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Blood component resuscitative strategies to mitigate endotheliopathy in a murine hemorrhagic shock model

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Abstract

Background: Resuscitation with plasma components has been shown to improve endotheliopathy induced by hemorrhagic shock, but the optimal resuscitation strategy to preserve the endothelial glycocalyx has yet to be defined. The aim of this study was to determine if resuscitation with lactated ringers (LR), whole blood (WB), packed red blood cells (RBC), platelet rich plasma (PRP), platelet poor plasma (PPP), balanced RBC:PRP (1:1), or day 14 RBC (d14) would best minimize endothelial damage following shock.

Methods: Male C57BL/6 mice were hemorrhaged to a goal mean arterial pressure (MAP) of 25 mm Hg for one hour. Unshocked sham mice served as controls. Mice were then resuscitated with equal volumes of LR, WB, RBC, PRP, PPP, 1:1, or d14 RBC and then sacrificed at 1-, 4-, or 24-hours (n=5). Serum was analyzed for syndecan-1, ubiquitin C-terminal hydrolase L1 (UCHL-1), and cytokine concentrations. Lungs underwent syndecan-1 immunostaining and lung injury scores were calculated after H&E stains. Proteolytic cleavage of the endothelial glycocalyx was assessed by serum matrix metalloprotease 9 (MMP-9) levels.

Results: Serum syndecan-1 and UCHL-1 levels were significantly increased following resuscitation with d14 RBC compared to other groups. Early elevation in lung syndecan-1 staining was noted in LR treated mice while d14 mice showed decreased staining compared to sham mice following shock. Lung injury scores were significantly elevated 4 hours after resuscitation with LR and d14 RBC compared to WB. Serum MMP-9 levels were significantly increased at 1 and 4-hours in d14 mice compared to sham mice. Systemic inflammation was increased in animals receiving LR, 1:1, or d14 RBC.

Conclusion: Resuscitation with WB following hemorrhagic shock reduces endothelial syndecan-1 shedding and mitigates lung injury. Aged RBC and LR fail to attenuate endothelial injury following hemorrhagic shock. Further research will be necessary to determine the effect of each of these resuscitative fluids in a hemorrhagic shock model with the addition of tissue injury.

Keywords: resuscitation, endotheliopathy, syndecan-1, whole blood

BACKGROUND

Traumatic injury continues to be the leading cause of death in young Americans (1). Exsanguination from hemorrhage accounts for a large proportion of early prehospital death, including up to 40% of patients within the first hour of injury (2). However, with advances in prehospital care, tourniquet usage, and early damage control surgery, early death from hemorrhage continues to decline. Patients surviving through the first 24 hours may succumb to later death due to an acute vascular inflammatory response to hemorrhagic shock. This is known as the endotheliopathy of trauma (EOT), which encompasses the combination of systemic inflammation, endothelial injury, hypocoagulability, and eventually multisystem organ failure. Studies have demonstrated that this phenomenon begins within 5-8 minutes of initial injury (3). While it is impossible to prevent such injury completely, research efforts over the past decade have focused on mitigating EOT to reduce post-traumatic complications and mortality.

Endothelial disruption primarily affects the glycocalyx, a protective layer which consists of many glycosaminoglycans and proteins functioning to maintain endothelial integrity (4). Following traumatic tissue injury or shock, proteases such as matrix metalloprotease 9 (MMP-9) and heparanase cleave extracellular portions of the proteoglycan syndecan-1, contributing to and signaling the breakdown of the endothelial barrier (5-11). Syndecan-1 is a transmembrane heparan sulfate proteoglycan found on the surface of vascular endothelial cells in addition to other epithelial cells throughout the body (8, 12). Shed syndecan-1 may be measured in the serum as a marker of endothelial damage (5-8, 10). Heparanase has also been shown to participate in activation of the coagulation system and contribute to acute pulmonary injury (13, 14) Another biomarker that has been historically studied as a marker of traumatic brain injury

and nerve injury is ubiquitin C-terminal hydrolase L1 (UCH-L1). Recent findings have suggested that UCH-L1 levels may be associated with endotheliopathy following shock and participate in the regulation of lung vascular permeability (15, 16). Each of these biomarkers may be measured in serum or tissue homogenates to quantify the level of endothelial damage present following traumatic injury or shock.

Previous literature has demonstrated that resuscitation with balanced blood products increases survival following hemorrhagic shock by decreasing systemic inflammation and end organ dysfunction (17-19). The mechanism of this benefit remains to be fully elicited. Kozar et al. and Torres et al. have shown that resuscitation with crystalloid solutions (normal saline or lactated Ringer's) fails to restore the integrity of endothelium following traumatic injury (7, 20). It has also been demonstrated that fresh frozen plasma (FFP) and cryoprecipitate attenuate the pulmonary endothelial disruption caused by hemorrhagic shock (7, 21, 22). With whole blood becoming more available in trauma centers across the country, it is important to evaluate the effects of whole blood and its individual components on the endothelium following traumatic injury. The aim of our study was to determine whether fresh whole blood (WB), packed red blood cells (pRBC), platelet rich plasma (PRP), platelet poor plasma (PPP), balanced resuscitation (1:1), or aged stored red blood cells (d14) would mitigate endotheliopathy and acute lung injury following hemorrhagic shock. Our goal was to determine which of the components of WB were most beneficial in endothelial glycocalyx restoration following hemorrhagic shock. Crystalloid and aged RBC were included in the study as these are some of the most common but potentially deleterious products given during resuscitation. We hypothesized that fresh WB and

balanced resuscitation (1:1) would minimize the endothelial dysfunction induced by hemorrhagic shock.

METHODS

Mouse Model

The local university Institutional Animal Care and Use Committee approved all animal experimentation. The ARRIVE guideline was used to ensure proper reporting of methods, results, and discussion (Supplemental Digital Content 1, http://links.lww.com/TA/C963). Animals were 9–10-week-old male mice C57BL6/J acquired from Jackson Laboratory (Bar Harbor, Maine). Mice were acclimated for 1 week after acquisition prior to experimentation in sterile housing conditions with unlimited access to food and water. Male mice were used in order to avoid the confounding effects of the estrous cycle on the inflammatory response (23).

Prior to experimentation mice were randomized into 8 groups (n=5 per group, total 120 animals used) and survived to 1, 4, or 24 hours: sham shock, shock + lactated Ringer's (LR), shock + shed whole blood (WB), shock + red blood cells (RBC), shock + platelet rich plasma (PRP), shock + platelet poor plasma (PPP), shock + 1RBC:1PRP, and shock + day 14 (d14) RBC. Strategies employed to reduce confounding data included assimilation of animals in each study cohort in the same cage prior to experimentation. Mice that did not acutely recover after hemorrhagic shock or ambulate independently were excluded from final serum analyses (n=8) (6% overall mortality rate 8/128).

