Metabolic Tissue Swelling and Local Microcirculation in Splanchnic Artery Occlusion Shock: Implications for Critical Illness*

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Nonstandard Abbreviations
ABG – Arterial Blood Gas
LVR – Low Volume Resuscitation
MFI – Mean Flow Index
OPSI – Orthogonal Polarization Spectral Imaging
PEG-20k – Polyethylene glycol 20,000 Da
PPV – Proportion of Perfused Vessels
SAO – Splanchnic Artery Occlusion
SIRS – Systemic Inflammatory Response Syndrome
SMA – Superior Mesenteric Artery

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Abstract

Trauma is a leading cause of death in the US. Advancements in shock resuscitation have been disappointing because the correct upstream mechanisms of injury are not being targeted. Recently, significant advancements have been shown using new cell impermeant molecules that work by transferring metabolic water from swollen ischemic cells to the capillary, which restores tissue perfusion by microcirculatory decompression. The rapid normalization of oxygen transfer improves resuscitation outcomes. Since poor resuscitation and perfusion of trauma patients also causes critical illness and sepsis, and can be mimicked by ischemia-reperfusion of splanchnic tissues, we hypothesized that inadequate oxygenation of the gut during trauma drives development of later shock and critical illness. We further hypothesized that this is caused by ischemia-induced water shifts causing compression no-reflow. To test this, the superior mesenteric artery (SMA) of juvenile anesthetized swine was occluded for 30 minutes followed by 8 hours of reperfusion to induce mild splanchnic artery occlusion (SAO) shock. One group received the impermeant polyethylene glycol 20,000 (PEG-20k) that prevents metabolic cell swelling, and the other a lactated Ringers vehicle. Survival doubled in PEG-20k treated swine along with improved macro-hemodynamics and intestinal mucosal perfusion. Villus morphometry and plasma inflammatory cytokines normalized with impermeants. Plasma endotoxin rose over time after reperfusion and impermeants abolished the rise. Inert osmotically active cell impermeants like PEG-20k improve intestinal reperfusion injury, SAO shock, and early signs of sepsis, which may be due to early restoration of mucosal perfusion and preservation of the septic barrier by reversal of ischemic compression no-reflow.
Significance Statement:

Significant advancements in treating shock and ischemia have been disappointing because the correct upstream causes have not been targeted. This study supports that poor tissue perfusion after intestinal ischemia from shock is caused by capillary compression no-reflow secondary to metabolic cell and tissue swelling, since selectively targeting this issue with novel PEG-20k based cell impermeant IV solutions reduces splanchnic artery occlusion shock, doubles survival time, restores tissue micro-perfusion, and preserves gut barrier function.
**Introduction**

**Hemorrhagic shock, critical illness and SAO:** For people under 45 years, trauma is the number one cause of death in the US. For all age groups in the US, trauma is the third leading cause of death. Trauma accounts for approximately 25% of all life-years lost, compared to cancer (12%), heart disease (11%), and HIV (2%)(Finkelstein et al., 2006; CDC, 2022). For traumatic injuries, hemorrhagic shock causes over 35% of pre-hospital deaths and over 40% of deaths within the first 24 hours.

Sepsis is best defined as multiple organ dysfunction caused by a global dysregulated host response to infection (Seymour et al., 2016; Shankar-Hari et al., 2016; Singer et al., 2016). The infection arises from a primary source such as a lung or urinary tract infection or a secondary infection usually downstream from severe traumatic or surgical tissue injury. In critical illness, the primary or secondary infection becomes systemic and causes a dysregulated immunological and inflammatory response typically characterized by hyperimmune reactions followed by later immunosuppression. Ultimately, reductions in organ perfusion establishes favorable conditions for critical illness in the ICU as poorly perfused vital organs begin to dysfunction and then fail cascading into whole systems failure and death. In fact, the mortality rate can run as high as 40% with survivors often experiencing delayed symptoms (Post-Sepsis Syndrome) for years later (Mostel et al., 2019; Vincent et al., 2019; Rudd et al., 2020).

The symptoms of shock, sepsis, and critical illness can be mimicked with splanchnic artery occlusion (SAO) shock where the splanchnic circulation is interrupted and after the tissues are allowed to undergo reperfusion injury. Lefer was the first to characterize this in detail and suggested that ischemia injures the gut and mucosal septic barrier, which allows enterotoxins and proteolytic enzyme release into the systemic circulation to cause myocardial depression, vasodilatation, sepsis, and shock (Glenn and Lefer, 1970; Lefer, 1970; Lefer and Verrier, 1970; Bridenbaugh et al., 1976). Because the gut after ischemic injury from trauma and hemorrhage has been termed the “motor” of critical illness (Carrico et al., 1986; Clark and
Coopersmith, 2007; Meng et al., 2017), reperfusion injury of the intestine and other splanchnic tissues should be considered as a primary causal mechanism of traumatic illness and targeted for treatment.

**Microcirculation:** Oxygen transfer from the lungs to the mitochondria in energy dependent tissues is accomplished by convection and diffusion. The macro-hemodynamic forces convect saturated hemoglobin and dissolved oxygen to the microcirculation where the last mile must be traveled by diffusion from the capillary to the mitochondria (Krogh, 1919; Pittman, 2000; Pittman, 2011). The microcirculation is optimized to this task by decreasing the capillary-to-cell diffusion distance and surface area to promote oxygen transfer (Pittman, 2013). Disruptions in the microcirculation can severely frustrate this process. One major hallmark of sepsis and septic shock is the development of significant defects in organ perfusion and microcirculatory problems. It is our preliminary conclusion that this ultimately causes critical illness and development of multiple organ failure through progressive loss of tissue and organ microcirculatory oxygen exchange and ischemic death. In sepsis, determinants of microcirculation such as reduced perfused capillary densities, lower flow through open capillaries, physical obstructions such as thrombi and inflammatory aggregates, intermittent flow patterns, and compressed capillaries are evident (De Backer et al., 2002; Ince, 2005). Furthermore, these changes are worse in patients with worse outcomes, suggesting a cause and effect relationship. Perfusion in the microcirculation and organ failure outcomes are correlated in septic patients (Legrand et al., 2019).

