Fresh frozen plasma and spray-dried plasma mitigate pulmonary vascular permeability and inflammation in hemorrhagic shock

Daniel R. Potter, PhD, Gail Baimukanova, MD, PhD, Sheila M. Keating, PhD, Xutao Deng, PhD, Jeffrey A. Chu, Stuart L. Gibb, PhD, Zhanglong Peng, PhD, Marcus O. Muench, PhD, Marina E. Fomin, PhD, Philip C. Spinella, MD, Rosemary Kozar, MD, PhD, *and* **Shibani Pati, MD, PhD,** *San Francisco, California*

BACKGROUND: In retrospective and prospective observational studies, fresh frozen plasma (FFP) has been associated with a survival benefit in massively transfused trauma patients. A dry plasma product, such as spray-dried plasma (SDP), offers logistical advantages over FFP. Recent studies on FFP have demonstrated that FFP modulates systemic vascular stability and inflammation. The effect of SDP on these measures has not been previously examined. This study compares SDP with FFP using in vitro assays of endothelial function and in vivo assays of lung injury using a mouse model of hemorrhagic shock (HS) and trauma.

METHODS: FFP, SDP, and lactated Ringer's (LR) solution were compared in vitro using assays of endothelial cell (EC) permeability, cytokine production and content, gene expression, as well as tight and adherens junction stability. All resuscitation products were also compared in a murine model of HS. Mean arterial pressures and physiologic measures were assessed. Pulmonary vascular permeability was measured using tagged dextran. Lung tissues were stained for CD68, VE-cadherin, and occludin.

RESULTS: Treatment of ECs with FFP and SDP, but not LR, preserved the integrity of EC monolayers in vitro and resulted in similar EC gene expression patterns and cytokine/growth factor production. FFP and SDP also reduced HS-induced pulmonary vascular permeability in vivo to the same extent. In mice with HS, mean arterial pressures and base excess were corrected by both FFP and SDP to levels observed in sham-treated mice. Treatment after HS with FFP and SDP but not LR solution reduce alveolar wall thickening, leukocyte infiltration, and the breakdown of EC junctions, as measured by staining for VE-cadherin, and occludin.

CONCLUSION: Both FFP and SDP similarly modulate pulmonary vascular integrity, permeability, and inflammation in vitro and in vivo in a murine model of HS and trauma. (*J Trauma Acute Care Surg*. 2015;78: S7–S17.

KEY WORDS: Hemorrhagic shock; lung injury; vascular permeability; spray-dried plasma; mice.

Trauma is the leading cause of death in the United States for individuals between the age of 1 and 44 years.[1] Approximately 90,000 people die each year from traumatic injury in the United States alone. In recent years, improved methods to stop bleeding, optimally resuscitate, and correct coagulopathy have increased the overall survival of severe traumatic injury with hemorrhage.[2] Aside from mortality, one of the main consequences of severe traumatic injury is multiorgan failure. Lung injury induced by hemorrhagic shock (HS) and trauma can result in adult respiratory distress syndrome, while acute kidney injury can lead to acute renal failure. Incidence of multiorgan failure in trauma can be as high as 17%.[3] There are few if any effective therapeutic measures that can prevent or mitigate these deleterious consequences of traumatic injury.

Submitted: October 30, 2014, Revised: February 2, 2015, Accepted: February 17, 2015.

From the Blood Systems Research Institute (D.R.P., G.B., S.M.K., X.D., J.A.C., S.L.G., M.O.M., M.E.F., S.P.); and Department of Laboratory Medicine (M.O.M., S.P.), University of California San Francisco, San Francisco, California; Department of Surgery (Z.P.), University of Texas-Houston Medical School, Houston, Texas; Division of Critical Care Medicine (P.C.S.), Department of Pediatrics, Washington School of Medicine, St. Louis, Missouri; and Shock Trauma Center (R.K.), University of Maryland, Baltimore, Maryland.

Presented at the 4th Annual Remote Damage Control Resuscitation Symposium of the Trauma Hemostasis and Oxygenation Research Network, June 9–11, 2014, in Bergen, Norway.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.jtrauma.com).

Address for reprints: Shibani Pati, MD, PhD, Blood Systems Research Institute, 270 Masonic Ave, San Francisco, CA 94118; email: spati@bloodsystems.org.

DOI: 10.1097/TA.0000000000000630

Blood products are routinely administered to patients with HS. The traditional use of fresh frozen plasma (FFP) in trauma and HS has been to provide a source of clotting factors that commonly become depleted or diluted with hemorrhage, massive transfusion, and fluid resuscitation. FFP is routinely administered to bleeding patients to treat coagulopathy, achieve hemostasis, and reduce death from hemorrhage.[4] Current studies, mostly retrospective and prospective observational, suggest that the early administration of FFP and red blood cells after injury is associated with dramatic increases in patient survival and reduced death in both military combat hospitals and civilian settings.[5–11] While the use of FFP is increasing with potential demonstrated survival benefits, little is known about FFP's exact mechanisms of action. Recent studies suggest that FFP's mechanisms of action in HS are not solely related to hemostasis but also due in part to plasma's ability to globally promote systemic vascular stability which mitigates the endotheliopathy of trauma (EOT). EOT may be loosely defined as increased endothelial cell (EC) permeability, dysfunctional coagulation, inflammation and hemodynamic instability that can lead to organ injury after HS.[4,12]

In this article, we sought to determine if the process of spray drying of a solvent detergent–treated pooled plasma product (SDP) affects the stabilizing effects of plasma on vascular endothelial integrity and inflammation induced by HS. In our previous work, we demonstrated that SDP and FFP were equivalent in several in vitro assays of EC function.[13] To date, the effects of SDP on pulmonary vascular integrity and

inflammation have not been studied in vivo. In this article, we hypothesized that the stabilizing effects of SDP and FFP on the endothelium in vitro and in vivo are equivalent and possibly superior to lactated Ringer's (LR) solution.

MATERIALS AND METHODS

Primary Cells

Human umbilical vein ECs (HUVECs) were purchased from Lonza (Walkersville, MD). HUVECs were maintained in EGM-2 media (Lonza). All cells were maintained at 37°C and 5% CO_2 in a humidified incubator.

Sourcing FFP and SDP

Three units of FFP were obtained by apheresis collection from Blood Centers of the Pacific, San Francisco, CA. Donor 1 was female Type O^+, Donor 2 was male Type O^+, and Donor 3 was male Type O^+. FFP was used freshly thawed on the same day as thaw. SDP (Resusix) was obtained from Entegrion, Inc. and is reconstituted with a supplied pH-adjusted phosphate buffer. SDP is multidonor and solvent detergent treated.[13] SDP tested was from one lot derived from more than 150 pooled Type AB donors.

In Vivo Immunocytochemistry

HUVECs were grown to confluence on 24-well plates. Culture medium was switched to a serum-free endothelial basal medium (EBM, Lonza Walkersville, Inc.) for 1 hour before the treatments were administered. Cells were then treated with culture media that contained 10% by volume of either Resusix (SDP), FFP, LR solution, or normal culture media. Heparin 10 U/mL was added to all groups to prevent the plasma from clotting. One hour following treatment, vascular EC growth factor (VEGF-A [165] Sigma Aldrich, St. Louis, MO) was added to the wells at a concentration of 50 ng/mL for 30 minutes before fixation and staining. In brief, cells were fixed with 4% paraformaldehyde for 10 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes, and blocked in Tris-buffered saline +2.5% normal goat serum. Antibodies used for immunofluorescence included a rabbit anti–VE-cadherin antibody (1:200 dilution, Cell Signaling #2500, Beverly, MA) and a mouse anti–β-catenin antibody (1:200 dilution, Cell Signaling 2677, Beverly, MA), which were applied in DAKO diluent at 4°C and detected using an Alexa Fluor 488 anti–rabbit antibody (1:500 dilution, A11008, Life Technologies, Carlsbad, CA) and an Alexa 568 anti-mouse (1:500 dilution, A11004, Life Technologies). Finally, ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI) was added, and monolayers were imaged using a Leica DM6000 fluorescent microscope (Leica Microsystems, Buffalo Grove, IL).