Blood Component Preparation

Mice in the shock + WB group were resuscitated with their own shed WB after 1 hour of hemorrhagic shock. Other resuscitation components were separated from WB (pooled from a separate group of male mice) on the morning of experimentation. All blood components were fresh (day 0) except for d14 RBC. Whole blood was collected from male C57BL6/J mice (9-10 weeks) via cardiac puncture. Blood was then anticoagulated with citrate phosphate double dextrose (CP2D), placed in serum separator tubes (BD Biosciences, Franklin Lakes, NJ) and centrifuged at 8000 rpm for 10 minutes. The supernatant plasma was then collected and used for the shock + PRP resuscitation group. The buffy coat was discarded and the packed RBC at the bottom of the serum separator tube were collected and pooled for the shock + RBC group. Approximately half of the collected plasma was then centrifuged a second time to remove the platelets producing PPP. Additive solution 3 (AS-3) was added to half of the collected RBC prior to storage for 14 days at 4° C to create the d14 RBC. The balanced resuscitation group (1:1) consisted of equal portions of day 0 RBC and PRP.

Hemorrhagic Shock Model

Hemorrhagic shock was induced by a pressure-controlled model utilizing a previously described protocol (18). Briefly, mice were anesthetized with 100 mg ketamine/16 mg xylazine via intraperitoneal injection. The left groin was prepped with povidone-iodine solution and alcohol then shaved allowing for exposure of left femoral vessels. A tapered polyethylene cannula (Becton Dickinson, Sparks, MD) was used to cannulate the left femoral artery and was connected to a pressure transducer for continuous hemodynamic monitoring. Mice were bled to a mean arterial pressure of 25 $\pm$ 5 mmHg for 1 hour and subsequently resuscitated to a mean

arterial pressure of 80 mmHg with either LR, WB, RBC, PRP, PPP, 1:1, or d14 RBC based upon experimental group allocation over a 30-minute period. Mice were then placed into a warmed recovery area until found to be eating, drinking, and ambulating independently. Sham shock mice were anesthetized and cannulated as described above but did not undergo controlled hemorrhagic shock.

Serum Biomarker Evaluation

Mice were sacrificed at 1, 4, or 24 hours for serum biomarker analysis. Mice were anesthetized with 100mg ketamine/16mg xylazine via intraperitoneal injection and whole blood was collected via sterile 22-gauge needle cardiac puncture. Whole blood was subsequently placed into serum separator tubes (BD Bioscience, San Diego, California) and centrifuged at 1000g for 10 minutes. Serum was then placed in -80°C freezer until further evaluation. Serum was analyzed for syndecan-1 and UCHL-1, known biomarkers of endothelial damage (My BioSource, San Diego, California) (24). Serum was also analyzed for MMP-9 by ELISA (R&D Systems, Minneapolis, MN) and inflammatory cytokines including interleukin 1a (IL-1a), interleukin 1b (IL-1b), interleukin 2 (IL-2), interleukin 3 (IL-3), interleukin 4 (IL-4), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 12 (IL-12), interleukin 17 (IL-17), monocyte chemoattractant protein 1 (MCP-1), tumor necrosis factor alpha (TNFα), macrophage inflammatory protein 1a (MIP-1a), granulocyte-macrophage colony-stimulating factor (GM-CSF), and regulated upon activation, normal T cell expressed and presumably secreted (RANTES) using a multiplex ELISA (Quansys, Logan, UT).

Pulmonary Immunohistochemistry

After euthanasia, whole lungs were harvested as previously described. Briefly, the left lung was immediately fixed at room temperature using 10% neutral buffered formalin (Thermo Scientific, Waltham, MA). Tissue was processed and embedded in paraffin for light microscopy. Lung tissues were cut to 5 µm sections and placed on glass slides. Antigen retrieval was performed via heat induced epitope retrieval in 1x citrate buffer (Thermo Scientific, Waltham, MA). Sections were blocked with ready to use Image IT-Fx signal enhancer (Invitrogen, Waltham, MA) for 30 minutes prior to washing (x2) with 1x TBS-T buffer (Thermo Scientific, Waltham, MA) for 5 minutes each. Sections were then blocked with 10% normal goat serum (Jackson Immuno Research, West Grove, PA), 0.3% Triton X 100, and PBS solution for 90 minutes at room temperature. Next, sections were incubated overnight at 4° C with a primary monoclonal antibody to murine syndecan-1 (1:100, rat anti-mouse CD138, BD Biosciences, Franklin Lakes, NJ) in a 5% normal goat serum (Jackson Immuno Research, West Grove, PA), and PBS-T solution. Sections are then washed x2 and incubated at room temperature with a goat anti-rat Alexa Fluor 594 secondary antibody (1:500, Life Technologies, Calsbad, CA) in a 1% normal goat serum (Jackson Immuno Research, West Grove, PA) and PBS-T solution. Lastly, slides were cover slipped using Vectashield Vibrance with DAPI (Vector Laboratories, Burlingame, CA) mounting media. A total of four random images of each slide were obtained using imaging software ZEN2012 version 1.1.2.0 on Axio Imager M2 microscope (Carl Zeiss AG, Jena, Germany). Images were taken at 40X magnification and analyzed using the image analysis software ImageJ version 1.49 (Wayne Rasband, National Institutes of Health, USA). Images were analyzed for total fluorescent cell intensity and background cell intensity. After obtaining these values the calculation of corrected total cell fluorescence or (CTCF) was

obtained via CTCF = integrated density- [area of selected cell x mean fluorescence of background readings] as previously described (25). Researchers who performed imaging and analysis were blinded to the animal groups being evaluated to ensure elimination of bias.

Lung Injury Score

Lung injury was estimated based upon hematoxylin and eosin-stained sections of the left lung. A previously published novel lung injury score was utilized, which scored lungs according to the following four items: alveolar congestion, hemorrhage, infiltration, or aggregation of neutrophils in airspace or vessel wall, and thickness of alveolar wall/hyaline membrane formation (26, 27). An overall score of lung injury was obtained from reviewers blinded to the treatment groups based upon the summation of all the scores with minimum score of 0 and a maximum score of 16.