**Metabolic Cell Swelling:** Cells swell in response to ischemia and hypothermia, both of which are associated with shock, sepsis, and critical illness. First described in detail in the context of organ preservation injury, cell swelling is primarily caused by failure of energy-dependent cell volume control mechanisms (Southard and Belzer, 1980; Mees et al., 1982; Lindell et al., 1989; Southard et al., 1990; Southard and Beltzer, 1993; Southard and Belzer, 1995). The sodium pump (Na/K ATPase) turns off or runs slower during shock, due to a lack of adequate ATP from altered oxygenation and cellular energetics (Barlet-Bas et al., 1990). This causes sodium ions to enter the cell down electrochemical gradients. The Na⁺ recruits Cl⁻ electrogenically, followed by passive water movements and the cell swells (Figure 1A). Hydropic degeneration from energy failure damages membrane and mitochondrial structures...
(Petit et al., 1998), which may lead to cell death. Swelling of parenchymal cells can also compress local capillaries (Figure 1B), leading to further reductions in capillary flow and oxygen delivery causing a self-amplifying cycle. Tissue and cell swelling during resuscitation can cause the “no reflow phenomenon”, which limits positive resuscitation outcomes (Reffelmann and Kloner, 2002; Rezkalla and Kloner, 2002; Kloner, 2011; Maksimenko and Turashev, 2012). Figure 1B shows how this mechanism may occur and how novel cell impermeant molecules can passively reverse this by osmotically holding water outside the cell (Figure 1C).

**Polyethylene Glycol:** Repeating units of ethylene glycol (polyethylene glycol-PEG) can range in size from 100-8,000,000 daltons. Polymers above 400 are nontoxic to animals and sizes above 500 are generally impermeant to cells and are called impermeants (Donovan et al., 1990; Ma et al., 1990; Gursahani et al., 2009). Polymers above 80,000 are generally confined to the capillary space where they act as colloids (Rudmann et al., 2013). Polymers of PEG between 20,000 and 35,000 are still cell impermeants but their variable permeability to the capillary gives them some variable oncotic strength also. PEG-20k (20,000 daltons) was found to have both impermeant and partial colloidal properties, as it distributes about 1/3 outside the capillary into the interstitial space (impermeant actions) and about 2/3 inside of the capillary where it has some oncotic actions (Parrish et al., 2014; Parrish et al., 2015). This unique size PEG (PEG-20k) is a true hybrid molecule possessing both impermeant and oncotic actions because of its unique size and molecular radius. The large biological effects of PEG-20k as an anti-shock molecule is due to its osmotic effects on water transfer in ischemic tissues and the follow-on restoration of capillary blood flow, oxygen transfer, and oxygen debt repayment (Parrish et al., 2015; Plant et al., 2017). Basically, PEG-20k capitalizes on Poiseuille’s law by decompressing the microcirculation, increasing capillary radius with transferred water, and augmenting flow (i.e. perfusion), as illustrated in Figure 1D. The unique osmotic reflection coefficients (Parrish et al., 2015) and hydrophilicity from oxo-ether bond of the PEG polymer (Tang et al., 2012) likely account for their superior water transfer properties.

**Impermeants in Hemorrhagic Shock:** Impermeant solutions, including solutions of PEG-20k (PM-208) were developed to treat severe hemorrhagic shock, which is a form of global
reperfusion injury. Several studies and publications from Mangino, et. al. over the last 10 years demonstrate how effective these solutions are in resuscitation and what the mechanism of action is (Parrish et al., 2014; Parrish et al., 2015; Parrish et al., 2016; Plant et al., 2016; Mangino et al., 2017; Plant et al., 2017; Liebrecht et al., 2018; Liebrecht et al., 2019; Wickramaratne et al., 2019; Khoraki et al., 2020). These data in hemorrhagic shock serve as justification and framework for using PEG-20k IV solution to treat the significant perfusion defects that occur in septic shock, SAO shock, and reperfusion injury in sensitive organs like kidney, heart, and splanchnic tissues.
Materials and Methods

Splanchnic Artery Occlusion Shock Model: Eight juvenile male Yorkshire swine (30-40 kg, Archer Farms, Inc., Darlington, Maryland, USA) were sedated with IM ketamine (25 mg/kg) and xylazine (0.5 mg/kg) combination, anesthetically induced with IV propofol (2-3 mg/kg), and anesthetically maintained by continuous inhalation with 1-2% isoflurane, 30% nitrous oxide, and 30% oxygen. Pressure controlled ventilation was used with a peak inspiratory pressure (PIP) of 25 cmH2O, positive end expiratory pressure (PEEP) of 7 cmH2O, and RR to maintain end-tidal CO2 to 35-55%. An 18g 4.25 inch catheter was placed in the right superficial femoral artery for recording blood pressure and pulse rate and for sampling arterial blood gases. A standard bore 20-inch IV extension tube catheter was cut and placed a few inches into the left external jugular vein for administration of fluids and drugs. A 7.5 Fr Swan-Ganz catheter was placed in the pulmonary artery via the right common jugular vein for continuous measurements of cardiac output and mixed venous oxygen saturation (SVO2). A midline laparotomy was made and the superior mesenteric artery (SMA) was isolated, and, at the appropriate time, clamped at the base of the aorta with a vascular clamp for 30 minutes. A few-inch segment of the distal ileum was exteriorized and isolated as a Thiry-Vella loop. A 2 cm enterotomy was made along the anti-mesenteric border for measurement of mucosal microcirculatory flow using Orthogonal Polarization Spectral Imaging (OPSI) using a MicroScan device. After baseline measurements of labs, arterial blood gases, central hemodynamics, and local capillary perfusion, the superior mesenteric artery was clamped for 30 minutes. Occlusion of the SMA was verified by observing a fall in OPSI signals to zero, which directly demonstrated lack of intestinal mucosal perfusion. After 30 minutes of ischemia to the small intestine, the clamp was released to immediately reperfuse the small bowel. Ten-minutes after reperfusion of the SMA, IV resuscitation was started with a bolus of either (randomized) LR or LR containing 10% polyethylene glycol 20,000 (PEG-20k) at 6.8 ml/kg over 5 minutes. All animals were allowed to survive up to 8 hours following SMA occlusion release without any further interventions except adjustments to anesthesia as needed to maintain sedation. Animals were euthanized by anesthetic overdose and potassium chloride bolus (100 mg/kg of a 150 mg/mL or 2mM or 2 mEq/L solution) at a pre-determined 8 hours (end of study) due to ventilator limitations of the non-survival study, or when their mean arterial pressure (MAP) dropped to and consistently remained at 30 mmHg (humane
endpoint). Arterial and venous blood samples were taken every hour or 2-4 hours, respectively, after reperfusion for analysis of lactate, metabolic panel, endotoxin, and cytokines. Samples of terminal ileum were taken at baseline and every two hours after gut reperfusion (i.e. SMA release) for histological analysis by light microscopy.

