAP of the saline and saline + rHuEPO groups remained significantly lower during the RP when compared with the blood-resuscitated groups ($p < 0.0001$).

The MAP of saline + rHuEPO group when compared with the saline group was not statistically significant ($p = 0.14$). The HR in all four groups decreased and increased accordingly during the blood shedding, shock, and RPs with no significant statistical differences between groups (Fig. 2).

The Hb levels in the four groups were similar at baseline with a mean of 12.9 g/dL ± 0.6 g/dL and similarly reduced at the end of the SP to a mean Hb of 9.7 g/dL ± 0.6 g/dL (Table 1). After resuscitation, the Hb levels for the blood and blood + rHuEPO groups returned to baseline, but the saline and saline + rHuEPO groups remained low and were further decreased after saline resuscitation.

### Lactate Levels

At the end of SP, the serum lactate level in all four groups increased ~10 times when compared with baseline.
lactate levels of <1 mmol/L (Fig. 3). After resuscitation using blood or blood + rHuEPO, the lactate levels returned to near-baseline values of <1 mmol/L and were significantly lower when compared with values observed in animals resuscitated with saline (p < 0.001). The 60-minute postresuscitation lactate levels in the saline + rHuEPO group compared with the saline group were not significantly different (3.49 mmol/L ± 0.48 mmol/L vs. 4.81 mmol/L ± 0.82 mmol/L; p = 0.20).

**Microcirculatory Perfusion and NADH Fluorescence**

The baseline perfused capillary density and RBC flow motion scores were similar in all four groups (Table 2). At the end of hemorrhagic SP, there were significant decreases in functional capillary density and RBC flow motion scores for all groups compared with baseline (p < 0.0001). After resuscitation of the shocked rats, the functional capillary density and RBC flow motion scores of the blood and blood + rHuEPO groups recovered to near normal values, whereas in the saline and saline + rHuEPO groups, only a partial recovery was noted. In the saline-treated groups, the postresuscitation comparison of functional capillary density between saline and saline + rHuEPO was not statistically different (23.03 Cap/mm ± 0.84 Cap/mm vs. 21.61 Cap/mm ± 0.88 Cap/mm; p = 0.40). However, the RBC flow motion scores in the saline + rHuEPO group compared with the saline group (2.32 Cap/mm ± 0.12 vs. 1.60 Cap/mm ± 0.13 Cap/mm; p < 0.01) were significantly higher. In all four experimental groups, after 60 minutes of shock, the NADH fluorescence of the ileal muscular layer of the small intestine significantly increased ranging from 27% to 33% above baseline (Fig. 4). Sixty minutes after resuscitation, the NADH fluorescence level for the blood- and blood + rHuEPO-resuscitated groups returned to near-baseline values, whereas the saline + rHuEPO group demonstrated significantly lower NADH fluorescence levels than the saline group relative to baseline values (110% ± 3.64% vs. 122% ± 4.26%; p < 0.05). In blood-resuscitated animals, the addition of rHuEPO did not appear to further improve microcirculatory flow and/or to tissue dysoxia.

**Venous Oxygen Saturation of Splanchnic Circulation**

The Svo₂ continuously monitored by catheter oximetry correlated well with the O₂ Sat assessed by co-oximeter for all three groups at baseline (Table 3). The Svo₂ values observed at the end of SP tended to be higher than the O₂ Sat measurement; however, there was a proportional drop in all three groups. After resuscitation, the Svo₂ in the saline + rHuEPO group was higher than the saline group, but this difference did not achieve statistical significance (45% ± 1% vs. 38% ± 3%; p = 0.06). In contrast, O₂ Sat measurements determined by co-oximetry demonstrated statistically higher saturations in the saline + rHuEPO when compared with the saline group (38% ± 2% vs. 29% ± 3%; p < 0.05). Similar changes in measured Hb levels occurred in the three groups.
The small intestine is highly sensitive to hypoperfusion injury because of higher mucosal cell critical oxygen requirements and a mucosal countercurrent microcirculation that limits extraction because of decreased oxygen gradients. Thus, patients subjected to hemorrhagic shock and resuscitation, trauma, and surgery often develop intestinal ischemia and tissue damage, which has been documented by both experimental and clinical studies. In a prehospital setting the battlefield where blood products are not available, the wound receive resuscitation with crystalloid/colloid fluids alone to establish hemodynamic stability while waiting for casualty evacuation to a field hospital for definitive care.