Statistical Analysis

The determination of sample size was based on the primary outcome of serum syndecan-1 based off our previous investigation of hemorrhagic shock. We estimated that the serum syndecan-1 levels would be 50% lower in whole blood compared to LR resuscitated animals with a 33.3% variance, so that a minimum sample size of 4 mice per groups was utilized (28). Other outcomes measured included serum UCHL-1 concentration, total serum MMP-9 concentration, pulmonary syndecan-1 levels, lung injury score, and serum cytokine levels. All statistical analyses were performed with Prism 9 (GraphPad Software, La Jolla, California). One way ANOVA analyses along with Tukey's multiple comparisons test were performed to compare cytokine concentrations between groups. To confirm significance an un-paired

student's t test was performed. Shapiro-Wilk test was utilized to confirm that data were normally distributed. Because the power analysis and sample size were based on a p-value for significance of ≤ 0.05, to minimize type 2 error, no corrections were made for multiple comparisons. A p value less than 0.05 was considered significant. All members of the research team were aware of treatment groups except lung injury score reviewers.

RESULTS

Whole blood and red blood cell resuscitation significantly reduce serum syndecan-1 release after hemorrhagic shock.

Syndecan-1 levels were significantly elevated in shock+1:1, shock+RBC, and shock+LR compared to sham shock mice (**Figure 1A**). Serum concentrations of syndecan-1 were significantly elevated in shock+d14 mice compared to sham shock, shock+LR, shock+WB, shock+RBC, shock+PRP, and shock+PPP mice at the 1-hour timepoint (**Figure 1A**). At 4-hours after shock, mice resuscitated with LR, 1:1, and d14 blood demonstrated significantly elevated serum syndecan-1 levels compared to sham shock animals (**Figure 1B**). Interestingly, reductions in serum syndecan-1 were also noted in shock+RBC mice compared to shock+PRP and shock+1:1 mice at 4-hours (**Figure 1B**). Those animals resuscitated with 1:1 also had elevated syndecan-1 levels compared to those resuscitated with RBC alone. Mice resuscitated with LR continued to have persistently elevated serum syndecan-1 levels at 24 hours compared to sham shock mice. (**Figure 1C**).

Serum UCHL-1 is increased with LR and d14 RBC resuscitation at 24 hours post-shock

Serum UCH-L1 was significantly elevated in mice receiving d14 RBCs compared to all other groups at 4-hours post-hemorrhagic shock (**Figure 2B**). UCH-L1 was also significantly elevated in the 1:1 group compared to the fresh WB group at 4-hours (**Figure 2B**). UCH-L1 levels continued to increase in all groups except sham, RBC, and 1:1 by 24 hours post-hemorrhagic shock (**Figure 2C**).

Pulmonary syndecan-1 shedding is increased with d14 and 1:1 resuscitation

One hour after shock, pulmonary vascular concentrations of syndecan-1 were significantly elevated within shock+LR lungs compared to the lungs of sham shock, shock+RBC, shock+PRP, shock+PPP, shock+1:1, and shock+d14 resuscitated mice (**Figure 3A**). Sham shock mice had significantly elevated pulmonary syndecan-1 compared to those resuscitated with d14 RBC at 1 hour. (**Figure 3A**). Four hours after shock, pulmonary endothelial concentrations of syndecan-1 were significantly lower in shock+LR, shock+WB, and shock+RBC compared to sham shock (**Figure 3B**). By 24 hours after shock, mice in the shock+PPP cohort displayed a significant elevation in pulmonary syndecan-1 compared to sham shock, shock+PRP, and shock+1:1 resuscitation groups (**Figure 3C**). Similarly, mice within shock+d14 cohort displayed significant elevation in pulmonary syndecan-1 expression 24 hours after shock compared to shock+PRP, and shock + 1:1 (**Figure 3C**). Animals resuscitated with 1:1 demonstrated decreased pulmonary endothelial syndecan-1 compared to those given WB or LR at 24 hours post-shock (**Figure 3C**).

MMP-9 levels are increased after d14 RBC resuscitation

Total serum MMP-9 was acutely elevated by 1 hour in mice resuscitated with d14 RBC compared to sham, LR, WB, RBC, PRP, PPP, 1:1 resuscitated mice (**Figure 4A**). The elevation in MMP-9 persisted to 4 hours in the d14 group compared to sham, LR, WB, and PPP groups (**Figure 4B**). By 24 hours post-injury, no groups demonstrated significant MMP-9 elevation compared to sham shock mice (**Figure 4C**).

Lung injury severity score decreased with whole blood resuscitation

Four hours after shock mice in the shock+WB cohort displayed a significant decrease in lung injury severity score compared to shock+PRP, shock+PPP, shock+1:1 and shock+d14 (**Figure 5A**). Notably in this group however, Shock+LR resuscitation did not demonstrate a higher lung injury than sham shock mice.

Systemic inflammation is increased with aged blood resuscitation

There were several additional nonsustained serum cytokine differences between resuscitation groups. IL-3 was acutely elevated in mice receiving d14 RBCs compared to 1:1 ($p<0.05$) resuscitation at 1-hour post-injury (Supplemental Digital Content 2, http://links.lww.com/TA/C964). IL-4 was significantly increased in mice treated with WB compared to pRBC ($p<0.05$) and 1:1 ($p<0.05$) resuscitation at 1-hour post-shock (Supplemental Digital Content 2, http://links.lww.com/TA/C964). Sham shock mice were noted to have significantly less serum IL-10 compared to LR ($p<0.01$), RBC ($p<0.05$), PRP ($p<0.01$), PPP ($p<0.01$), 1:1 ($p<0.05$), and d14 ($p<0.01$) mice at 1 hour (**Figure 6D**). MIP-1α (vs. sham, LR, RBC, PPP, 1:1, $p<0.0001$; vs. PRP, $p<0.001$; vs. WB, $p<0.01$) and TNF-α (vs. sham, LR, WB,

RBC, p<0.001); vs. PPP, 1:1, p<0.01) both demonstrated elevation at 1 hour post shock in mice resuscitated with d14 RBCs (**Figure 6A,** Supplemental Digital Content 2, http://links.lww.com/TA/C964). IL-17 was elevated in RBC resuscitated mice at 1 hour compared to LR (p<0.05), PPP (p<0.01), and d14 RBC (p<0.05) resuscitation (Supplemental Digital Content 2, http://links.lww.com/TA/C964).

Serum cytokine analysis revealed significant elevation of IL-1a (vs. all groups, p<0.0001), IL-1b (vs. all groups, p<0.0001), IL-12 (vs. all groups, p<0.05), TNF-α (vs. all groups, p<0.01), MIP-1α (vs. sham, LR, WB, RBC, PPP, 1:1, d14, p<0.001) in mice resuscitated with d14 RBCs at 4 hours post-injury (**Figure 6A, 6C,** Supplemental Digital Content 2, http://links.lww.com/TA/C964). Mice resuscitated with PPP were noted to have increased IL-10 at 4 hours post injury compared to sham shock (p<0.01), 1:1 (p<0.05), and RBC (p<0.05) (**Figure 6D)**. Resuscitation with d14 RBCs led to increased IL-10 compared to sham shock (p<0.05) mice at 4 hours (**Figure 6D**). RANTES was decreased in LR treated mice at 4 hours compared to PRP (p<0.01), 1:1 (p<0.05), and d14 (p<0.001) treatment (Supplemental Digital Content 2, http://links.lww.com/TA/C964**)**.