**Clinical Laboratory Analysis:** Arterial blood gas (ABG) labs were measured on an ABL800 FLEX blood gas analyzer (Radiometer, Copenhagen, Denmark) after drawing 0.5 ml arterial blood into a 1.0 ml syringe primed with 0.05 ml heparin sodium injection (1,000 USP units per ml) solution (Henry Schein Medical, Melville, NY, USA). Complete metabolic panels (CMP) were measured on a Vetscan VS2 Chemistry Analyzer (Zoetis, Parsippany-Troy Hills, NJ, USA) using their Comprehensive Diagnostic Profile disks with 100 ul of whole venous blood drawn into a lithium heparin tube.

**Orthogonal Polarization Spectral Imaging (OPSI):** Measurements of mucosal ileal perfusion were obtained using a MicroScan USB3 (MS-U) handheld video microscope with sidestream dark field imaging (MicroVision Medical Inc., Amsterdam, Netherlands) by holding the device on the surface of the mucosa to obtain a series of short video clips of the flow patterns in the microvessels. The OPSI videos were obtained with MicroScan AVA software version 4.3c and then analyzed offline by a separate blinded investigator (RD) to determine the mean flow index (MFI) and proportion of perfused vessels (PPV), which signify capillary perfusion, following the method of De Backer (De Backer et al., 2002). To determine the MFI, the video was divided into 4 quadrants and rated according to the flow in the vessels smaller than 20 µm (0: no flow, 1: intermittent (flow periodically appears and disappears), 2: sluggish (slow flow) and 3: normal). To determine the PPV, the video was divided into 16 quadrants and the number of vessels with absent flow, intermittent flow (≥ 50% time without flow), and normal flow (continuous flow) that crossed the quadrant lines were counted. PPV was then calculated by: 100 x (total number of vessels – [absent flow + intermittent flow])/total number of vessels.

**Endotoxin Assay:** Endotoxin in venous blood samples taken at baseline and after 2 and 4 hours after release of SMA occlusion in both groups were determined by a chromogenic kit (Pierce, A395525, ThermoFisher Scientific) according to the instructions. Briefly, authentic E. Coli endotoxin standards (1.0-0.01 EU/ml) were added to wells of a 96-wellplate or the same
volume of plasma diluted 1:1 with buffer. After adding amebocyte lysate to each well, a chromogenic substrate was added for 30 minutes and the reaction was stopped by addition of acetic acid solution. The plates were read at 405 nm and the unknown endotoxin in the plasma samples were derived by extrapolation from the standard curve using known amounts of endotoxin standard. Samples were done in triplicate and averaged. Results were expressed as mEU endotoxin / ml plasma.

**Cytokine Assays:** Plasma concentrations of porcine IL-6, IL-8, and TNFα were measured on a 96-well plate bead-based multiplex assay using a Luminex platform (ThermoFisher Scientific) according to the manufacturers assay protocol. The plates were read on a Luminex Technologies, xMAP Multiplex analyzer.

**Light Microscopy:** The collected ileum specimens were fixed in 10% formalin and embedded in paraffin. Tissue samples were then cut into 5-µm serial tissue sections and stained with hematoxylin and eosin (H&E). The images of the slides were captured using a digital microscope camera (Olympus, Tokyo, Japan) and the ZEN microscope software (Zeiss Microscopy, Jena, Germany). Next, using the Image J system (National Institute of Health, Bethesda, Maryland, USA), height of the villi and depth of the crypts were measured and calibrated to a lens micrometer.

**Statistical Analysis:** Data were analyzed using IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY) and Prism software (V 9.5.1, GraphPad Software, Boston, MA). Two tailed unpaired t-test was used to compare outcomes between groups. Multiple unpaired t-tests per time point with post-hoc Holm-Šídák’s multiple comparisons test were used to compare differences of means between treatment groups over time. One-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction was used to compare within groups over time of repeated measures as compared to baseline. A power analysis to detect changes in end plasma lactate of 30% was conducted using variance data from previous studies, an alpha of 0.05, and a beta of 0.8. Correlation between two variables (standard curves) was determined using the Pearson correlation coefficient. Statistical significance was determined if p < 0.05.
Results

Baseline weights, treatment volumes, and surgical times are shown in Table 1. There were no differences with any of these variables between treatment groups except for survival time and total anesthesia time, which includes survival time.

Survival time of the swine from the time of resuscitation after SAO release was significantly longer (68.8%) in the PEG-20k treated group relative to the LR treated controls (8.14 ± 0.06 vs 4.8 ± 0.98 hours, respectively). These data are shown in Figure 2. It should be considered that all of the swine in the PEG-20k treated group were euthanized at 8 hours after SAO release because of the pre-determined 8-hour end-of-study-rule in the protocol, so their true survival time was not measured. In the LR group, however, all four subjects met the blood pressure euthanasia criteria (i.e. MAP <30 mmHg) before the end of study.