Gene Expression and Cytokine/Growth Factor Assays

HUVECs were grown to 80% confluence in T175 flasks, serum starved for 1 hour, and then treated with culture media that contained 10% by volume of SDP, FFP, LR solution, or normal culture media, all containing 10 U/mL heparin (n = 3 per group). For gene expression experiments, cells were allowed to incubate for 16 hours and 8 hours for cytokine assays. Media was collected and frozen for cytokine assays, while cells destined for gene expression were harvested via a cell scraper. The cells was pelleted into RNAse later (Life Technologies) and stored at −80°C. Later, the cells' RNA was harvested using a *mir*Vana RNA Isolation kit (Life Technologies) and analyzed using a custom real-time polymerase chain reaction (PCR) array (Qiagen, Inc., Redwood City, CA). Statistical analysis was conducted using RT^2 Profiler PCR Array Data Analysis (Qiagen, Inc.). The conditioned media were run on a Bio-Rad, Bio-Plex Pro (Hercules, CA). Panels run were Human Cancer Biomarker Panel 1 (16 plex), Panel 2 (18 plex), and Cytokine/Chemokine panels (21 and 27 plex). Standard curves were tested in duplicate, and samples were tested in singlet. Standard curves measured a 5-log pg/mL range. Values that were out of range below the standard curve were assigned ½ the lowest calculated value for each analyte. Data were analyzed and graphed using GraphPad Prism 6.0 (La Jolla, CA). Comparisons were made by analysis of variance (ANOVA), multiple comparisons were corrected by Tukey or Bonferroni, and the adjusted p values are reported.

In Vitro Endothelial Monolayer Resistance and Permeability

HUVEC cell barrier function was assessed by measuring changes in transendothelial electrical resistance (TEER) in real time using an electric cell substrate impedance sensing (ECIS) system (ECIS 1600, Applied BioPhysics, Troy, NY) as described.[14,15] A change in TEER across the cell monolayer indicated increased or decreased paracellular permeability. HUVECs were grown to confluence on L-cysteine reduced, fibronectin-coated 96W10E electrodes (Applied Biophysics) and serum starved for 1 hour in EBM media before treatment. We treated confluent HUVECs with culture media that contained 10% volume of either Resusix (SDP), FFP, LR solution, or normal culture media, all with 10 U/mL heparin, and measured the TEER at 4/16/64 kHz continuously for 2.5 hours afterward (n = 6 for each group). Resistance measurements at 4 kHz were normalized by dividing each well by its baseline resistance. For a period of approximately 20 minutes after the plasma addition period, changes in TEER from each well's baseline (ratio of posttreatment to pretreatment) were computed, averaged for each group (with average control changes subtracted), and expressed as the area under the curve for 2 hours.

Animals

The described research was performed with approval of the Institutional Animal Care and Use Committee at ISIS Services LLC (San Carlos, CA). The experiments were conducted in compliance with the National Institutes of Health guidelines on the use of laboratory animals. All animals were housed at a controlled room temperature with a 12:12-hour light-dark cycle with access to food and water ad libitum. Male C57BL6 mice, 8 weeks to 12 weeks old, were used (Harlan Laboratories, Livermore, CA). Under inhaled isoflurane anesthesia, animals were placed on a heating plank to maintain a body temperature of 35°C to 37°C. A 2.5-cm midline laparotomy was performed and then sutured. Femoral arterial catheters were flushed with 1,000-U/mL heparin and then placed into the femoral arteries of both legs. No additional heparin was used. The left catheter was connected to the corresponding fluid

reservoir, and the right, to a blood pressure monitor (PowerLab 8, AD Instruments, Dunedin, New Zealand).

In Vivo Lung Vascular Hyperpermeability

With the use of an established model of HS and trauma, mice were bled to a mean arterial blood pressure (MAP) of 35 mm Hg for 60 minutes.[16,17] After the shock period, mice received fluid treatments of FFP, SDP, or LR solution. FFP and SDP mice were resuscitated with volumes equal to shed blood at a rate of 25 μL/min. LR solution mice received a volume equal to three times the shed blood. To correct for this extracellular fluid deficit, infusion of isotonic crystalloid fluid in a ratio of three volumes of crystalloid to one of blood is required.[18,19] Shock controls were allowed to self-resuscitate. Mice were monitored for an additional 30 minutes after shock and then they received 200 μL of 10 mg/mL Alexa Fluor 680 dextran 10 kD through the cannula. After 30 minutes, the animal was then perfused with 30 mL of ice cold phosphate-buffered saline via the left ventricle. The lungs and the heart were then removed and then perfused with an additional 30 mL of ice cold phosphate-buffered saline, taking care that the lungs inflate and all blood is flushed from the lungs. The lungs were then harvested, inflated with OCT Tissue Tek (Andwin Scientific, Schaumburg, IL), and placed on a Odyssey scanner (LI-COR Biosciences, Lincoln, NE), and the fluorescence read at 700 nm. With the use of an image studio software (LI-COR Biosciences), an average fluorescence intensity per lung was quantitated and averaged with others from the same treatment group after being normalized to sham. Then, the lungs were frozen in OCT. Statistical differences were determined by two-tailed *t* tests.

In Vivo Physiologic Assessment of Mice

With the use of an established model of HS and trauma, mice were hemorrhaged to a MAP of 35 mm Hg for 90 minutes.[16,17] Sixty-five microliters of blood was used for initial blood gas measurement via ABL Flex blood gas analyzer (Radiometer America, Westlake, OH). After the shock period, mice were resuscitated as in the hyperpermeability experiments. This practice is based on the laboratory findings demonstrating that extracellular fluid redistributes during shock into both the intravascular and intracellular spaces. Mice were monitored for MAPs for an additional 30 minutes after shock. Catheters were then removed and allowed to freely ambulate for 60 minutes, after which the mouse was reanesthetized. Arterial blood was sampled for physiologic measurements of base excess, and then the mouse was exsanguinated via cardiac blood draw. The left main bronchus of the lungs from the mice that underwent the 90-minute shock procedure was tied off, and the right lobes were inflated with OCT. The left lobes were placed in a 1.5-mL centrifuge tube and snap frozen on dry ice for future studies.

Immunofluorescence and Histologic Staining of Lungs for Endothelial and Inflammatory Markers

Lung sections 5 μm in thickness were cut on a CM1859 UV cryostat (Leica Microsystems) and stained with antibodies to VE-cadherin (1:200 dilution) (H-72, Santa Cruz Biotechnology, Dallas, TX), CD68 (C-18, 1:10 dilution, Santa Cruz Biotechnology), occludin (M-20, 1:50 dilution, Santa Cruz Biotechnology), and Alexa Fluor–labeled secondary antibodies (A21208, A11057, A21206 Life Technologies). Sections were mounted with FluorGold with DAPI. Images were taken on a DM6000 (Leica Microsystems). Manual quantification of $CD68^+$ cells was performed using ImageJ (US National Institutes of Health, Bethesda, MD). Intensity quantification of VE-cadherin and occludin was performed using Cell Profiler's automatic thresholding (Broad Institute, Cambridge, MA). Statistical analysis was performed using a two-tailed unpaired *t* tests. Hematoxylin and eosin staining was performed using a kit from IHC World, LLC (Woodstock, MD) and imaged on Nikon Labophot-2 microscope (Nikon Corp, Tokyo, Japan).

Data Analysis and Tests of Statistical Significance

Significance of TEER data was assessed as $p < 0.05$, and data were analyzed using ANOVA with Tukey test for post hoc analysis. Statistical tests of gene expression were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA), while statistical differences between cytokines and growth factor were determined by an ANOVA with Bonferroni group comparisons. Statistical analysis for staining of tissue was performed using a two-tailed unpaired *t* tests.

RESULTS

FFP and SDP Decreases Endothelial Permeability In Vitro

Previously, we demonstrated that FFP and SDP decrease endothelial permeability in transwell assays using fluorescein isothiocyanate–labeled dextran.[13] Using novel methodology, herein we investigated the potential of FFP and SDP to increase endothelial monolayer electrical resistance, which is directly correlated to the permeability of an endothelial monolayer.[14,15,17] Using the ECIS system, we found that both FFP and SDP increased endothelial monolayer resistance. Figure 1*A* demonstrates that FFP is more effective at increasing EC monolayer resistance than SDP. Figure 1*B* shows that two other donors of FFP display increases in resistance similar to SDP, suggesting that donor variability exists in single-donor FFP on these outcome measures of EC permeability. In contrast, LR solution displayed no capacity to increase the EC monolayer resistance over normal media controls.