By 24 hours after shock, a significant decrease in serum IL-6 was noted in the sham, pRBC, and 1:1 resuscitation groups compared to the PPP and LR groups (**Figure 6B, p<0.05**). Resuscitation with pRBC lead to a significant increase in IL-2 at 24 hours compared to all other groups (p<0.001) (Supplemental Digital Content 2, http://links.lww.com/TA/C964). An elevation in RANTES was observed in LR resuscitated mice compared to sham (p<0.0001), RBC (p<0.0001), 1:1 (p<0.0001), WB (p<0.05) at 24 hours (Supplemental Digital Content 2,

http://links.lww.com/TA/C964**).** RANTES was also elevated in PPP resuscitated mice compared to sham ($p<0.05$) and RBC ($p<0.05$) at 24-hours post-shock (Supplemental Digital Content 2, http://links.lww.com/TA/C964).

DISCUSSION

The goal of this study was to determine the optimal resuscitation fluid for mitigating endotheliopathy following hemorrhagic shock. Serum analysis revealed that resuscitation with fresh WB or RBC leads to reduced syndecan-1 shedding. UCH-L1 elevation is persistently increased in mice resuscitated with d14 RBCs following hemorrhagic shock. Serum MMP-9 levels were significantly increased at 1 and 4-hours in shock+d14 mice compared to sham mice. Mice resuscitated with fresh WB demonstrated the lowest lung injury scores while those receiving aged RBCs had the highest scores. Lastly, systemic inflammation was increased in animals receiving LR, 1:1, or aged RBC. Taken together, these findings suggest an endothelial benefit when resuscitating with fresh WB and significant disadvantage in administering LR or d14 RBC.

Syndecan-1 shedding was found to be worse in mice resuscitated with LR, 1:1, and d14 RBC. Resuscitation with WB and fresh RBC resulted in decreased serum syndecan-1 levels. These findings correlate with previous work by Barry et. al. which demonstrated an increase in syndecan-1 shedding in mice resuscitated with LR compared to FFP or cryoprecipitate (21, 22). Torres et. al. demonstrated reduced glycocalyx degradation when using blood products as compared to crystalloid or colloid fluids for resuscitation (19). The thickness of the glycocalyx correlated inversely with heparan sulfate and syndecan-1 serum levels (19). Although we did not

assess the glycocalyx directly by microscopy, serum syndecan-1 levels as well as lung injury scores would support the finding that fresh blood product resuscitation causes less endothelial damage than crystalloid or aged blood product resuscitation. Our results correlate with previous microfluidics work by Diebel and Liberati which demonstrated increased glycocalyx degradation when aged RBC were administered compared to fresh RBC (29). This effect has been hypothesized to be caused by increased breakdown in the glycocalyx of RBC during storage. Another biomarker, MMP-9, participates in the endotheliopathy process by cleaving the extracellular domain of syndecan-1 to release it from the endothelial glycocalyx to the serum (9). Upon evaluation of serum MMP-9, we found that mice resuscitated with d14 RBC had increased concentrations at 1- and 4- hours compared to sham mice. This early MMP-9 elevation, along with the trend in 24-hour MMP-9 levels correlating with serum syndecan-1 levels, further supports our findings. Early MMP-9 elevation has been associated with respiratory failure in COVID patients further emphasizing that increased MMP-9 contributes to or is at least associated with acute lung injury after shock. (30).

The evaluation of pulmonary syndecan-1 staining did not inversely correlate with serum syndecan-1 levels as expected in our study. This may be due to the presence of syndecan-1 on epithelial cells as well as endothelial cells. We were specifically anticipating pulmonary endothelial damage following hemorrhagic shock. The alveoli consist of epithelial cells which have also been shown to stain for syndecan-1, potentially confounding our results (8, 12). When evaluating H&E stains, lung injury scoring demonstrated a benefit in fresh WB resuscitation compared to PRP, PPP, 1:1, and d14 RBC resuscitation at 4-hours post injury. Potter et. al. has previously demonstrated increased vascular permeability and endothelial breakdown within the

lungs of mice treated with LR (31). Our study revealed worsened lung injury in mice resuscitated with LR at 4 hours post-shock, however this was not statistically significant. Although our results do not specifically support the previous finding of acute lung injury with LR administration, the results of this study did demonstrate the benefit of fresh WB in mitigating lung injury following hemorrhagic shock. These findings are supported by previous work demonstrating decreased alveolar thickening following hemorrhagic shock when resuscitated with WB instead of crystalloid (32). When assessed as a whole, these results all support the conclusion that fresh WB mitigates EOT while LR and aged blood products worsen endothelial damage following hemorrhagic shock. In addition, the findings of this study suggest that RBCs may be more critical to the preservation of endothelial integrity than the plasma products.

Although UCH-L1 has been previously studied with neuronal and central nervous system injury, there is recent evidence to suggest that this biomarker is indicative of endotheliopathy, ischemia, and pulmonary vascular permeability (15, 16, 28). Limited research has been completed regarding the role of UCH-L1 in endothelial damage, however Mitra et. al. found that this biomarker may be protective for vascular permeability within the lungs (16). Our results support this correlation as the mice resuscitated with 1:1 and d14 RBCs had higher levels of UCH-L1 by 24 hours post-shock. This may be a response to worsened lung endothelial injury as those groups also had higher syndecan-1, MMP-9 levels, and worsened lung injury scores. UCH-L1 levels continued to increase through the 24-hour timepoint for most groups suggesting that this is a response to hemorrhagic shock or acute lung injury. Further research is necessary to determine the specific relationship between this biomarker and endothelial dysfunction (15).