The macro-hemodynamic responses to SAO shock and treatment with polymer based cell impermeants (PEG-20k IV solution) vs. LR control is shown in Figure 3. Mean arterial pressure (MAP, Panel 3A) increased in both groups with ischemia (SMA occlusion), then immediately decreased with clamp release of SMA back to baseline or lower levels. Within 5 minutes of LVR, there was a significant difference between treatment groups from 15 to 240 minutes. The PEG group MAP also increased significantly higher than its baseline and was sustained at this level for one hour, after which a gradual decline towards baseline is seen. Comparatively, the LR group MAP did not increase above baseline after LVR, and in fact was significantly lower than baseline starting two hours after LVR until the end of study. Heart rate (HR, Panel 3B) followed a similar pattern to MAP increasing after SMA occlusion, and while largely nonsignificant due to higher intragroup variations, there was a clear post-treatment trend where LR rates were higher than PEG rates (over vs under 200 bpm, respectively). Cardiac output (CO, Panel 3C), unlike MAP and HR, very minimally increased with SAO shock. However, within 1 hour after release of SAO, CO for PEG-20k treated swine almost doubled from baseline but began to fall in the LR group at the same time. Cardiac output at all times starting 50 minutes after resuscitation was statistically higher in the PEG-20k resuscitated group compared to the LR group. Mixed venous oxygen saturation (SvO2, Panel 3D) similar to CO showed a nonsignificant increases in both groups with SAO, then a drop
with release, and finally a significant difference between PEG and LR groups starting at 10 minutes after LVR until the end of the procedure.

The blood volume expansion effects of LR solution compared to PEG-20k IV solution is shown in Figure 4A, with hemoglobin changes over time shown in 4B. Volume expansion was calculated by hemoglobin dilution using Fick as previously described (Plant et al., 2017; Khoraki et al., 2020), and simplified with a percent change from baseline formula in this model. One to four hours after resuscitation from SAO with LR solution, a significant negative volume expansion effect was seen (i.e. hemoconcentration) compared to baseline, from -18.3% ± 13.8, p=0.0046, to -26.2% ± 10.3, p<0.0001, as seen in figure 4A. This corresponds with the increase from baseline in the LR group seen in the Hb Figure 4B. Up to two hours after resuscitation from SAO with PEG-20k solution, a significant positive volume expansion effect was seen (i.e. hemodilution) with 19.8% ± 4.1, p=0.0017. However, neither a significant expansion nor contraction was observed two hours and after (from 9.1% ± 6.2, p=0.3492, to -10.8% ± 10.4, p=0.1882). This corresponds with the decrease from baseline in the PEG group seen in the Hb Figure 4B, which then trends back toward baseline. The paired differences in volume expansion between the groups at all time points for the first four hours after LVR were significant, as were the first three hours after LVR for Hb.

Other signs of volume expansion (PEG-20k group) and/or hemoconcentration (LR group) from hepatic blood tests can be seen in Supplemental Figure 1 (S1), including total protein (S1A), albumin (S1B), globulin (S1C), alkaline phosphatase (S1E), and uncorrected calcium (S1H), which all show significant differences both between and within groups after SAO shock and treatment. While similar trends were seen in alanine transaminase (S1D) and amylase (S1F), the results were not significant. It is notable that once corrected for changes in serum albumin using the formula “corrected calcium = serum calcium + 0.8 * (3.1 - serum albumin)”, calcium showed no differences between or within groups throughout the study (S1I). The normal expected swine albumin level of 3.1 g/dL was used as this was the average baseline value for all eight subjects. Also, there were no changes to total bilirubin (S1G) with either group.

Renal markers and electrolyte results can be found in Supplemental Figure 2 (S2), notable for significant increases in both groups for creatinine (S2A), blood urea nitrogen (S2B),
potassium (S2E), and phosphorus (S2G). While there is no difference between or within groups for BUN to creatinine ratio (S2C), there is a clear difference between groups in rate of change for both creatinine and BUN, with at least a 4-hour delay in the PEG group rise as compared to LR group. Additionally, while potassium and phosphorus rates rise similarly in both groups due to the ischemic intestinal injury, PEG is able to maintain survival for an additional four hours as compared to LR group despite having similar levels. Sodium (S2D) levels did not differ between or within groups, while chloride (S2F) showed a minor difference at one hour after LVR with p=0.0469, and calcium (S2H) also with a minor difference at three hours after LVR with p=0.0348. Finally, glucose (S2I) shows a significant downward trend within each group, but again the PEG group lags compared to LR and ultimately has significantly higher levels than LR group at both three and four hours post-LVR.

Acid-base results from arterial blood gases are shown in Supplemental Figure 3 (S3). PEG-20k resuscitated swine showed a nonsignificant but distinct trend in maintaining a normal pH (S3A) from 2-8 hours after a small dip at 1 hour, while the LR group maintained the lower pH thereafter. Congruently, lactate (S3B) showed a small rise at hour one but then fell to below baseline levels thereafter in the PEG group before starting to climb back toward baseline by the end of study, while the LR group lactate continued to rise. Bicarbonate (S3E) and base excess (S3F) showed similar nonsignificant intergroup differences, where PEG maintained more normal levels while LR group drifted lower. Finally, arterial pCO₂ (S3C) showed a significant increase from 1 to 3 hours in the LR group after SMA release (note that respiratory frequency was not changed due to study protocol at this time), but this only lasted for 1 hour in the PEG group. Congruently, pO₂ (S3D) showed a significant drop for the first two hours only in the LR group.

