A Comparison of Red Cell Rejuvenation *versus* Mechanical Washing for the Prevention of Transfusion-associated Organ Injury in Swine

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ABSTRACT

Background: We evaluated the effects of two interventions that modify the red cell storage lesion on kidney and lung injury in experimental models of transfusion.

Methods: White–landrace pigs (n = 32) were allocated to receive sham transfusion (crystalloid), 14-day stored allogeneic red cells, 14-day red cells washed using the red cells washing/salvage system (CATS; Fresenius, Germany), or 14-day red cells rejuvenated using the inosine solution (Rejuvesol solution; Zimmer Biomet, USA) and washed using the CATS device. Functional, biochemical, and histologic markers of organ injury were assessed for up to 24 h posttransfusion.

Results: Transfusion of 14 day red cells resulted in lung injury (lung injury score *vs.* sham, mean difference –0.3 (95% CI, –0.6 to –0.1; *P* = 0.02), pulmonary endothelial dysfunction, and tissue leukocyte sequestration. Mechanical washing reduced red cell–derived microvesicles but increased cell-free hemoglobin in 14-day red cell units. Transfusion of washed red cells reduced leukocyte sequestration but did not reduce the lung injury score (mean difference –0.2; 95% CI, –0.5 to 0.1; *P* = 0.19) relative to 14-day cells. Transfusion of washed red cells also increased endothelial activation and kidney injury. Rejuvenation restored adenosine triphosphate to that of fresh red cells and reduced microvesicle concentrations without increasing cell-free hemoglobin release. Transfusion of rejuvenated red cells reduced plasma cell-free hemoglobin, leukocyte sequestration, and endothelial dysfunction in recipients and reduced lung and kidney injury relative to 14-day or washed 14-day cells.

Conclusions: Reversal of the red cell storage lesion by rejuvenation reduces transfusion-associated organ injury in swine. (ANESTHESIOLOGY 2018; 128:375-85)

ORGAN injury associated with red cell transfusion has been attributed in part to the "storage lesion," a progressive disruption of erythrocyte homeostasis associated with depletion of high energy phosphates during storage that results in the accumulation of microvesicles and other inflammatory substances in the supernatant of red cell units.[1] Experimental studies in animal models have shown that these changes cause organ injury *via* complex mechanisms including platelet and monocyte activation, altered iron metabolism, endothelial injury, and the loss of microcirculatory autoregulation.[2–5] Mechanical washing of aged stored red cells effectively removes the supernatant. This attenuates inflammation and organ injury attributable to transfusion in experimental models[2,6] and has been shown to attenuate inflammation in children undergoing cardiac surgery.[7] The storage lesion in red cells can also be reversed by rejuvenation, whereby coincubation with a solution rich in inosine (Rejuveso solution; Zimmer Biomet, USA) activates glycolysis and restores cellular adenosine triphosphate (ATP) and 2,3-diphosphoglycerate to normal or even supranormal levels[8] that is followed by removal of the red cell supernatant in a washing step, before transfusion, to

What We Already Know about This Topic

- Erythrocyte (red blood cell) transfusions are associated with a multifactorial storage lesion that is associated with the potential for organ injury
- Multiple experimental models have evaluated methods to prevent or minimize these effects, including pharmacologic approaches of rejuvenation to restore intracellular energy levels

What This Article Tells Us That Is New

- In a porcine model, red blood cell washing along with an inosine rejuvenation solution restored red cell energy stores, reduced inflammatory responses, and reduced transfusion-associated organ injury in swine in 14-day-old stored blood

remove residual inosine. Transfusion of rejuvenated red cells has been shown to improve renal microvascular blood flow in a rodent exchange transfusion model.[9] We recently described a porcine model in which transfusion of allogeneic red cells causes acute lung and kidney injury in a storage-dependent manner.[4,10] We hypothesized that mechanical washing of red cells would reduce organ injury in the swine model. We further

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hypothesized that the effects of red cell rejuvenation on endothelial function in transfusion recipients would confer additional benefits beyond those observed with washing alone. We explored the processes underlying our observations in swine in a complementary *in vitro* human red cell transfusion model.

Materials and Methods

Detailed methods are described in the Supplemental Digital Content (http://links.lww.com/ALN/B563).

Red Cell Storage

Allogeneic human (from volunteers) and porcine (from 11 adult female Large White–Landrace crossbred pigs weighing 80 to 100 kg) whole blood was collected in citrate–phosphate–dextrose; the buffy coat was removed; and the blood was leukodepleted and stored in sucrose–adenosine–glucose–mannitol using the Leukotrap WB system (Pall Medical, UK) and stored at 4°C for either 14 days (porcine) or 35 days (human) according to National Health Service Blood and Transplant Standards for Human Blood.[11] Our previous work has demonstrated that these storage times result in comparable storage lesions in human and porcine red cells.[4,10] Washing procedures were performed using the quality mode of the red cells washing/salvage system (CATS; Fresenius, Germany) and resuspended in 0.9% normal saline. Rejuvenation was performed according to the manufacturer's instructions, the cells were washed as described using the red cells washing system as above and resuspended in saline. Red cells were transfused immediately after the washing/rejuvenation procedure. In transfused pigs, donor and recipient blood was cross-matched using a visual agglutination test as described previously.[4,10]

Porcine *In Vivo* *Transfusion Model*

Animals received care in accordance with and under license (Project Licence 30/2522) of the Animals (Scientific Procedures) Act 1986 and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (publication No. 85-23, revised 1996). The study received local institutional review board approval and was conducted over a 2-yr period.