We used a hemorrhagic shock rat model to study the effects of saline + rHuEPO resuscitation on the microcirculation and tissue bioenergetics of the small intestine. We found that addition of rHuEPO to saline produced significant improvements in the RBC flow motion and Svo₂, coinciding with decreased bioenergetic impairment as measured by diminished NADH fluorescence in the ileal muscle layers.

The improved microcirculatory perfusion observed with rHuEPO cannot be explained by cardiovascular hemodynamics or changes in Hb levels alone. The MAP after resuscitation and the Hb levels were similar for both saline groups. As expected, isovolemic anemia was induced by resuscitation with normal saline. As well any potential anti-inflammatory effects of heparin, which was used to prevent blood clotting during blood withdrawal and reinfusion, would be expected to similarly affect all four experimental groups. The use of citrate as an anticoagulant in this model resulted in a higher mortality rate with fewer animals completing the assessment by intravital microscopy (data not shown).

Thus far, the mechanisms that improve microcirculation have not been defined. Besides the regulation of erythropoiesis by EPO, recent studies indicate that this hormone exerts multiple actions promoting the maintenance of homeostasis of the organism under hypoxia-mediated stressors such as oxidative stress during ischemic-reperfusion injury. The secretion and production of EPO and distribution of the EPOR occur in a wide variety of organs. Besides the kidney and liver, the cells of the cardiovascular, neurologic, and gastrointestinal systems including cardiomyocytes, vascular smooth muscle, endothelial cells, neurons, astrocytes, microglia, and rat gastric mucosal/intestinal epithelial cells expresses functional EPOR that can mediate antiapoptotic, anti-inflammatory, and angiogenic signaling in a variety of tissue injury models.

### DISCUSSION

The small intestine is highly sensitive to hypoperfusion injury because of higher mucosal cell critical oxygen requirements and a mucosal countercurrent microcirculation that limits extraction because of decreased oxygen gradients. Thus, patients subjected to hemorrhagic shock and resuscitation, trauma, and surgery often develop intestinal ischemia and tissue damage, which has been documented by both experimental and clinical studies. In a prehospital setting such as the battlefield where blood products are not available, the wounded receive resuscitation with crystalloid/colloid fluids alone to establish hemodynamic stability while waiting for casualty evacuation to a field hospital for definitive care.

### TABLE 2. Functional Capillary Density and Red Blood Cell Flow Motion in the Terminal Ileum: Results From Experiment 1

<table>
<thead>
<tr>
<th>Group (n = 6 per Group)</th>
<th>Capillary Density (Cap/mm)</th>
<th>RBC Flow Motion Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Shock</td>
</tr>
<tr>
<td>Saline</td>
<td>28.08 ± 1.16</td>
<td>19.80 ± 0.24</td>
</tr>
<tr>
<td>Saline + rHuEPO</td>
<td>27.80 ± 0.58</td>
<td>18.87 ± 1.34</td>
</tr>
<tr>
<td>Blood</td>
<td>28.27 ± 1.32</td>
<td>19.65 ± 1.39</td>
</tr>
<tr>
<td>Blood + rHuEPO</td>
<td>27.67 ± 1.28</td>
<td>18.52 ± 1.03</td>
</tr>
</tbody>
</table>

Functional capillary density and RBC flow motion scores in the muscular layer of the ileum at baseline, end of hemorrhagic shock, and after resuscitation periods.