Local microcirculatory perfusion to the distal ileal mucosa was measured before SAO shock and at hourly time points after reperfusion in both control (LR treated) and in PEG-20k treated swine (Figure 5). Treatment with a 6.8 ml/kg, IV infusion of either solution started 10 minutes after release of the superior mesenteric artery was used. Two outcomes of perfusion measured by OPSI were the percentage of perfused capillaries (PPV, Panel 5A) and mean flow index (MFI, Panel 5B). Both perfusion outcomes steadily declined after reperfusion in the control group, with PPV being about 20-30% of baseline (29.8% ± 7.9 to 22.6% ± 8.3 in hours
three and four, respectively) and MFI being half of baseline after 4 hours (1.4 ± 0.1). Further
time points were not measured in the control group because all of the swine died from shock.
Perfusion outcomes measured at the same times in the PEG-20k treated groups, however,
had significantly higher perfusion outcomes relative to the LR control (Figure 5). In fact, PEG-
20k showed 2-3 times higher PPV (5A) than LR, around 60% of baseline (60.6 ± 5.0 to 58.3 ±
9.0), and 52.6% higher MFI (5B) than LR at the same comparative timepoints (2.4 ± 0.3). The
swine in the PEG-20k group also lived to end of study, which was 8 hours after release of
SAO with perfusion outcomes that plateaued and stabilized from values seen after 2 hours.

Serum from peripheral venous blood removed before SAO shock and after 2 and 4 hours
from release of SMA occlusion were used to measure endotoxin release over time in swine in
both treatment groups (Figure 6). The LR treated control swine produced significantly higher
amounts of endotoxin compared to baseline (i.e. before SAO shock). Over the 4-hour period,
the rise in endotoxin concentrations assumed a logarithmic progression and ended at 4 hours
because the comparative control animals died. The swine treated with the impermeant
polymer (PEG-20k) did not show any significant changes in endotoxin concentrations from
baseline and were statistically lower in the corresponding values measured in the LR control
group.

Since the only injury created in this model was ischemia to the small intestine, a histological
evaluation of the distal ileum was assessed in samples of the bowel in both groups removed
at baseline (before SAO shock) and after 2 and 4 hours from reperfusion and treatment.
Figure 7A shows light microscopy of the distal ileum in both groups at the three time periods.
At baseline, the samples appeared normal and not differ between the two groups. After
reperfusion, however, the untreated bowel experienced classic ischemic injury (Mangino et
al., 1989) characterized by villus height reduction, villus “clubbing”, and loss of
epithelialization from the tip of the villus down, as well as cellular inflammation and
hemorrhage in the lamina. In PEG-20k treated swine however, the histological picture
appears closer to normal (at baseline) with maintained villous height and less inflammation
and hemorrhage. In fact, morphometric analysis of villus height using image analysis (Figure
7B) showed no reduction in height after SAO release in the PEG-20k treated swine relative to
baseline before SAO shock. The values in the treated group were also significantly higher
compared to the corresponding values observed in the LR control group at each time after reperfusion. The villous height difference between the two groups was about 30% at both 2 and 4 hours after reperfusion.

Finally, plasma cytokine production after 4 hours was assessed relative to baseline in both groups (Figure 8). Baseline values for IL-6 (8A), IL-8 (8B), and TNFα (8C) were assessed and found to be no different between groups. However, the LR control group showed significantly elevated levels after 4 hours from release of SAO, relative to the polymer treated swine group.
Discussion

Trauma with severe hemorrhage and/or splanchnic ischemia causes global hypoperfusion and further ischemia to vital tissues with reperfusion injury at resuscitation. This contributes to acute hemorrhagic shock or mesenteric ischemia injury and downstream life-threatening disease seen in the surgical-trauma ICU. These include development of SIRS, sepsis, septic shock, and critical illness. The strategy in treating these patients is to rapidly reverse the cause of tissue injury, which is ATP depletion (ischemia) from frank blood loss and/or anatomical interruption of blood flow from major arteries. Therefore, resuscitation and surgical strategies should aim to resolve the oxygen debt accumulated in vital organs and tissues as quickly as possible, since it is known that rapid debt repayment results in better outcomes immediately after resuscitation and later in the ICU (Rixen and Siegel, 2005; Barbee et al., 2010; Khoraki et al., 2020). This study aimed to test this principle in a severe global SAO shock model by using novel anti-shock resuscitation tools that target compression no reflow of the microcirculation.

We have recently demonstrated a novel maneuver that maintains capillary perfusion early in severe hemorrhagic shock and resuscitation, which produces striking acute and chronic downstream benefits in outcomes because it causes rapid oxygen transfer and debt repayment. Prevention of compression capillary no reflow after resuscitation with a novel osmotic polymer solution (PEG-20k IV solution) corrects metabolic cell and tissue swelling, which secondarily allows for efficient capillary perfusion and oxygen transfer at resuscitation (Parrish et al., 2015; Parrish et al., 2016; Plant et al., 2017). Since we believe this mechanism of tissue reperfusion injury is universal to all forms of ischemia and not just limited to global ischemia with hemorrhagic shock, then it should effectively prevent or lessen injury to splanchnic tissues per se during shock, which we believe cause both acute and downstream critical illness and sepsis in the ICU. These studies were designed to test this. We hypothesize that clinical use of these IV crystalloid solutions targeting compression no reflow from metabolic tissue swelling will not only improve short term outcomes but prevent, delay, or attenuate the follow-on critical illness commonly observed later in the surgical ICU. The main mechanism being preservation of small bowel integrity by preventing long term tissue oxygen debt accumulation after resuscitation. Therefore, we studied the effects of these new
anti-shock molecules in a classic model of splanchnic artery occlusion (SAO) shock where only ischemia to the small bowel occurs.

Classic experiments in the 1970’s studying splanchnic artery occlusion (SAO) shock clearly demonstrated the lethal nature of reperfusion of large amounts of ischemic gut or splanchnic tissue (pancreas, stomach, large and small bowel). The deterioration in cardiovascular function after reperfusion in these models was attributed to the release of cardio-depressant molecules from the ischemic gut tissue forming a local toxic milieu. Mediators are released from deterioration of the gut from ischemia and reperfusion injury, which leads to severe inflammation of the gut mucosa with production of pro-inflammatory mediators able to wash out into the systemic circulation. Breakdown of the gut septic barrier also occurs. This allows gram negative bacteria and enterotoxins to absorb from the septic luminal side into the blood and cause secondary infection and inflammation. Therefore, the key to preventing downstream systemic effects in SAO shock is to protect the mucosa from ischemia and reperfusion injury. The best way to do that is to limit compression no reflow after reperfusion to pay back oxygen debt quickly to limit ongoing ischemia after reperfusion. This was our hypothesis. The results indicate microcirculation is improved with PEG-20k IV solution administration after gut reperfusion. This is associated with much improved gut histology, which are likely causally related and support our hypothesis. These data also indicate that metabolic cell and tissue swelling and secondary compression no reflow occurs in other ischemic tissues and is not unique to global shock. If this mechanism of tissue reperfusion is as universal as suspected, then specific tools leveraging this mechanism should also be protective in other tissues like acute kidney injury, cerebral ischemia with stroke, and myocardial injury after myocardial infarction. This needs to be tested in future studies, as these specific patient populations may be more sensitive to plasma volume expansion.