To further understand at the molecular level the effects of FFP and SDP on endothelial junctional stability, we investigated the effects of both EC adherens junctions, composed of β-catenin and VE-cadherin. Adherens junctions are critical regulators of vascular endothelial junctional stability.[20–24] Monolayers of cells that had been stimulated with VEGF-A were stained for both β-catenin and VE-cadherin (Fig. 1*C*). FFP and SDP both qualitatively restored junctional integrity as depicted by an enhanced and more organized pattern of VE-cadherin (*green*) and β-catenin (*red*) staining of the cells. LR solution did not prevent EC junctional breakdown induced by VEGF-A.

FFP and SDP Induce Similar Patterns of Gene Expression and Cytokine Production in ECs

One of the first cell types that plasma comes into contact with after transfusion is vascular ECs. The molecular and genetic effects of plasma on the endothelium have not been

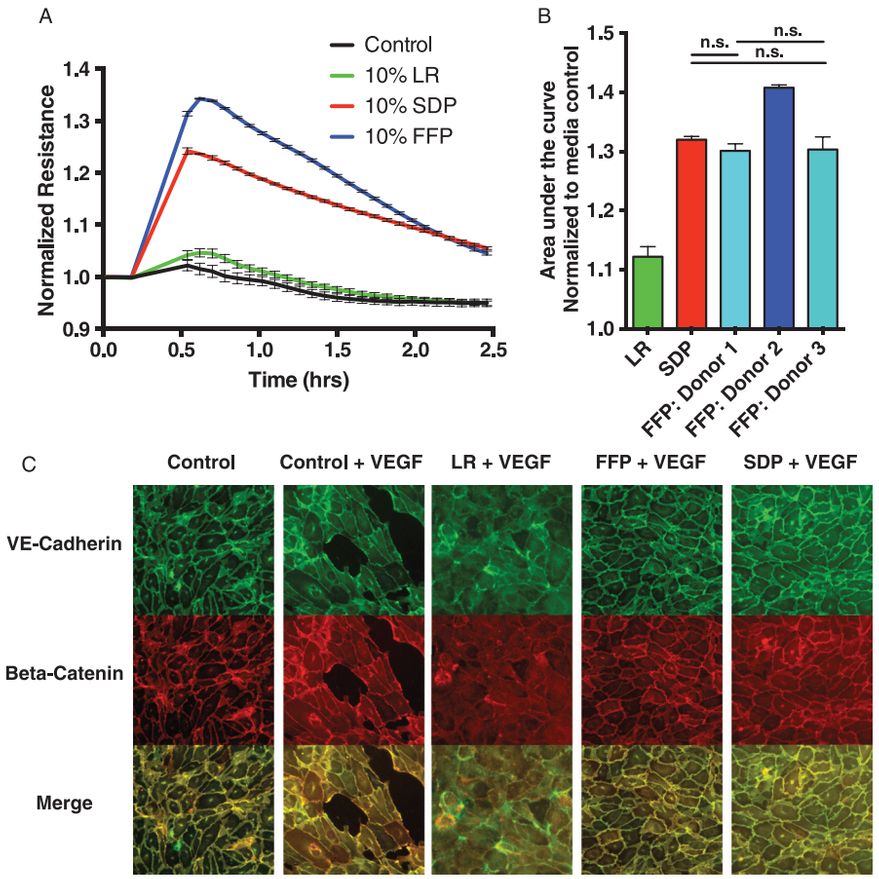

Figure 1. A) Mean average ECIS generated traces of the TEER of HUVECs treated with 10% media (control), LR, FFP or SDP B) Comparison of the TEER normalized values at 1 hour post treatment with three different FFP donors. Black lines indicate significant difference ($p < 0.05$). C) Representative images of endothelial cells stained with antibodies reactive to VE-Cadherin (green) and b-Catenin (red). Merged staining (yellow). Qualitatively, cells treated with FFP and SDP display reconstituted intact adherens junctions post VEGF-A treatment. (Representative images are selected).

characterized to date. To determine how FFP and SDP modulate gene expression and gene product secretion in ECs, we treated ECs with the three fluid groups (FFP, SDP, and LR solution). The expression of 89 genes were measured using a real-time PCR array of genes selected for their relevance to permeability and inflammation of ECs. Cells treated with either FFP or SDP displayed a number of significantly up-regulated and down-regulated genes (Table, Supplemental Digital Content 1, http://links.lww.com/TA/A555). Volcano plots of the gene expression changes indicate that SDP and FFP do indeed alter overall gene expression within treated ECs compared with media alone (Fig. 2*B*). Gap junction A4 (GJA4) and angiotensin converting enzyme (ACE) were down-regulated by both FFP and SDP, while VCAM-1 was up-regulated. Comparison of FFP to SDP reveals few significant changes indicating their overall similarity; however, VCAM-1 was significantly unregulated in FFP-treated ECs by twofold compared with SDP (Fig. 2*C*). LR solution showed few significant differences from control (Fig. 2*D*).

To determine the effects of FFP and SDP on EC soluble factor production, we treated ECs with the different fluid groups (Fig. 3*A*). A similar experiment assayed the concentration in the conditioned media of 61 soluble factors, including cytokines and chemokines after 8 hours of treatment with 10% FFP, SDP, LR solution, or control medium (Table, Supplemental Digital Content 2, http://links.lww.com/TA/A556). Figure 3*B* shows factors that displayed similar expression level changes in ECs between FFP and SDP. Notably, the expression levels for VEGF-A, VEGF-C, VEGF-D, and soluble Tie2 (sTie2), which all play a role in EC permeability (Fig. 3*C*), are altered by both FFP and SDP treatment of ECs.[25,26] Supplementary Table 3 (http://links.lww.com/TA/A557) lists the differences in content of cytokines, chemokines, and growth factors present in the plasma itself.

SDP and FFP Equivalently Reduce Pulmonary Vascular Leak Induced by HS

To evaluate the ability of FFP and SDP to reduce the vascular leak associated with HS, we studied permeability in the lungs of shocked mice (Fig. 4*A*). Mice were subjected to 60 minutes of shock, a time frame when active lung permeability is maximal. Shock-induced mice were not resuscitated. The lungs were harvested, and the leakage of the dye into the lung tissue was quantitated (Fig. 4*B*). The data, expressed as average pixel intensity, are normalized to the presence of dye in