The relationship of serum and tissue syndecan-1 to pro- and anti-inflammatory cytokines following hemorrhagic shock has yet to be definitively and consistently defined across species. Previous research by Haywood-Watson et. Al. demonstrated IL-10 to be negatively correlated with serum syndecan-1 levels while IL-1b was positively correlated (33). Our results are similar, demonstrating reduced IL-1b in LR treated mice at 1 hour when syndecan-1 is increased. We also found the relationships between groups for IL-10 were similar to the relationship between groups with syndecan-1 levels, especially at 24 hours, suggesting that there may be a relationship between this specific cytokine and endothelial degradation. IL-1b is a pro-inflammatory cytokine while IL-10 is anti-inflammatory. Syndecan-1 may reduce the systemic pro-inflammatory effects following traumatic injury by inducing production of IL-10 (33). IL-6 has been previously demonstrated to cause local infiltration of polymorphonuclear nucleophilic granulocytes (PMN) within the lung parenchyma while exhibiting anti-inflammatory effects within the systemic circulation (34). A study by Meng et. al. found that the pro-inflammatory effects of IL-6 within the pulmonary tissue dominate over the anti-inflammatory effects systemically (35). We did not specifically analyze cytokine levels within the lung tissue, but our study did show increased systemic IL-6 in mice resuscitated with LR, PPP, and d14 RBCs at 24 hours post-shock. Overall, systemic inflammation was consistently exacerbated in mice resuscitated with d14 RBCs.

Our results would suggest that fresh WB, RBC, or 1:1 resuscitation are superior in promoting recovery of the endothelial glycocalyx layer following hemorrhagic shock. These data correlate with clinical studies demonstrating the benefit of administering fresh WB in trauma. McCoy and colleagues have demonstrated that WB resuscitation is equivalent or better than balanced blood product resuscitation (36). Cotton et al. demonstrated in a small randomized

controlled pilot trial that WB administration reduced transfusion volumes compared to component therapy (37). Another recent study by Torres et al. found that administration of WB as an adjunct to massive transfusion led to decreased mortality compared to those that received component therapy alone (38). These results further support our findings that WB is beneficial in restoring the endothelial glycocalyx leading to fewer post-traumatic complications and reduced mortality.

Our study does have limitations which must be addressed. Previous research by Kozar et. al. and others included both a hemorrhagic shock and tissue injury component whereas this study only focuses on the hemorrhagic shock aspect of injury, with the only tissue injury being the cutdown on the femoral vessels. This may contribute to the severity of endotheliopathy as previous work in our laboratory has demonstrated tissue injury is strongly associated with systemic endothelial disruption (28). Second, the sham shock groups in our study still underwent femoral artery cutdown and cannulation. This tissue injury, although mild, could have caused some measurable endothelial dysfunction. Third, we utilized 1-, 4-, and 24-hour timepoints focusing on the acute effects of endotheliopathy. Our study does not comment on the lasting effects of endothelial damage depending on which resuscitation fluid was administered. Previous research has suggested that recovery time of the endothelial barrier may require 5-7 days (31). Fourth, while mice in shock groups were all shocked to a mean arterial pressure of 25 mmHg, each mouse received a different volume of resuscitation to return to 80 mmHg. Varying volumes of resuscitative fluid may impact the biomarker levels observed in the serum. Fifth, while we examined pulmonary histochemistry as well as systemic serum changes, we did not directly evaluate glycocalyx integrity as other studies have completed in the past. Finally, these

initial studies were performed in male mice and will need to be replicated in models of female mice as well as in polytrauma models including more intentional tissue injury.

In conclusion, fresh WB may be the optimal resuscitation fluid to administer for mitigating the endotheliopathy of trauma. Aged RBC and LR crystalloid fluid fail to attenuate endothelial injury following hemorrhagic shock. Based on this study and previous work regarding the benefits of plasma resuscitation, fresh WB, fresh RBC or 1:1 is preferred following hemorrhagic shock instead of LR or aged blood products. Future efforts will include the addition of a laparotomy and rectus muscle crush tissue injury to further simulate polytrauma instead of hemorrhagic shock alone. We plan to evaluate each of the resuscitation fluids to determine if the addition of tissue injury changes the benefits observed with whole blood, and how the benefit of RBCs compares to that of plasma in establishing resuscitation algorithms.

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Figure Legends

Figure 1: Serum syndecan-1 levels (n=5 per group at each timepoint) post-hemorrhagic shock and resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: A) 1-hour: sham vs. 1:1 (p<0.01), sham vs LR, RBC (p<0.05), d14 vs. sham (p<0.0001), d14 vs. WB, PRP, PPP (p<0.01), d14 vs. LR, RBC (p<0.05). B) 4-hour: sham vs. 1:1 (p<0.001), sham vs. LR (p<0.01), sham vs. d14 (p<0.05), RBC vs. 1:1 (p<0.01). C) 24-hour: sham vs. LR (p<0.05).

Figure 2: Serum UCHL-1 levels (n=5 per group at each timepoint) post-hemorrhagic shock and resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: A) 1-hour. B) 4-hour: d14 vs. sham, LR, WB, RBC, PPP, PRP (p<0.0001), d14 vs. WB (p<0.05), d14 vs. 1:1 (p<0.001). C) 24 hour.

Figure 3: Pulmonary syndecan-1 corrected total cell fluorescence (n=5 per group at each timepoint) post-hemorrhagic shock and resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: A) 1-hour: LR vs. RBC, PRP, PPP, 1:1, d14 (p<0.0001), LR vs. sham (p<0.05), d14 vs. sham, WB (p<0.0001). B) 4-hour: sham vs. LR, WB (p<0.01), sham vs. RBC (p<0.05). C) 24-hour: PPP vs. 1:1 (p<0.001), PPP vs. PRP (p<0.01), d14 vs. 1:1 (p<0.01), 1:1 vs. LR, WB (p<0.05), PPP vs. sham (p<0.05), PRP vs. d14 (p<0.05).

Figure 4: Serum MMP-9 levels (n=5 per group at each timepoint) post-hemorrhagic shock and resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: A) 1-hour: sham vs. d14 (p<0.01), d14 vs. LR (p<0.05), d14 vs. WB (p<0.05), d14 vs. RBC (p<0.05), d14 vs. PRP (p<0.05), d14 vs. PPP (p<0.01), d14 vs. 1:1 (p<0.01). B) 4-hours: d14 vs. sham (p<0.0001), d14 vs. LR, WB, PPP (p<0.001) C) 24-hours.

Figure 5: A) Lung injury scores (n=5 per group at each timepoint) at 4-hours post hemorrhagic shock/resuscitation and scoring rubric. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: WB vs PRP (p<0.01), PPP (p<0.05), 1:1 (p<0.01), d14 (p<0.01) B) H&E lung staining of sham shock mice at 4-hours. C) H&E lung staining of shock+LR mice at 4-hours. D) H&E lung staining of shock+1:1 mice at 4-hours. E) H&E lung staining of shock+d14 mice at 4-hours.