Fixing persistent no reflow through this novel osmotic mechanism to restore local capillary perfusion preserves mucosal architecture. If the ischemic gut is the motor for the secondary downstream systemic changes that cause shock and critical illness, as hypothesized, then early repair or prevention of post-reperfusion injury should also modify the follow on SAO shock. The study clearly indicates this occurred. Survival time doubled in the PEG-20k group, which likely would have been longer had the treated animals been allowed to survive past the
arbitrary end of study time period (8 hours). Central hemodynamics were more preserved compared to the control group too, with the ability of PEG-20k IV solution to maintain microcirculation MAP and HR while enhancing cardiac output and microcirculation (OPSI) to repay the ischemic debt, as indicated by lowered lactate and higher SvO₂. Finally, the logarithmic appearance of endotoxin in the blood in control swine after release of SAO, presumably caused from the translocation of endotoxin from the gut, was reversed in swine treated with PEG-20k solution. This likely occurred because perfusion was maintained in the mucosa and gut architecture was protected (Figure 7). Better tissue perfusion probably protected the septic barrier to limit movement of endotoxin into the blood. Plasma IL-6, IL-8, and TNFα cytokine production was also prevented with PEG-20k treatment, which is consistent with less endotoxin priming of mononuclear cells.

The animals in the LR control group displayed clinical signs of secondary septic shock following SAO and release in this model. They clearly had cardiovascular collapse consistent with the Lefer model since their arterial blood pressure and cardiac output fell over time after release of SAO, which terminated in euthanasia once their MAP reached 30 mmHg. Furthermore, they demonstrated biochemical signs of acute inflammation because Th1 cytokines accumulated in the plasma of the untreated group. Finally, the appearance of endotoxin in the systemic venous blood indicates gram negative infection, presumably secondary to gut reperfusion injury since that is the only tissue injured in this model (infection source). These are the hallmark symptoms leading to diagnosis of secondary septic shock. All of these indicators were mitigated in the PEG-20k treated group suggesting that this anti-shock molecule prevented secondary septic shock after SAO release. So, if the mechanism of action of PEG-20k predominantly is to improve tissue perfusion in ischemic tissues, which occurred in the small bowel mucosa in this model, then it is reasonable to conclude that reperfusion of ischemic bowel can lead to secondary septic shock and that targeting early restoration of ischemic gut perfusion can ameliorate or prevent this from occurring.

Severe hemorrhagic shock, common in civilian and military trauma, produces global ischemia with global reperfusion injury at resuscitation. Some organ beds are affected more than others as redistribution of blood flow in shock is variable, depending on the tissue. Furthermore, the sensitivities of these tissues and the consequences of reperfusion also varies depending on
the specific tissue. The local distribution of blood flow and oxygen delivery to the splanchnic organs is the first to go in hypovolemic shock. The metabolic rate of the small intestinal mucosa is particularly high, and therefore, sensitive to oxygen delivery reductions. Finally, the consequences of reperfusion of the bowel with critical amounts of ischemic injury and critical amounts of bowel mass can produce dangerous effects in the patient as seen from SAO shock studies and from patients with SMA occlusion following restoration of flow. We and others propose that the splanchnic tissues, especially small bowel mucosa, are a critical target organ of injury in hemorrhagic shock and critical illness (Clark and Coopersmith, 2007; Klingensmith and Coopersmith, 2016; Meng et al., 2017; Kang et al., 2023). We go one step further and postulate that the ischemic small bowel that inevitably occurs in hemorrhagic shock after trauma drives critical illness and secondary sepsis in the surgical-trauma ICU following damage control resuscitation due to a poor tissue perfusion mechanism. This study, having isolated the injury largely to only the small bowel mucosa and showing effects of restoring microcirculation, supports this hypothesis and provides a road map to successfully treat the acute and downstream secondary effects of resuscitation by targeting early effective restoration of ischemic gut tissue perfusion by reversing metabolic cell swelling. The new anti-shock polymer solution (PEG-20k IV solution, PM-208) is the prototype new drug that does this and while not yet FDA approved, first in human trials will soon be underway.

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Data Availability Statement
The data that support the findings of this study are available on request from the corresponding author, including raw hemodynamic, laboratory, and video data as well as their analyses.
Authorship Contributions

Participated in research design: Liebrecht, Khoraki, Mangino

Conducted experiments: Liebrecht, Khoraki, Li, Archambault, Broadway, Bane, Deitch, Mangino

Contributed new reagents or analytic tools: Liebrecht, Eldering, Mangino

Performed data analysis: Liebrecht, Khoraki, Li, Bane, Deitch, Mangino

Wrote or contributed to the writing of the manuscript: Liebrecht, Khoraki, Li, Bane, Deitch, Mangino
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**Footnotes**

* This work was supported by the Department of Defense US Army Medical Research and Development Command (USAMRDC) Grant W81XWH-18-1-0759 to Dr. Mangino.