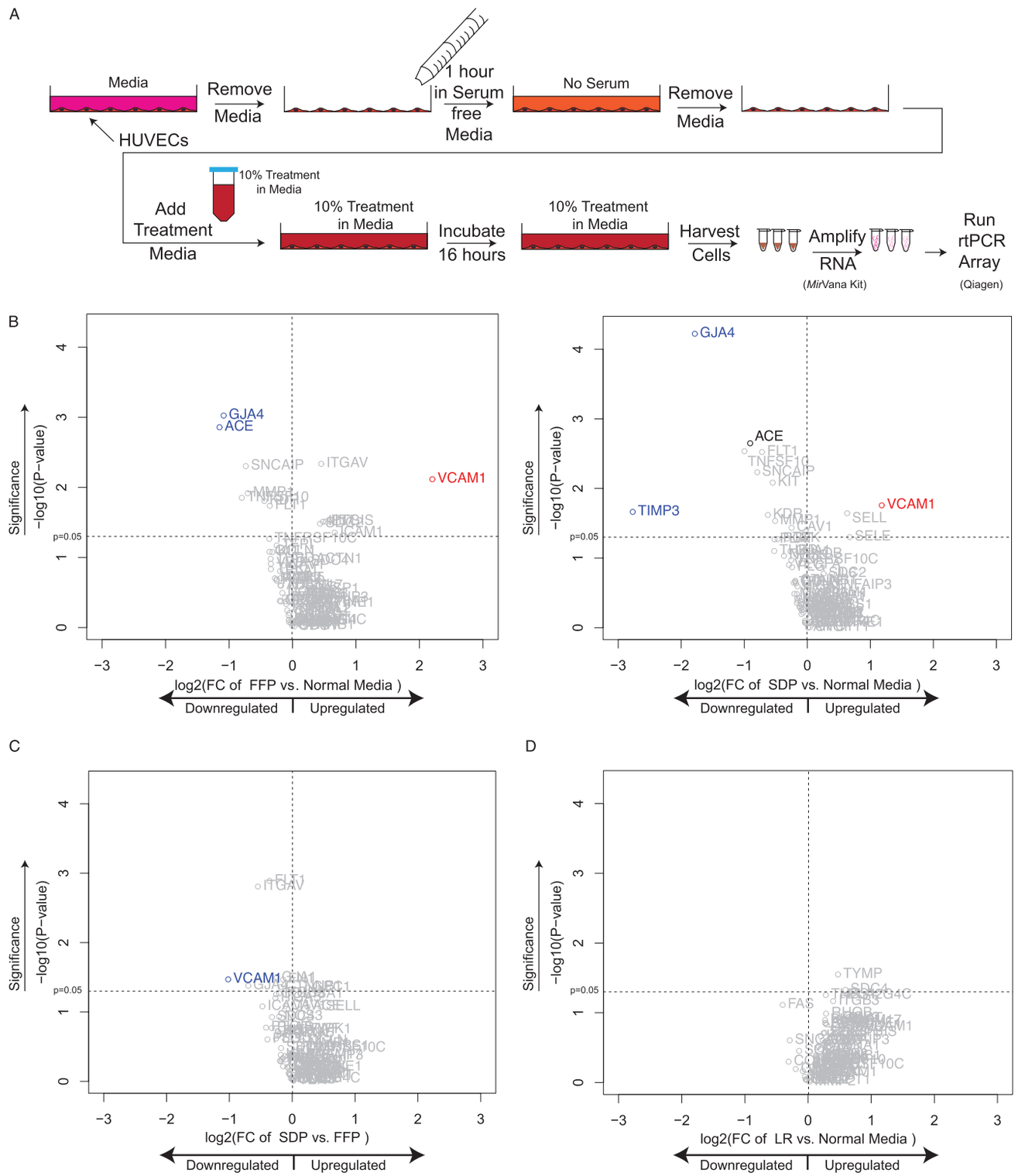

Figure 2. A) RT-PCR array protocol and scheme. Endothelial cells were cultured in individual wells and starved for one hour prior to treatment to decrease variability. 4 arrays were run for each treatment group. Cells were treated with 10% of each treatment group for 16 hours. Cells were harvested, RNA was amplified and analyzed on targeted endothelial RT PCR arrays (Qiagen Inc). B, C, D and E Volcano plots of C) FFP vs. control, D) SDP vs. control, E) SDP vs. FFP and F) LR vs. Control. The horizontal line indicates p = 0.05, genes above it are considered significant. Genes in red indicate a more than two fold up regulation, blue is a two fold or more down regulation. SDP and FFP are similar to each other in profile. LR is similar to control.

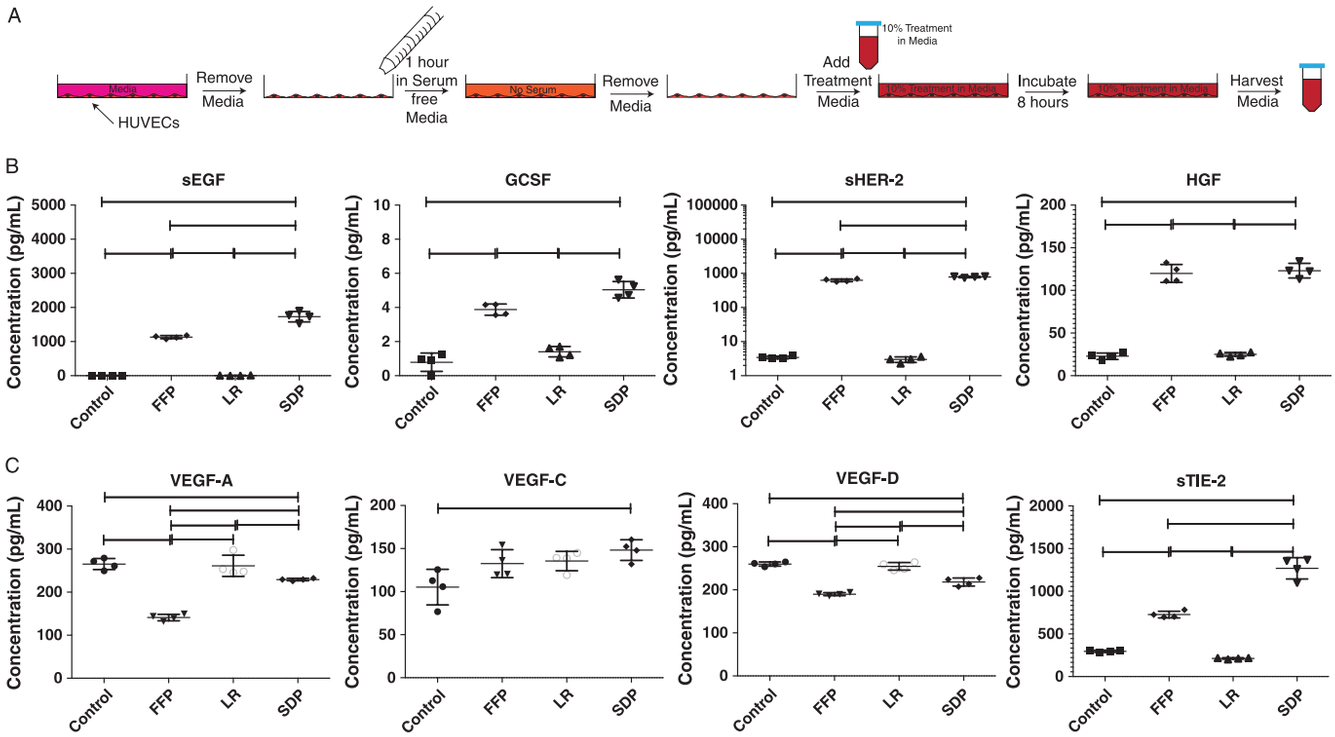

Figure 3. A) Scheme of EC cytokine/growth factor response to resuscitation products. Endothelial cells were cultured in individual wells and starved for one hour to decrease variability. Four wells were run for each treatment group and cells were treated with 10% of each treatment group for 8 hours. Conditioned media was harvested and run on a series of custom Luminex (Billerica, MA, USA) panels. B) Graphs of selected cytokine concentrations, C) Graphs of a factors that influence permeability. Bars indicate significant differences ($p < 0.05$). Each point indicates a single measurement.

the lungs of the sham animals, which have been through the entire procedure (cannulation, anesthesia) without the hemorrhage or injury. Animals resuscitated with either of the FFP and SDP resulted in significantly lower dye permeability compared with shock-induced animals (shock, 3.37 ± 1.18; FFP, 1.56 ± 0.21; SDP, 1.61 ± 0.13; and LR solution, 2.16 ± 0.73; n = 4 per group). FFP and SDP groups were significantly different from shock and LR solution groups but not from each other (Fig. 4*B*).

SDP and FFP Equivalently Correct MAPs and Base Excess in HS

To evaluate the effects of FFP and SDP transfusion on HS and lung injury, we studied hemodynamic measures, lung inflammation, and lung vascular integrity in HS mice (Fig. 5*A*). Mice were subjected to 90 minutes of hypotension to increase the severity of the injury, necessary to study histopathologic changes. The MAPs for all groups are depicted in graph form in Figure 5*B*. MAP traces from FFP and SDP mice were similar.

Radiometer measurements revealed that the human plasma products significantly attenuated the depth of the shock as measured by the base excess of the blood (Fig. 5*C*). A low base excess is strongly correlated to mortality in patients who have experienced both trauma and hemorrhage.[27] Both FFP and SDP but not LR solution significantly corrected base excess to normal levels. Base excess values determined at the time of sacrifice 3 hours after initiation of hemorrhage are as follows: (preshock, −1.05 ± 2.35; sham, −1.93 ± 1.55; shock, −9.46 ± 2.19; FFP, −2.78 ± 2.139; SDP, −3.44 ± 1.71; LR solution, −13.38 ± 4.08 mmol/L). Other blood parameters were monitored (calcium, potassium, sodium, chloride, and glucose). The only significant difference between FFP and SDP was glucose levels found in the plasma after resuscitation. This is likely caused by the lower levels of glucose present in SDP compared with FFP. SDP is apheresis derived versus whole blood derived for FFP, which results in higher glucose levels due to differences in the storage buffer[28] (Figures, Supplemental Digital Contents 4 and 5, http://links.lww.com/TA/A558 and http://links.lww.com/TA/A559).