Figure 6: Serum analysis (n=5 per group at each timepoint) of A) TNFα, B) IL-6, C) IL-1β, and D) IL-10 at 1-, 4-, and 24-hours post hemorrhagic shock/resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: A) TNFα: 1-hour; d14 vs. PPP (p<0.0001), d14 vs. sham (p<0.001), d14 vs. RBC (p<0.01), d14 vs. LR, PRP (p<0.05). 4-hour; d14 vs. Sham, LR, WB, RBC (p<0.001), d14 vs. PPP, 1:1 (p<0.01). B) IL-6: 24-hour; Sham vs. LR, PPP (p<0.05), RBC vs. LR, PPP (p<0.05), 1:1 vs. LR, PPP (p<0.05). C) IL-1β: 1-hour; d14 vs. all groups (p<0.0001) D) IL-10: 1-hour; sham vs. LR, PRP, PPP, d14 (p<0.01), sham vs. RBC, 1:1 (p<0.05). 4-hour; PPP vs. sham (p<0.01), PPP vs. RBC, 1:1 (p<0.05), sham vs. d14 (p<0.05).

Supplemental Digital Content:

SDC 1. ARRIVE checklist

SDC2. Serum analysis of IL-1α, IL-2, IL-3, IL-4, IL-12, IL-17, MCP-1, MIP-1α, GM-CSF, and RANTES at 1-, 4-, and 24-hours post hemorrhagic shock/resuscitation. Horizontal lines represent significant (p<0.05) differences between denoted groups as follows: IL--1α: 1-hour; d14 vs. all groups (p<0.0001). IL-2: 24-hours; RBC vs. all groups (p<0.0001). IL-3: 1-hour; 1:1 vs. d14 (p<0.05). IL-4: 1-hour; WB vs. RBC, 1:1 (p<0.05). IL-12: 1-hour; RBC vs. d14 (p<0.05). 4-hour; d14 vs. RBC, 1:1 (p<0.001), d14 vs. PPP (p<0.01), d14 vs. sham, LR, WB, PRP (p<0.05). IL-17: 1-hour; RBC vs. PPP (p<0.01), RBC vs. LR, d14 (p<0.05). MCP-1: 1-hour; PPP vs. d14 (p<0.0001), PPP vs. sham (p<0.001), PPP vs. WB (p<0.01), d14 vs. LR, RBC (p<0.01), d14 vs. 1:1 (p<0.05). MIP-1α: 1-hour; d14 vs. sham, LR, RBC, PPP, 1:1 (p<0.0001), d14 vs. PRP (p<0.001), d14 vs. WB (p<0.01), WB vs. PPP (p<0.05). 4-hour; d14 vs. sham, LR, WB, RBC, 1:1 (p<0.0001), d14 vs. PPP (p<0.001). RANTES: 4-hour; LR vs. d14 (p<0.001), LR vs. PRP (p<0.01), LR vs 1:1 (p<0.05), WB vs. PRP (p<0.05). 24-hour; LR vs. sham, RBC, 1:1 (p<0.0001), LR vs. WB (p<0.05), PPP vs. sham, RBC (p<0.05).