* M.J.M. is co-founder and Chief Scientific Officer of Perfusion Medical, Inc., a biotechnology company commercializing PEG-20k IV solution. G.E. is co-founder and Chief Executive Officer of Perfusion Medical, Inc. L.K.L. is a volunteer scientist for and former interim Chief Medical Officer of Perfusion Medical, Inc. M.J.M, G.E., L.K.L., and Virginia Commonwealth University all have ownership interest in Perfusion Medical, Inc., which has licensed the intellectual property utilized in this study. No other authors have any actual or perceived conflict of interest with the contents of this article.
Figure Legends

**Figure 1**: Mechanism of action of cell impermeants in ischemic compression capillary no reflow. Normal cell volume control mechanisms are dependent on ATP. Ischemia causes ATP depletion due to lack of oxygen, which limits activity of the basolateral sodium pump (Na/K ATPase). This leads to sodium flux into the cell followed by osmotic water. This causes metabolic cell and tissue swelling (A). Parenchymal swelling causes compression of the capillaries moving through the tissues. Endothelial cell swelling further reduces the lumen of the capillary. This results in ischemic compression no reflow at reperfusion (B). Loading the extracellular space with cell impermeant molecules creates an osmotic force that pulls metabolic water out of the cells and tissues to limit cell and tissue swelling and resultant compression no reflow (C).

**Figure 2**: Survival time of swine after superior mesenteric artery (SMA) occlusion and release comparing resuscitation with lactated Ringers (LR) solution as the vehicle control and LR containing 10% polyethylene glycol 20,000 (PEG-20k), each given at 6.8 ml/kg over five minutes. The pre-determined end of the non-survival surgical study was 8 hours. Values are mean ± SD, n=4 per group. ***P = 0.0005 using unpaired parametric two-tailed t-test.

**Figure 3**: Cardiovascular macro-hemodynamic outcomes in splanchnic artery occlusion (SAO) shocked swine after resuscitation with LR solution (control) or the same volume of PEG-20k IV solution. Mean Arterial Pressure (MAP) (A), Heart Rate (HR) (B), Cardiac Output (CO) (C), and Mixed Venous Oxygen Saturation (SvO₂) (D) are shown at baseline, after SMA occlusion (ischemia, “Clamp on”) and after release of SAO (“Clamp off”), and continued after low volume resuscitation (LVR) until either the MAP reached 30 mmHg (euthanasia endpoint) or 8 hours after release (end of study). Shaded boxes dictate typically normal ranges of those outcomes in swine. Values are expressed as mean ±SD, n=4 per group. *P<0.05 denotes intergroup comparison (LR vs PEG) using multiple unpaired t-tests per time point with the Holm-Šídák’s multiple comparisons test. †P<0.05 for LR and §P<0.05 for PEG denote intragroup comparisons (baseline value vs repeated measure) using one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction.
**Figure 4**: Plasma volume (A) and hemoglobin (B) changes during the study in both LR and PEG-20k treated groups in hours from release of splanchnic artery occlusion (SAO). Values are expressed as mean ± SD, n=4 per group. *P<0.05 denotes intergroup comparison (LR vs PEG) using multiple unpaired t-tests per time point with the Holm-Šídák’s multiple comparisons test. †P<0.05 for LR and §P<0.05 for PEG denote intragroup comparisons (baseline value vs repeated measure) using one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction.

**Figure 5**: Microcirculatory perfusion in the ileal mucosa as measured by OPSI in LR and PEG-20k resuscitated groups before and after release of SAO. Perfusion data include the Percent Perfused Capillary (PPV) values (A) and the Mean Flow Index (MFI) value (B). All data expressed as mean ± SEM, n=4 per group. *P<0.05 relative to the corresponding value in the LR vs. PEG resuscitated group using unpaired t-tests.

**Figure 6**: Serum endotoxin concentrations measured in venous blood from both LR and PEG-20k resuscitated swine both before SMA occlusion and then 2 and 4 hours after release. All data expressed as mean ± SD, *P<0.05 of LR group relative to the corresponding value in the PEG-20k resuscitated group using unpaired t-tests.

**Figure 7**: Histological assessment of distal ileal biopsies from both LR and PEG-20k resuscitated swine before and 2 and 4 hours after release of SAO. Light microscopy with H&E staining at 100x magnification are shown in (A). Morphometric analysis of villous height is shown in (B). Values are expressed as mean ±SD, *P<0.05 of PEG group relative to the corresponding value in the LR resuscitated group using unpaired t-tests.

**Figure 8**: Plasma cytokine concentrations including Interleukin-6 (IL-6, A), Interleukin-8 (IL-8, B), and Tumor Necrosis Factor-alpha (TNFα, C) from both LR and PEG-20k resuscitated swine before (Baseline, BL) and 4 hours after release of SAO. All data expressed as mean ± SD, with *P<0.05 of LR group relative to the corresponding value in the PEG-20k resuscitated group using unpaired t-tests.
Tables

Table 1:
Baseline weights, treatment volumes, and surgical times are listed comparing LR control vs. PEG-20k groups. Formulas to calculate weight-based volumes and treatment rates are listed. Values are mean ± SD, n=4 per group. Bolded p-value denotes significance of p<0.05 using multiple unpaired t-tests per variable with the Holm-Šidák’s multiple comparisons test. Abbreviations: Wt (weight), EBV (estimated blood volume), LVR (low volume resuscitation).

<table>
<thead>
<tr>
<th>Variable (units)</th>
<th>Formula</th>
<th>PEG-20k</th>
<th>LR</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (kg)</td>
<td>n/a</td>
<td>33.5 ± 2.2</td>
<td>33.6 ± 1.5</td>
<td>0.9426</td>
</tr>
<tr>
<td>Estimated Total Blood</td>
<td>Wt (kg) * 68 (ml/kg)</td>
<td>2278.0 ± 150.5</td>
<td>2281.4 ± 100.8</td>
<td>0.9713</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loading LR Volume (ml)</td>
<td>Wt (kg) * 10 (ml/kg)</td>
<td>335.0 ± 22.1</td>
<td>335.5 ± 14.8</td>
<td>0.9712</td>
</tr>
<tr>
<td>LVR Volume (ml)</td>
<td>EBV (ml) * 0.1</td>
<td>227.8 ± 15.1</td>
<td>228.1 ± 10.1</td>
<td>0.9747</td>
</tr>
<tr>
<td>LVR Rate (ml/min)</td>
<td>LVR Vol (ml) / 5 (min)</td>
<td>45.6 ± 3.0</td>
<td>45.6 ± 2.0</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Surgery Prep Time (hr)</td>
<td>n/a</td>
<td>2.7 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>0.1482</td>
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<tr>
<td>SMA Occlusion Time (hr)</td>
<td>n/a</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
<td>n/a</td>
</tr>
<tr>
<td>Survival Time (hr)</td>
<td>n/a</td>
<td>8.14 ± 0.06</td>
<td>4.80 ± 0.98</td>
<td>*0.0005</td>
</tr>
<tr>
<td>Total Anesthesia Time</td>
<td>Intubation +</td>
<td>11.7 ± 0.3</td>
<td>8.6 ± 1.7</td>
<td>*0.0106</td>
</tr>
<tr>
<td></td>
<td>Surgery +</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Monitoring time</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2