SDP and FFP Equivalently Inhibit Leukocyte Infiltration and Breakdown of Endothelial Adherens and Tight Junctions Induced by HS

To study lung inflammation, lung tissue from all groups of mice were sectioned and stained for CD68 and myeloperoxidase (MPO) as markers of infiltrating leukocytes. FFP and SDP significantly attenuated the number of $CD68^+$ cells compared with the total cells (quantitated via DAPI staining) compared with shock and LR solution (sham, 0.15 ± 0.07; shock, 0.50 ± 0.11; FFP, 0.22 ± 0.12; SDP, 0.23 ± 0.09; LR solution, 0.43 ± 0.095; n = 4 mice per group; Fig. 6*A* and *C*). Yet, across all groups, there was no change in MPO^+ cells (Figure, Supplemental Digital Content 6, http://links.lww.com/TA/A560). There seems to be a baseline level of MPO in sham mice likely due to the initial instrumentation that they are subjected to before HS.

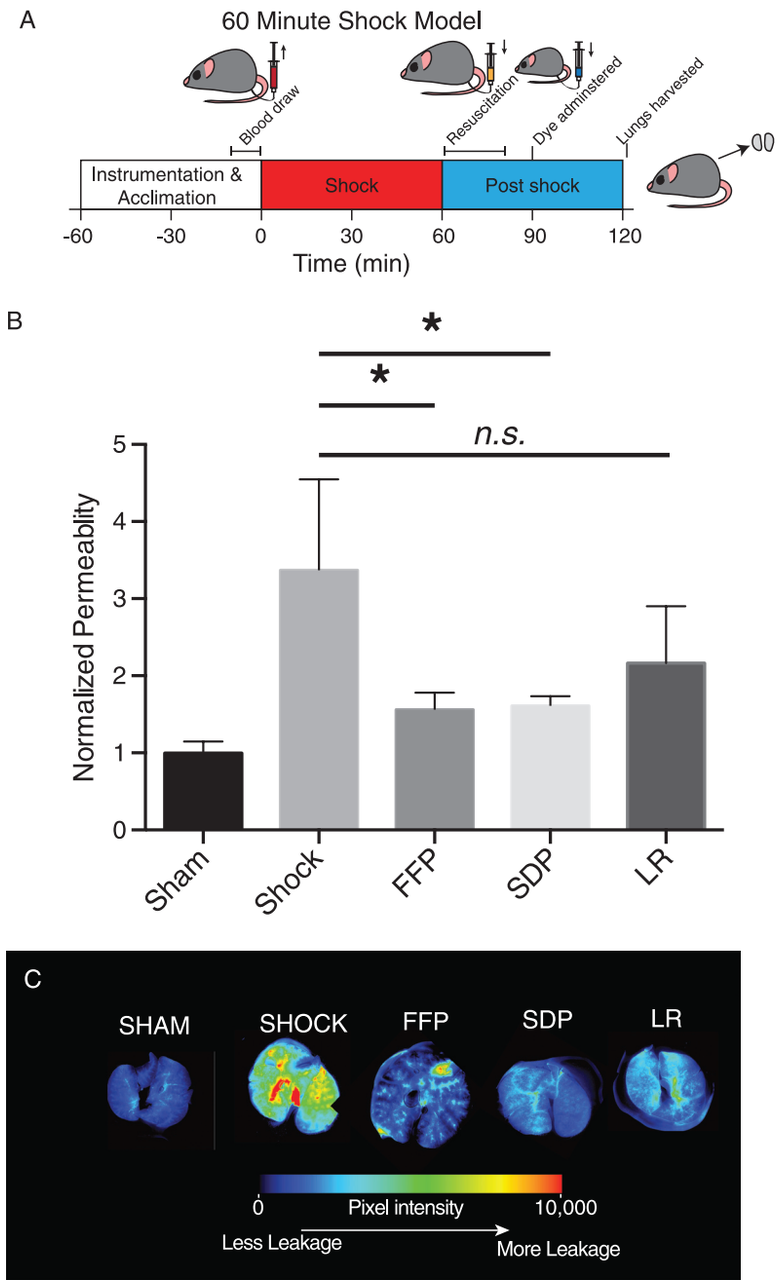

Figure 4. A) Timeline of 60 minute shock experiments. Sixty minutes shock is the optimal time point for assessment of vascular permeability in the lungs. B) Normalized pulmonary vascular permeability of mouse lungs to 10 kD Alexa Fluor 680 conjugated dextran after mice underwent 60 minutes of shock followed by resuscitation. SDP and FFP groups are significantly decreased in lung vascular permeability compared to LR treated mice and Shock mice. Sham animals were instrumented but not subjected to shock or trauma. Experimental animals were either allowed to auto-resuscitate or resuscitated with plasma products equal to shed blood (FFP or SDP) or 3x LR. (* = $p < 0.5$, n = 4 for all groups) LR. C) Representative scans of mouse lungs from each group.

To study endothelial junctional integrity proteins, VE-cadherin and occludin were stained for in lung sections, and they were both compromised as measured by fluorescence intensity in shock and LR solution mice compared with sham. FFP and SDP preserved staining for both EC junctions. Occludin staining values were as follows: sham, 1.00 ± 0.29; shock, 0.36; FFP, 0.66 ± 0.070; SDP, 0.77 ± 0.26; LR solution, 0.22 ± 0.16. VE-cadherin staining values were as follows: sham, 1.0 ± 0.23; shock, 0.72 ± 0.903; FFP, 1.247 ± 0.33; SDP, 0.99 ± 0.27; LR solution, 0.70 ± 0.19. Although the trend for increased staining of VE-cadherin was present in SDP mouse lungs, it was not significantly different from HS mice ($p = 0.105$, Fig. 6). Histopathologic staining of lung tissue sections with hematoxylin and eosin reveal signs of lung injury in shock animals (see Figure, Supplemental Digital

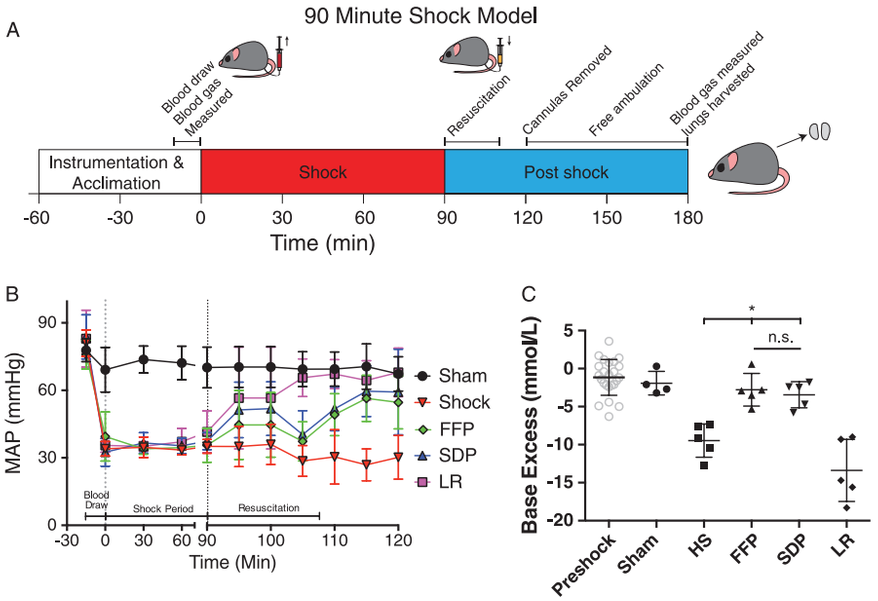

Figure 5. A) Timeline of 90 minute shock experiments. B) Resuscitation mean arterial pressures (MAPs) for shock mice are shown. N = 7 mice/group. Note: Mice that received human SDP or FFP experienced an initial rise in blood pressure in response to resuscitation but then a transient drop followed by MAP recovery after resuscitation ended. Error bars indicate +/- SEM. C) Base excess obtained from blood gas measurements. Pre-shock is all the measurements taken before hemorrhage from all groups. Each point is a single measurement, error bars show standard deviation. All readings were assessed on a hospital grade ABL90 Flex analyzer (Radiometer America Inc.) for physiologic measures.

Content 7, http://links.lww.com/TA/A561). Alveolar wall thickening and increased inflammatory infiltrates were present in shock mice compared with sham mice. FFP, SDP, and to a lesser extent, LR solution attenuated these inflammatory changes.