Figure 1

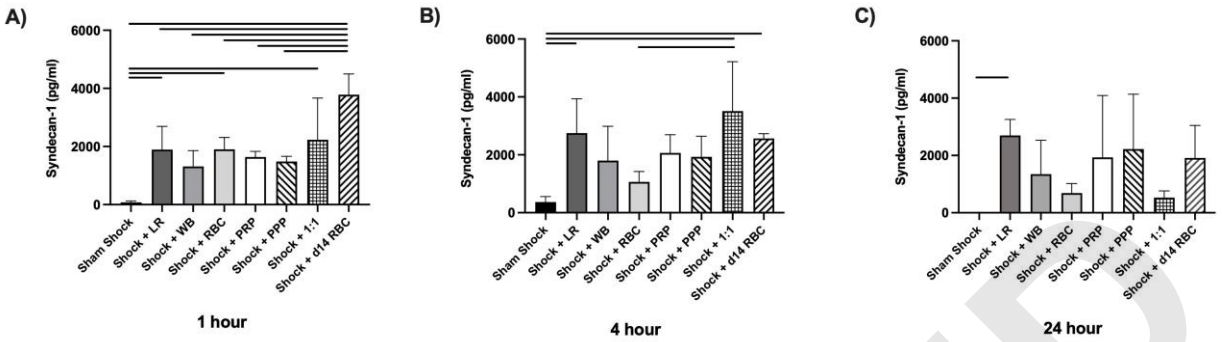


Figure 2

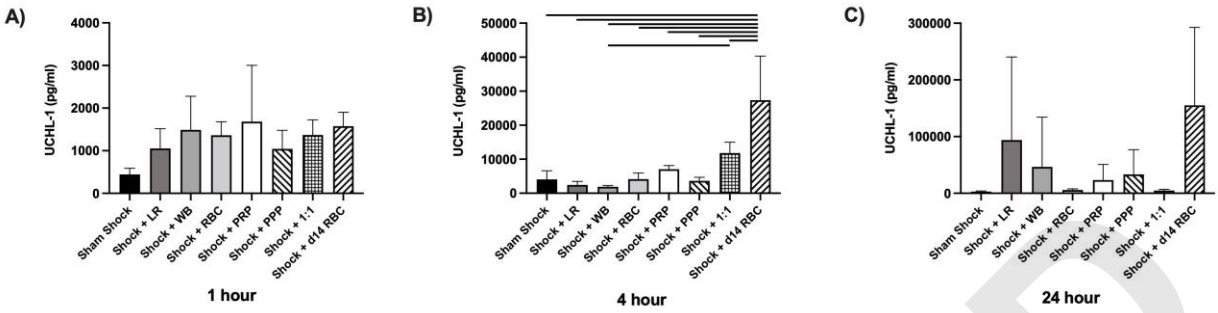


Figure 3

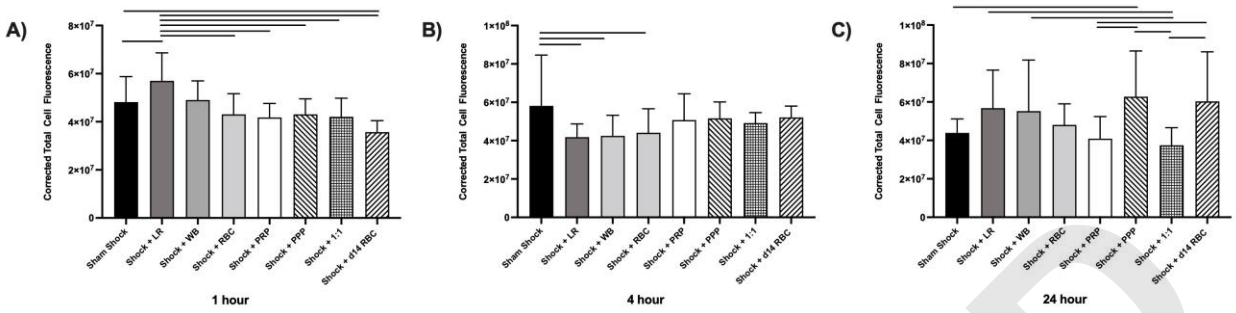


Figure 4

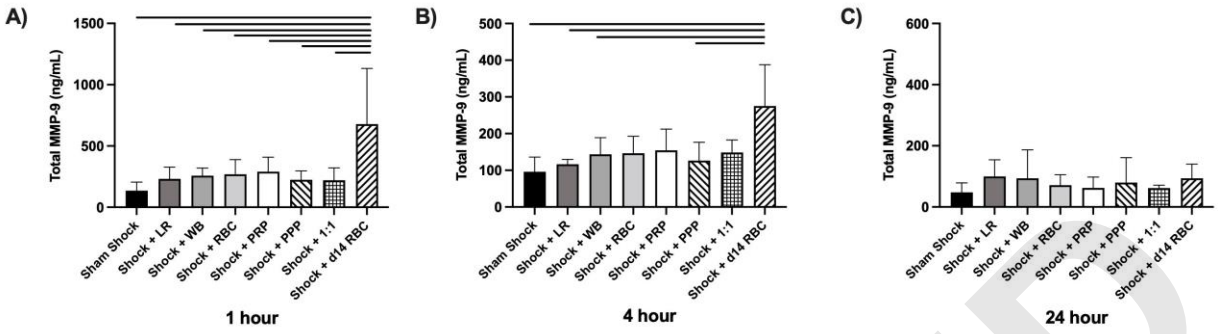
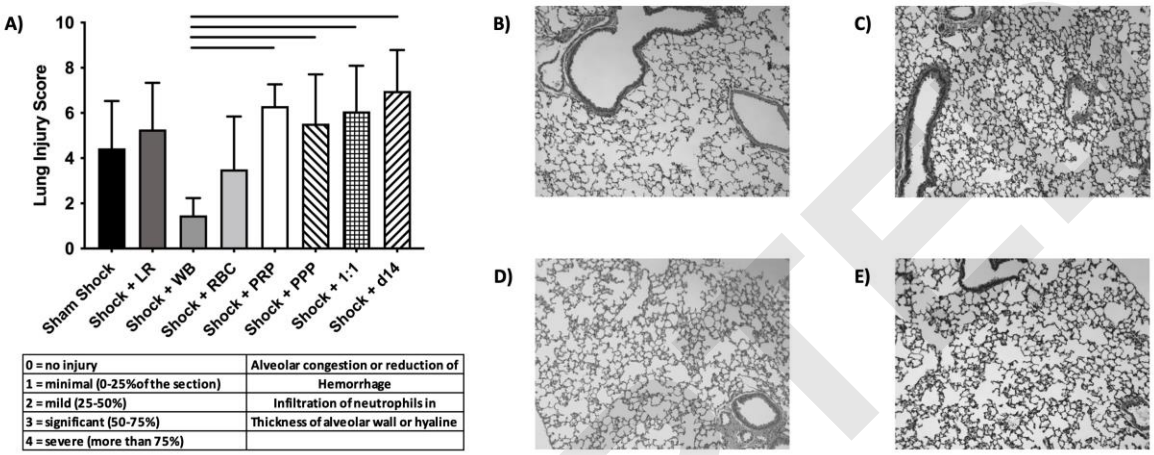
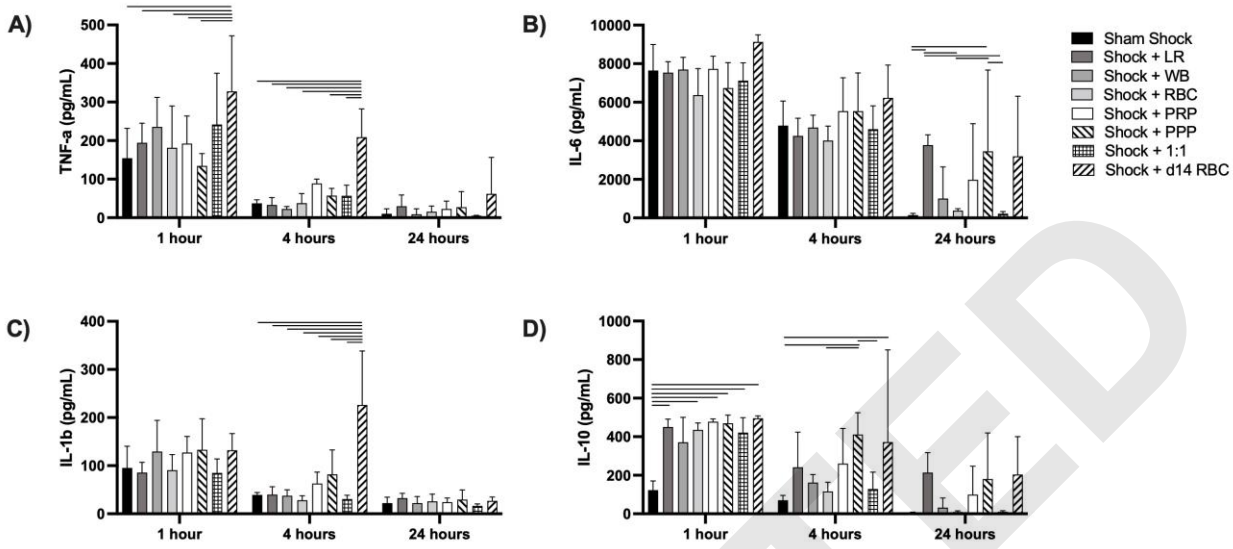


Figure 5



0 = no injury	Alveolar congestion or reduction of
1 = minimal (0-25% of the section)	Hemorrhage
2 = mild (25-50%)	Infiltration of neutrophils in
3 = significant (50-75%)	Thickness of alveolar wall or hyaline
4 = severe (more than 75%)	