Survival Time (Hours)

End of Study

LR Solution: 4.8

PEG-20k Solution: 8.1

* Indicates a significant difference.
Figure 3

A. Mean Arterial Pressure (MAP)

- LR
- PEG-20k

B. Heart Rate (HR)

- LR
- PEG-20k

C. Cardiac Output (CO)

- LR
- PEG-20k

D. Mixed Venous Oxygen Saturation (SvO2)

- LR
- PEG-20k

Time (min)

Ischemia | Reperfusion

Normal Range

Clamp On, Clamp Off, LVR

* Significant difference

Additional symbols and annotations as indicated in the figure.
Figure 4

A. Blood Volume Expansion by Hemoglobin Dilution Method

- **LR**
- **PEG-20k**

<table>
<thead>
<tr>
<th>Time after Resuscitation (Hrs)</th>
<th>Volume Expansion (% Change from Hb Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>-10</td>
</tr>
<tr>
<td>6</td>
<td>-20</td>
</tr>
<tr>
<td>7</td>
<td>-30</td>
</tr>
<tr>
<td>8</td>
<td>-40</td>
</tr>
</tbody>
</table>

B. Hemoglobin (Hb)

- **LR**
- **PEG-20k**

<table>
<thead>
<tr>
<th>Time after Resuscitation (Hrs)</th>
<th>Hemoglobin (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BL</td>
<td>12</td>
</tr>
<tr>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
</tr>
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<tr>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
</tr>
</tbody>
</table>

*Normal Range*

- * indicates statistically significant difference from baseline.
- † indicates statistically significant difference between groups.

DOI: 10.1124/jpet.123.001831
Figure 5

(A) Percent Perfused Capillaries (PPV)

(B) Mean Flow Index (MFI)

- **LR Control**
- **PEG-20k IV Solution**
Figure 6

SAO Shock Endotoxin

Serum Endotoxin (mEU/ml)

<table>
<thead>
<tr>
<th>Time (Hrs)</th>
<th>LR</th>
<th>PEG-20k</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significant difference compared to control.
Figure 7

(A) Histological images of gut tissue from different treatments. The left column shows baseline images, the middle column shows images taken 2 hours after resuscitation, and the right column shows images taken 4 hours after resuscitation. The comparison is between LR and PEG-20k treatments.

(B) Graph showing villus height (µM) over time after resuscitation. The x-axis represents time after resuscitation in hours (BL, 2 Hrs, 4 Hrs). The y-axis represents villus height in µM. The graph compares LR and PEG-20k treatments.
Figure 8

A  IL-6

B  IL-8

C  TNFα

Time after SAO Release

* indicates significant difference between LR and PEG-20k groups.

IL-6 (ng/ml)  LR  PEG-20k

IL-8 (ng/ml)  LR  PEG-20k

TNFα (ng/ml)  LR  PEG-20k
Supplemental Figure 1: Hepatic Laboratory Panel

Hepatic panel for laboratory blood analysis before (i.e. baseline, BL) and after SAO shock (splanchnic artery occlusion via superior mesenteric artery occlusion for 30 minutes) in LR and PEG-20k treated swine, n=4 per group. *P<0.05 denotes intergroup comparison (LR vs PEG) using multiple unpaired t-tests with the Holm-Šidák’s multiple comparisons test. †P<0.05 for LR and §P<0.05 for PEG denotes intragroup comparisons (baseline value vs repeated measure) using one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction.
Supplemental Materials, Journal of Pharmacology and Experimental Therapeutics JPET-AR-2023-001831

Title: Metabolic Tissue Swelling and Local Microcirculation in Splanchnic Artery Occlusion Shock: Implications for Critical Illness.

Loren K. Liebrecht, Jad Khoraki, Ru Li, Caitlin Archambault, John Bane, Rebecca Deitch, Michael Broadway, Erika Martin, Gerard Eldering, and Martin J. Mangino.

Supplemental Figure 2: Renal and Electrolyte Laboratory Panel

Renal and electrolyte panel for laboratory blood analysis before (i.e. baseline, BL) and after SAO shock (splanchnic artery occlusion via superior mesenteric artery occlusion for 30 minutes) in LR and PEG-20k treated swine, n=4 per group. *P<0.05 denotes intergroup comparison (LR vs PEG) using multiple unpaired t-tests with the Holm-Šidák’s multiple comparisons test. †P<0.05 for LR and §P<0.05 for PEG denotes intragroup comparisons (baseline value vs repeated measure) using one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction.
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Supplemental Figure 3: Arterial Blood Gas Laboratory Panel

Arterial blood gas laboratory analysis before (i.e. baseline, BL) and after SAO shock (splanchnic artery occlusion via superior mesenteric artery occlusion for 30 minutes) in LR and PEG-20k treated swine, n=4 per group. *P<0.05 denotes intergroup comparison (LR vs PEG) using multiple unpaired t-tests with the Holm-Šídák’s multiple comparisons test. †P<0.05 for LR and §P<0.05 for PEG denotes intragroup comparisons (baseline value vs repeated measure) using one-way analysis of variance (ANOVA) with Dunnet’s multiple comparison correction.