DISCUSSION

Previously, we have demonstrated that freshly thawed FFP and SDP (spray-dried, solvent detergent–treated plasma) have potent protective effects on endothelial permeability and inflammation in vitro.[17,29] In this article, we hypothesized that the stabilizing effects of SDP and FFP on the endothelium in vitro and in vivo in a mouse model of HS are equivalent and superior to LR. We demonstrate that FFP and SDP modulate EC permeability and gene expression in vitro and mitigate pulmonary vascular permeability and lung inflammation induced by HS in vivo.[17] In vivo, we specifically chose, as we have in the past, an early 3-hour time point after HS to evaluate lung injury. This time point was based on past studies demonstrating partial restoration of the endothelial glycocalyx at 3 hours.[17,30] It is important to note that this early time point primarily addresses the development of pulmonary hyperpermeability, inflammation, and not the full manifestation of acute lung injury or adult respiratory distress syndrome.[17]

In vitro, our data support our previously published results that FFP and SDP, but not LR solution, reduce the permeability of the EC monolayer. With the use of a novel approach, our data in Figure 1 demonstrate that both FFP and SDP increase transendothelial resistances (TEER) as measured by an endothelial impedance system (ECIS). TEER measurements indicate the electrical resistance of the EC monolayer, where increased TEER is associated with decreased EC permeability.[14,15] SDP is a pooled product in which only one lot was tested. It is not surprising that there are individual FFP donors that are more or less effective at increasing the resistance of an EC monolayer and decreasing endothelial permeability. The clinical consequences of this variability in FFP and lot-to-lot variability in a pooled product such as SDP are of interest and have yet to be determined.

The gene expression studies suggest the FFP and SDP are relatively equivalent in their effects on EC gene expression (Fig. 2*E*). These data also generate several interesting targets that may allow us a starting point for elucidating the mechanism of plasma's action on ECs. Interestingly, VCAM-1 was up-regulated by both FFP and SDP after 16 hours of treatment, suggesting a possible proinflammatory effect of plasma on ECs. VCAM-1 mediates leukocyte and platelet binding and is generally associated with an inflammatory phenotype.[31] This is contrary to our previously reported findings where VCAM-1 levels and leukocyte-endothelial binding are unchanged on ECs after plasma treatment.[13] This difference may be caused by the effect of longer treatment with plasma (16 hours) in a static, no-flow system. Down-regulation of GJA4 is of interest since it codes for connexin 37, a gap junction protein involved in nitric oxide signaling and is inhibitory to vascular remodeling.[32] It is also of interest that ACE is down-regulated by plasma. The downstream effect of ACE at low levels could be peripheral end-organ vasodilation and increased perfusion as less angiotensin I is converted to angiotensin II.[31] Taken together, these data suggest that plasma may regulate cell-cell communication and vasoreactivity of the endothelium. All of these data are hypotheses generating and warrant further investigation.

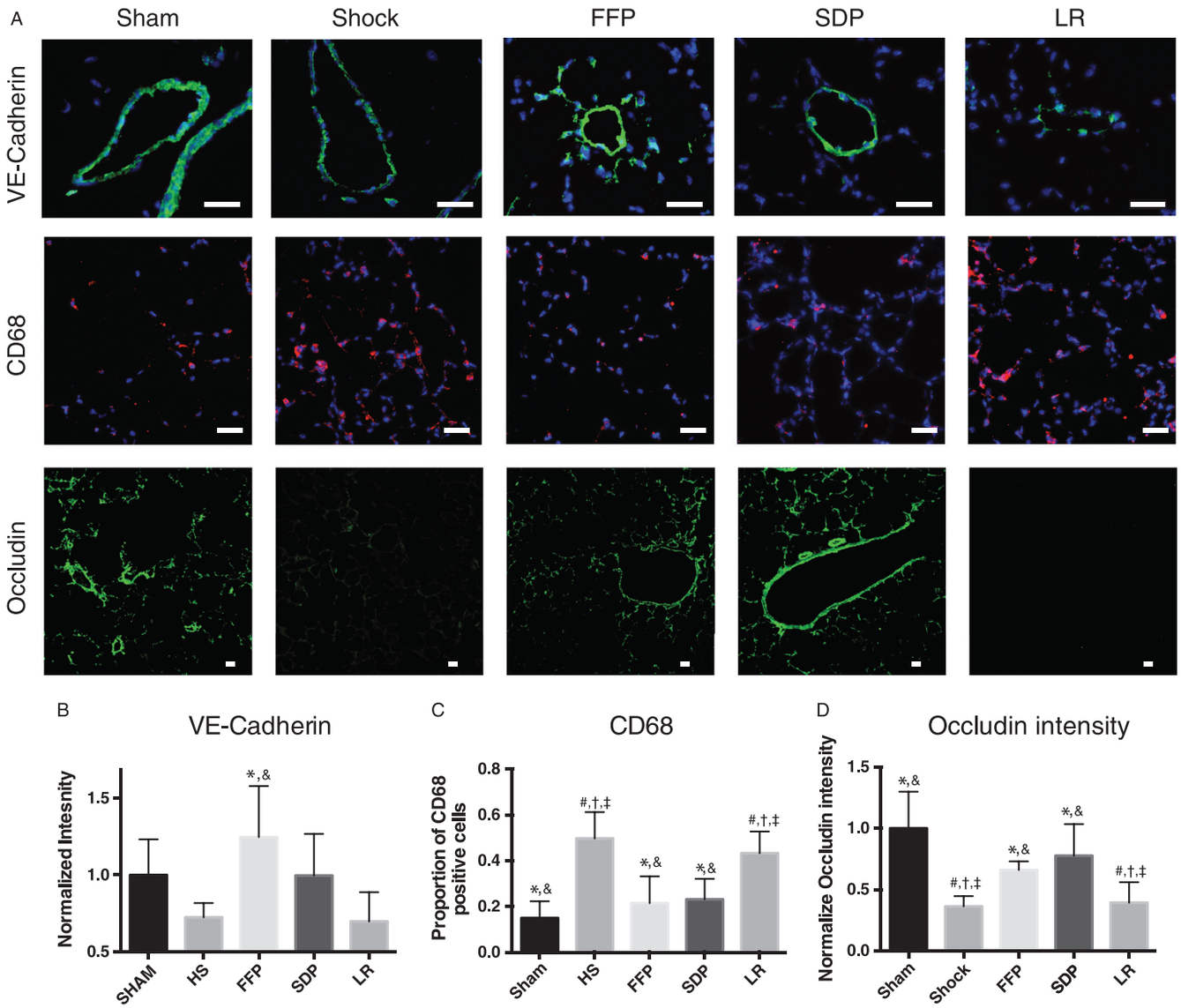

Figure 6. A) Representational images of lung tissue sections stained with antibodies against VE-Cadherin, CD68 and Occludin. All scale bars are 100 μm. N = 4 mice/group for all quantification. B) Average mean intensity normalized to sham for VE-Cadherin positive vessels is identified through cell profiler's automatic threshold generation. C) The proportion of CD68 positive cells in the lung sections were compared to DAPI positive cells, thereby giving us a relative value of inflammatory infiltrating CD68 cells to all cells in the region assessed. Cells near or within vessel walls were excluded. D) Average mean intensity normalized to sham of Occludin positive vessels, identified through automatic threshold generation. Error bars represent STD, significance of $p < 0.05$ designated & vs. Sham, * vs HS, # vs. FFP, † vs. SDP and ‡ vs. LR.)

Cytokine and chemokine profiles between FFP- and SDP-treated cells were also similar, particularly for gene products that have an impact on EC permeability (Fig. 3*C*). VEGF-A and VEGF-D are both significantly reduced compared with controls, which can contribute to reduced EC permeability.[25,33] However, other markers that may modulate endothelial activation and promote permeability are increased, such as increased sTie2, which modulates angiopoietin 1 and 2 signaling.[26,34,35] Although these results are mixed, the net result is likely caused by the balance between stabilizing and destabilizing factors, which in the case of FFP and SDP results in a net decrease in EC permeability in vitro and in vivo. Some differences were noted between FFP and SDP, including expression of RANTES, leptin, and interleukin 4 (Supplementary Table 2). These differences may be caused by inherent donor variability in FFP, a topic of future investigation for our laboratory.