Data are presented as the mean ± SE for n = 6 animals/group.

* p < 0.01 vs. saline resuscitation alone.

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of inducible nitric oxide synthase. Thus, rHuEPO may regulate blood flow within the microcirculation through yet undetermined endothelium-dependent mechanisms.

Coinciding with an increase in the functional capillary density and improved RBC flow motion in the saline + rHuEPO group when compared with the saline group, an improved tissue bioenergetic state was demonstrated with techniques we have recently validated. Treatment with rHuEPO after saline resuscitation resulted in a sustained decrease of tissue NADH fluorescence when compared with the saline-only resuscitated group. An elevated level of mitochondrial NADH signifies impairment of electron transport chain (ETC) function. NADH in the mitochondria reduces and oxidizes adjacent cytochrome complexes, creating a proton gradient that drives ATP production. Cytchrome c oxidase, the terminal complex of the ETC, reduces molecular oxygen to water allowing this series of redox reactions of the ETC to continue. Cessation of electron transfer anywhere along this pathway will halt the production of ATP resulting in NADH accumulation within the mitochondria and further affect pyruvate metabolism contributing to metabolic acidosis as well as impaired oxidative phosphorylation. Therefore, we infer that increased NADH fluorescence in the gut reflects impaired function of the ETC in the cells of the muscularis mucosa and thus a bioenergetic impairment of this tissue. Although the NADH fluorescence levels in the blood-resuscitated groups were less elevated compared with the saline groups, they did not completely normalize after resuscitation. This observation is consistent with findings from other studies suggesting that traditional methods of resuscitation often fail to adequately restore mesenteric perfusion despite stabilization of MAP and HR. Progressive deterioration of mesenteric blood flow postresuscitation may contribute to hypoxia-induced intestinal inflammation and the loss of gut mucosal barrier function both of which can trigger of multiorgan dysfunction. Currently, we do not have any data that allows us to determine the relation between the magnitude of change in NADH fluorescence observed in this study and degree of cellular dysfunction or tissue injury. However, the significant reduction of tissue NADH fluorescence associated with improvement of the microcirculation after EPO treatment is a new observation in hemorrhagic shock. Because we did not measure NADH, we cannot rule out a change in the total NADH pool size contributing to the decrease in NADH fluorescence. The activation of poly(ADP-ribose) polymerase (PARP) by oxidative stress in hemorrhagic shock has been proposed as a pathway that could lead to NADH-NADH depletion. In our study, at the end of the 60-minute SP, it is unlikely that depletion of the NADH-NADH pool size occurred because there was a consistent elevation of NADH fluorescence levels relative to the baseline in all four groups, which returned to new baseline levels at 60 minutes postresuscitation with blood. Depletion of the NADH-NADH pool size by increased levels of PARP appears to be dependent on the duration of oxidative stress and noted to occur in a porcine model of controlled arterial hemorrhage after 4 hours, whereas in our study, a shorter SP was used.

The improvement in the splanchnic SvO2 in the saline + rHuEPO group versus saline group may not only be due to improved functional capillary density and RBC flow motion but also due to the result of additional effects of EPO on the brain stem and the carotid bodies, which control hypoxic ventilation, a parameter not assessed in this study because the animals used were not ventilated. However, an increase in respiratory frequency and tidal volume has only been noted after direct injection into the central nervous system, whereas i.v. infusions of EPO appear to have no significant effects in anesthetized rats.

In conclusion, addition of rHuEPO to saline resuscitation for the treatment of hemorrhagic shock improves RBC flow motion in the gut with a concomitant decrease in NADH tissue fluorescence. Thus, rHuEPO may improve mitochondrial oxidative phosphorylation and pyruvate metabolism in this animal model, in part, by improving tissue microcirculation. Further studies are warranted to determine the potential mechanisms underlying these observations and to determine whether these effects can lead to less tissue injury, improved organ function, and reduced morbidity and mortality in the treatment of patients with hemorrhagic shock.

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