Figure 6



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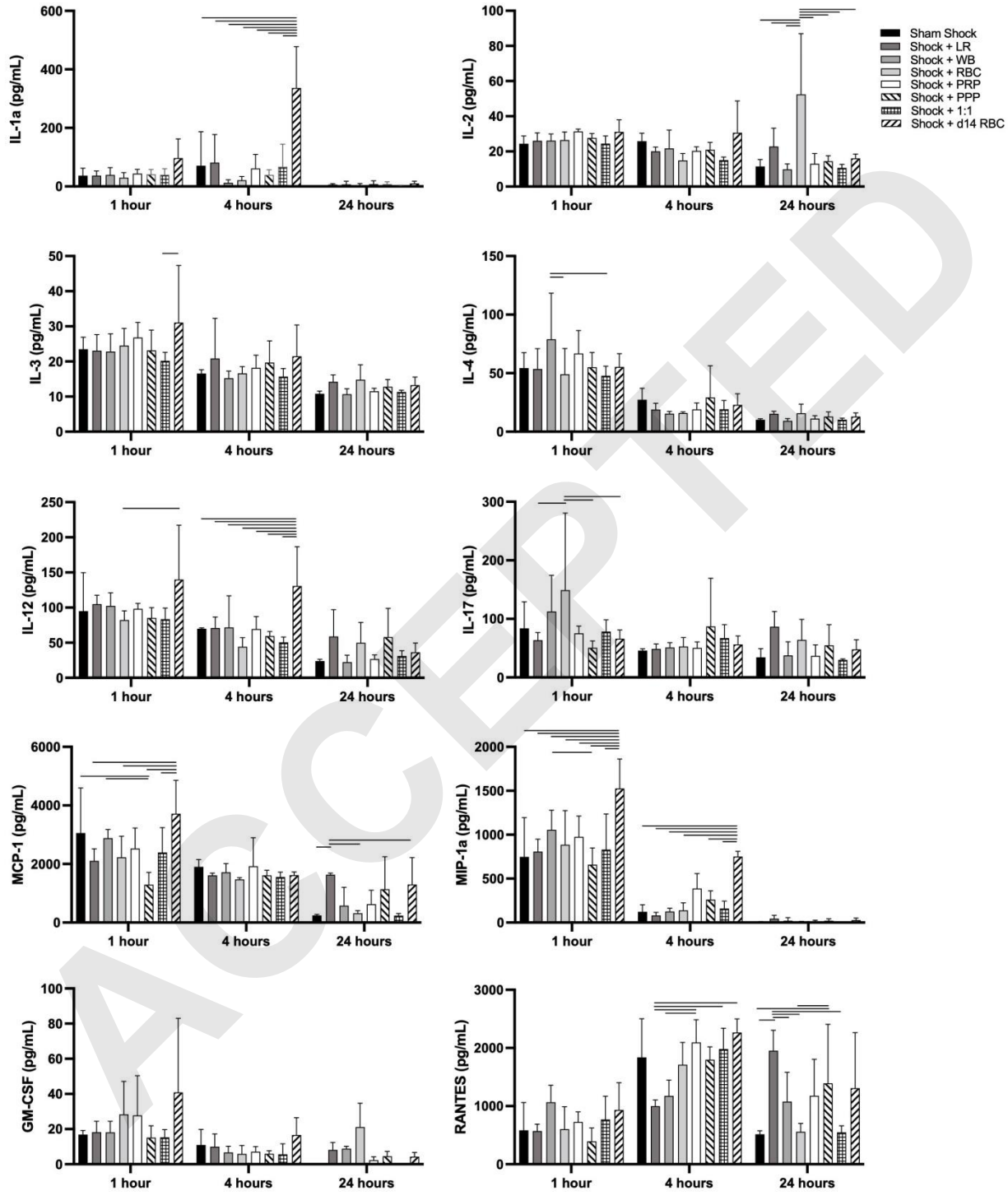
The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

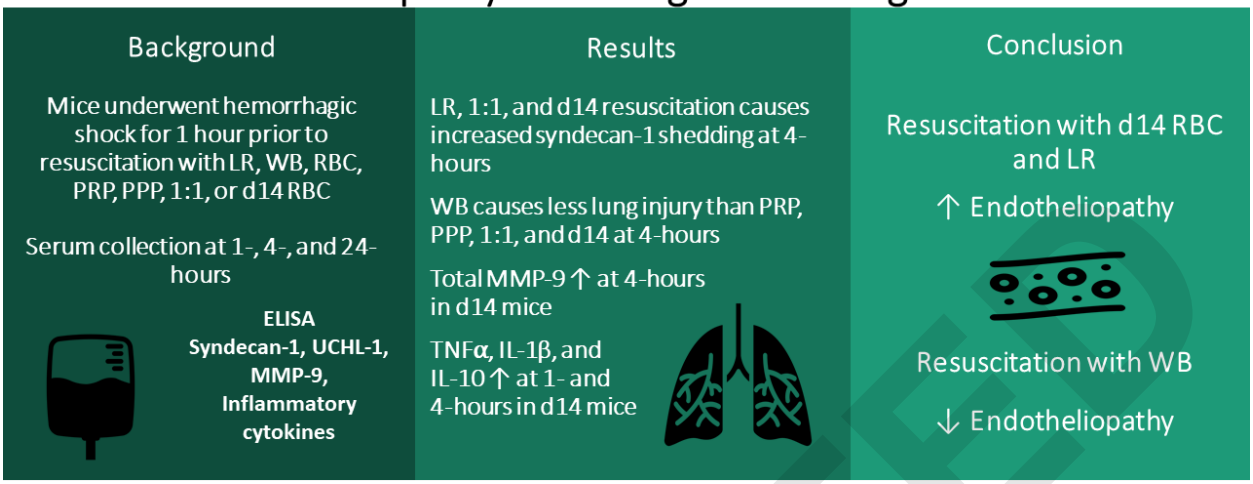
These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including:	Methods/Page 3 - line 3
		a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated.	Methods/Page 3- line 3
		b. The experimental unit (e.g. a single animal, litter, or cage of animals).	
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used.	Methods/Page 3- line 3
		b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	Methods/Page 6 line 11
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori*. If no criteria were set, state this explicitly.	Methods/Line 194
		b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so.	Methods/Page 3 Line 9
		c. For each analysis, report the exact value of *n* in each experimental group.	Methods/Page 3 Line 3
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence.	Methods/Page 3 Line 3
		b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Methods/Page 3 Line 7
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	Methods/Page 6 Line 20
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes).	Methods/Page 6 Line 14
		b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	Methods/Page 6 Line 10
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used.	Methods/Page 6 Line 15
		b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	Methods/Page 6 Line 18
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight.	Methods/Page 2 Line 23
		b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	N/A
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including:	Methods/Page 3-6
		a. What was done, how it was done and what was used.	Methods/Page 3-6
		b. When and how often.	Methods/Page 3-6
		c. Where (including detail of any acclimatisation periods).	Methods/Page 3-6
		d. Why (provide rationale for procedures).	
Results	10	For each experiment conducted, including independent replications, report:	Page 7-10
		a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range).	N/A
		b. If applicable, the effect size with a confidence interval.	

SDC



Blood Component Resuscitative Strategies to Mitigate Endotheliopathy Following Hemorrhagic Shock



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