In vivo, our model of shock aptly demonstrates that both SDP and FFP equally attenuate acute lung injury–induced permeability. In this model, we find that SDP and FFP, but not LR solution, equivalently decrease pulmonary inflammation and vascular integrity. We found a transient suppression of MAP after the start of resuscitation with SDP and FFP. This

effect may be caused by a xenogeneic reaction in the mouse to the human plasma and seemed to be transitory as has been noted in our past studies with human FFP.[17] LR solution resuscitation exhibited no such decline. Our main reason for using human plasma was to test the actual clinical product that would be used in human trials. This is an acute model of injury (3 hours), and in this short time, we have not noted any deleterious effects aside from the dip in MAP after infusion. However, we acknowledge that in a more chronic model of injury, this may not be the case. We have published in our past work the effects of human FFP in this same acute shock model in mice and have not found any deleterious consequences.[17] In future studies, we will consider the use of immunodeficient mouse models to avoid this potential complication.[36]

Our studies are based on the central hypothesis that FFP has the capacity to repair injured endothelium and the EOT by inhibiting and repairing the damage from a number of detrimental processes induced by HS: prolonged vascular permeability, nonspecific initiation of coagulation, endothelial contraction and death, interstitial edema, leukocyte infiltration, inflammation, and tissue hypoxia.[4,12,29] FFP resuscitation in an HS rat model has also been shown to partially restore a damaged endothelial glycocalyx and syndecan-1 expression.[17,37,38] Restoration of the glycocalyx physically reinforces the endothelial barrier and attenuates leukocyte-EC adhesion.[17,37,38] Work by Alam et al. demonstrated that FFP administration can decrease mortality, blood-brain barrier compromise, and cerebral edema in a swine model of traumatic brain injury.[39–41] Furthermore, our group has shown that the beneficial effects of FFP on endothelial function and hemodynamic stability in vitro and in vivo decrease after just 5 days of storage at 4°C.[29] Increased transforming growth factor β in Day 5 FFP may be the reason for these changes, which potentially result in diminished repair of injured vessels during trauma and HS.[42]

Our data support the premise that plasma (both FFP and SDP) has potent effects on the vascular endothelium and mitigates the EOT.[4,12] One of the hallmarks of EOT is the development of vascular hyperpermeability. Vascular permeability has been implicated in HS to lead to end-organ dysfunction and increased mortality, suggesting that this may be one of the mechanisms by which increased plasma use decreases mortality in trauma. We hypothesize that the plasma's effects are systemic and not solely limited to mitigating pulmonary vascular injury in HS.

In conclusion, the need for a dry plasma product for clinical use is clear. The main goal is rapid implementation of plasma in bleeding patients, an early intervention that retrospective and prospective observational studies support.[5,10,43,44] Despite its potential benefits, the use of FFP in traumatic injury comes with the significant burden of shipping and storage of frozen blood products around the world. In addition, the delay required for thawing plasma at non–high-volume trauma centers may increase mortality secondary to hemorrhage. Conversely, SDP, which is still in the clinical development stage, can be stored at room temperature, transported easily, and reconstituted rapidly. Because SDP can be administered rapidly after injury, much earlier than frozen FFP, logistical benefits of SDP could potentially lead to cost benefits and improved outcomes in traumatically injured patients at risk of death.[13] Lyophilized or freeze-dried plasma has also been shown to have similar beneficial effects in reducing inflammation and mortality after HS.[20,21,45–47] The significance of some of the differences found between FFP and SDP warrant further investigation and may be caused by fact that single-donor FFP was compared with SDP. Clinical efficacy and safety of SDP and lyophilized plasma products require additional study. The drying phase of development alters its protein content, and as a result, it may affect its hemostatic capacity and safety profile. The logistical benefits of dried plasma products will need to be balanced with its efficacy and safety compared with FFP or other standard plasma products.

AUTHORSHIP

All authors participated in the manuscript preparation. D.R.P. performed the animal experimentation, performed the statistical analysis, interpreted the data, and drafted the article. G.B. performed the intravital experiments and wrote the article. J.A.C. imaged and analyzed sections. Z.P., S.M.K., and X.D. performed statistical analysis. M.E.F. stained sections. M.O.M., P.C.S., and R.K. contributed to the conception and design of the research. P.C.S. initiated and designed the experiments and collaborated on the article draft.

DISCLOSURE

The Pati laboratory and BSRI have a grant from Entegrion, Inc. (Durham, NC) to investigate the use of Resusix.

REFERENCES

1. Norton R, Kobusingye O. Injuries. *N Engl J Med*. 2013;368(18):1723–1730.
2. Eastridge BJ, Mabry RL, Seguin P, Cantrell J, Tops T, Uribe P, Mallett O, Zubko T, Oetjen-Gerdes L, Rasmussen TE, et al. Death on the battlefield (2001–2011): implications for the future of combat casualty care. *J Tauma Acute Care Surg*. 2012;73(6 Suppl 5):S431–S437.
3. Sauaia A, Moore EE, Johnson JL, Chin TL, Banerjee A, Sperry JL, Burlew CC. Temporal trends of postinjury multiple-organ failure: still resource intensive, morbid, and lethal. *J Tauma Acute Care Surg*. 2014;76(3):582–592.
4. Holcomb JB, Pati S. Optimal trauma resuscitation with plasma as the primary resuscitative fluid: the surgeon's perspective. *Hematology Am Soc Hematol Educ Program*. 2013;2013:656–659.
5. Borgman MA, Spinella PC, Perkins JG, Grathwohl KW, Repine T, Beekley AC, Sebesta J, Jenkins D, Wade CE, Holcomb JB. The ratio of blood products transfused affects mortality in patients receiving massive transfusions at a combat support hospital. *J Trauma*. 2007;63(4):805–813.
6. Maegele M, Engel D, Bouillon B, Lefering R, Fach H, Raum M, Buchheister B, Schaefer U, Klug N, Neugebauer E. Incidence and outcome of traumatic brain injury in an urban area in Western Europe over 10 years. *Eur Surg Res*. 2007;39(6):372–379.
7. Maegele M, Lefering R, Paffrath T, Tjardes T, Simanski C, Bouillon B. Red-blood-cell to plasma ratios transfused during massive transfusion are associated with mortality in severe multiple injury: a retrospective analysis from the Trauma Registry of the Deutsche Gesellschaft fur Unfallchirurgie. *Vox Sang*. 2008;95(2):112–119.
8. Gonzalez EA, Moore FA, Holcomb JB, Miller CC, Kozar RA, Todd SR, Cocanour CS, Balldin BC, McKinley BA. Fresh frozen plasma should be given earlier to patients requiring massive transfusion. *J Trauma*. 2007;62(1):112–119.
9. Kashuk JL, Moore EE, Johnson JL, Haenel J, Wilson M, Moore JB, Cothren CC, Biffl WL, Banerjee A, Sauaia A. Postinjury life threatening coagulopathy: is 1:1 fresh frozen plasma:packed red blood cells the answer? *J Trauma*. 2008;65(2):261–270.
10. Holcomb JB, del Junco DJ, Fox EE, Wade CE, Cohen MJ, Schreiber MA, Alarcon LH, Bai Y, Brasel KJ, Bulger EM. The prospective, observational, multicenter, major trauma transfusion (PROMMTT) study: comparative effectiveness of a time-varying treatment with competing risks. *JAMA Surg*. 2013;148(2):127–136.

11. del Junco DJ, Holcomb JB, Fox EE, Brasel KJ, Phelan HA, Bulger EM, Schreiber MA, Muskat P, Alarcon LH, Cohen MJ, et al. Resuscitate early with plasma and platelets or balance blood products gradually: findings from the PROMMTT study. *J Tauma Acute Care Surg*. 2013;75(1 Suppl 1): S24–S30.
12. Jenkins DH, Rappold JF, Badloe JF, Berséus O, Blackbourne L, Brohi KH, Butler FK, Cap AP, Cohen MJ, Davenport R, et al. Trauma hemostasis and oxygenation research position paper on remote damage control resuscitation: definitions, current practice, and knowledge gaps. *Shock*. 2014; 41(Suppl 1):3–12.
13. Wataha K, Menge T, Deng X, Shah A, Bode A, Holcomb JB, Potter D, Kozar R, Spinella PC, Pati S. Spray-dried plasma and fresh frozen plasma modulate permeability and inflammation in vitro in vascular endothelial cells. *Transfusion*. 2013;53(Suppl 1):80S–90S.
14. Giaever I, Keese CR. Use of electric fields to monitor the dynamical aspect of cell behavior in tissue culture. *IEEE Trans Biomed Eng*. 1986;33(2): 242–247.
15. Keese CR, Bhawe K, Wegener J, Giaever I. Real-time impedance assay to follow the invasive activities of metastatic cells in culture. *Biotechniques*. 2002;33(4):842–844, 6, 8–50.
16. Chesebro BB, Rahn P, Carles M, Esmon CT, Xu J, Brohi K, Frith D, Pittet JF, Cohen MJ. Increase in activated protein C mediates acute traumatic coagulopathy in mice. *Shock*. 2009;32(6):659–665.
17. Peng Z, Pati S, Potter D, Brown R, Holcomb JB, Grill R, Wataha K, Park PW, Xue H, Kozar RA. Fresh frozen plasma lessens pulmonary endothelial inflammation and hyperpermeability after hemorrhagic shock and is associated with loss of syndecan 1. *Shock*. 2013;40(3):195–202.
18. Moore FA, McKinley BA, Moore EE. The next generation in shock resuscitation. *Lancet*. 2004;363(9425):1988–1996.
19. Tollofsrud S, Elgjo GI, Prough DS, Williams CA, Traber DL, Kramer GC. The dynamics of vascular volume and fluid shifts of lactated Ringer's solution and hypertonic-saline-dextran solutions infused in normovolemic sheep. *Anesth Analg*. 2001;93(4):823–831.
20. Sawant DA, Tharakan B, Hunter FA, Smythe WR, Childs EW. Role of beta-catenin in regulating microvascular endothelial cell hyperpermeability. *J Trauma*. 2011;70(2):481–487.
21. Tharakan B, Hellman J, Sawant DA, Tinsley JH, Parrish AR, Hunter FA, Smythe WR, Childs EW. β-catenin dynamics in the regulation of microvascular endothelial cell hyperpermeability. *Shock*. 2012;37(3):306–311.
22. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci*. 2008;121(Pt 13):2115–2122.
23. Dejana E, Spagnuolo R, Bazzoni G. Interendothelial junctions and their role in the control of angiogenesis, vascular permeability and leukocyte transmigration. *Thromb Haemost*. 2001;86(1):308–315.
24. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. *Dev Cell*. 2009;16(2):209–221.
25. Li X, Eriksson U. Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *Int J Biochem Cell Biol*. 2001;33(4):421–426.
26. Benest AV, Kruse K, Savant S, Thomas M, Laib AM, Loos EK, Fiedler U, Augustin HG. Angiopoietin-2 is critical for cytokine-induced vascular leakage. *PLoS One*. 2013;8(8):e70459.
27. Rutherford EJ, Morris JA Jr, Reed GW, Hall KS. Base deficit stratifies mortality and determines therapy. *J Trauma*. 1992;33(3):417–423.
28. Simon TL, Snyder EL, Stowell CP, Strauss RG, Solheim BG, Petrides M. *Rossi's Principles of Transfusion Medicine*. Hoboken, NJ: Wiley-Blackwell; 2011.
29. Pati S, Matijevic N, Doursout MF, Ko T, Cao Y, Deng X, Kozar RA, Hartwell E, Conyers J, Holcomb JB. Protective effects of fresh frozen plasma on vascular endothelial permeability, coagulation, and resuscitation after hemorrhagic shock are time dependent and diminish between days 0 and 5 after thaw. *J Trauma*. 2010;69(Suppl 1):S55–S63.
30. Kozar RA, Peng Z, Zhang R, Holcomb JB, Pati S, Park P, Ko TC, Paredes A. Plasma restoration of endothelial glycocalyx in a rodent model of hemorrhagic shock. *Anesth Analg*. 2011;112(6):1289–1295.
31. Pacurari M, Kafoury R, Tchounwou PB, Ndebele K. The renin-angiotensin-aldosterone system in vascular inflammation and remodeling. *Int J Inflam*. 2014;2014:689360.
32. Derouette JP, Wong C, Burnier L, Morel S, Sutter E, Galan K, Brisset AC, Roth I, Chadjichristos CE, Kwak BR. Molecular role of Cx37 in advanced atherosclerosis: a micro-array study. *Atherosclerosis*. 2009;206(1):69–76.
33. Chen XL, Nam JO, Jean C, Lawson C, Walsh CT, Goka E, Lim ST, Tomar A, Tancioni I, Uryu S, et al. VEGF-induced vascular permeability is mediated by FAK. *Dev Cell*. 2012;22(1):146–157.
34. Ganter MT, Cohen MJ, Brohi K, Chesebro BB, Staudenmayer KL, Rahn P, Christiaans SC, Bir ND, Pittet JF. Angiopoietin-2, marker and mediator of endothelial activation with prognostic significance early after trauma? *Ann Surg*. 2008;247(2):320–326.
35. London NR, Whitehead KJ, Li DY. Endogenous endothelial cell signaling systems maintain vascular stability. *Angiogenesis*. 2009;12(2):149–158.
36. Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. *Nat Rev Immunol*. 2007;7(2):118–130.
37. Haywood-Watson RJ, Holcomb JB, Gonzalez EA, Peng Z, Pati S, Park PW, Wang W, Zaske AM, Menge T, Kozar RA. Modulation of syndecan-1 shedding after hemorrhagic shock and resuscitation. *PLoS One*. 2011; 6(8):e23530.
38. Peng Z, Ban K, Sen A, Grill R, Park P, Costantini TW, Kozar R. Syndecan 1 plays a novel role in enteral glutamine's gut-protective effects of the postischemic gut. *Shock*. 2012;38(1):57–62.
39. Alam HB, Hamwi KB, Duggan M, Fikry K, Lu J, Fukudome EY, Chong W, Bramos A, Kim K, Velmahos G. Hemostatic and pharmacologic resuscitation: results of a long-term survival study in a swine polytrauma model. *J Trauma*. 2011;70(3):636–645.
40. Imam AM, Jin G, Duggan M, Sillesen M, Hwabejire JO, Jepsen CH, DePeralta D, Liu B, Lu J, deMoya MA, et al. Synergistic effects of fresh frozen plasma and valproic acid treatment in a combined model of traumatic brain injury and hemorrhagic shock. *Surgery*. 2013;154(2): 388–396.
41. Imam AM, Jin G, Sillesen M, Duggan M, Jepsen CH, Hwabejire JO, Lu J, Liu B, DeMoya MA, Velmahos GC, et al. Early treatment with lyophilized plasma protects the brain in a large animal model of combined traumatic brain injury and hemorrhagic shock. *J Tauma Acute Care Surg*. 2013; 75(6):976–983.
42. Duan C, Cao Y, Deng X, Wang W, Yang W, Liu X, Chen Z, Pati S, Kozar RA, Gonzalez EA, et al. Increased transforming growth factor beta contributes to deterioration of refrigerated fresh frozen plasma's effects in vitro on endothelial cells. *Shock*. 2011;36(1):54–59.
43. Holcomb JB, Wade CE, Michalek JE, Chisholm GB, Zarzabal LA, Schreiber MA, Gonzalez EA, Pomper GJ, Perkins JG, Spinella PC, et al. Increased plasma and platelet to red blood cell ratios improves outcome in 466 massively transfused civilian trauma patients. *Ann Surg*. 2008;248(3): 447–458.
44. Holcomb JB, Zarzabal LA, Michalek JE, Kozar RA, Spinella PC, Perkins JG, Matijevic N, Dong JF, Pati S, Wade CE, et al. Increased platelet:RBC ratios are associated with improved survival after massive transfusion. *J Trauma*. 2011;71(2 Suppl 3):S318–S328.
45. Sailliol A, Martinaud C, Cap AP, Civadier C, Clavier B, Deshayes AV, Mendes AC, Pouget T, Demazeau N, Chueca M, et al. The evolving role of lyophilized plasma in remote damage control resuscitation in the French Armed Forces Health Service. *Transfusion*. 2013;53(Suppl 1):65S–71S.
46. Martinaud C, Ausset S, Deshayes AV, Cauet A, Demazeau N, Sailliol A. Use of freeze-dried plasma in French intensive care unit in Afghanistan. *J Trauma*. 2011;71(6):1761–1764; discussion 4–5.
47. Martinaud C, Tourtier JP, Pasquier P, Ausset S, Sailliol A. The French freeze-dried plasma. *J Trauma*. 2011;71(4):1091–1092.