Interventions. Thirty-two pigs were allocated to the following groups: Sham, neck dissection for great vessel cannulation plus 1,000-ml crystalloid infusion; Day 14, neck dissection plus 4 units (approximately 1,000 ml) of 14-day-old stored porcine red cell units; Washed, neck dissection plus 4 units of washed 14-day-old stored porcine red cell units; and Rejuvenated, neck dissection plus 4 units of inosine solution–treated washed 14-day-old stored porcine red cell units. Postintervention, all animals were recovered and then reanesthetized and reevaluated after 24 h. Staff undertaking the animal experiments were not blinded to allocation; however, all laboratory assessments were performed in a blinded manner.

Anesthesia, Analgesia, Monitoring, and Sample Size Calculations. Anesthesia, analgesia, monitoring, and sample size calculations are described in detail in the Supplemental Digital Content (http://links.lww.com/ALN/B563).

Outcomes

Organ Injury and Endothelial Function. Pulmonary endothelial function, acute kidney injury, and renal endothelial function were determined as described previously[4,12] and in the Supplemental Digital Content (http://links.lww.com/ALN/B563). The lung injury score[13] was calculated from lung compliance, and the P_{AO_2}/F_{IO_2} ratio was measured *in vivo* at baseline, 1.5 h, and 24 h postintervention using the SERVO-i Universal Ventilator (Maquet, Germany) using volume-controlled ventilation with a tidal volume of 10 ml/kg, F_{IO_2} of 0.5, respiratory rate of 12 breaths/min, and peak end-expiratory pressure of 5 cm/H_2O. Chest radiographs were not performed. Leukocyte invasion was assessed in cryosections of porcine lung and renal biopsies with antibodies against CD14 and CD16. All histologic methods are described in detail in the Supplemental Digital Content (http://links.lww.com/ALN/B563).

Microvesicle Analysis. Microvesicle analysis was performed in citrated plasma samples spun twice at 1,500*g* and red blood cells bags with supernatant spun at 1,850*g* after 1:1 dilution with phosphate-buffered saline. Concentration and size distribution were estimated using NanoSight NS500, a nanoparticle tracking device (Malvern Instruments, UK). Derivation of microvesicles and phosphatidylserine exposure were determined with fluorescein isothiocyanate (FITC)-coupled CD235a, and annexin V (phycoerythrin-coupled) (Affymetrix, USA): 20- to 50-μl samples were labeled with antibodies at 1:20 dilution in annexin V–binding buffer for 25 min at room temperature in a total volume of 100 μl. The samples were analyzed by flow cytometry (CyAn ADP; Beckman Coulter, USA). To separate larger microvesicles, red blood cell supernatant was further spun at 28,000*g* for 35 min. The pellet was resuspended in phosphate-buffered saline (microvesicle, 1:4 original volume, average microvesicles concentration was 5 to 7×10^8 particles/ml) and stored at –80°C, as was the remaining high-speed supernatant (HS), for later analyses (Supplemental Digital Content, http://links.lww.com/ALN/B560).

In Vitro Flow Assay. Human microvascular pulmonary endothelial cells or human umbilical vein endothelial cells growing on 35-mm culture dishes were sealed with rubber gaskets and a flow deck. The flow chambers were connected to a peristaltic pump, which pushed the whole blood through the system at 5 dynes/cm². Circulating fresh blood was obtained from healthy donors and anti-coagulated with citrate phosphate dextrose–adenine. Leukocytes were labeled with nuclear dyes SYTO11 or SYTO64 and FITC-coupled CD42a. Leukocyte rolling, adhesion, and platelet aggregation were imaged with an inverted Zeiss Axio Observer Z1 microscope, equipped with 20×, N/A 0.4 objective, Hamamatsu Flash 4.0 camera, and Collibri LED illumination system at 10 frames/s; 301 frames were acquired every 10 min. Images were analyzed with ImageJ and Mtrack2 plugin.[14] The region encoding annexin V for blocking experiments was cloned into pTrcHisA bacterial expression vector (Thermo Fisher Scientific, USA). Protein was isolated from

bacterial cultures, adjusted to 1 mg/ml, and stored at –80°C. For blocking, equal volumes of microvesicles and annexin V were incubated for 30 min and used in the assays.

Red Blood Cell Survival. Red blood cell survival was measured using a modified version of the methods of Mock *et al.*[15] Briefly, the cells were washed with phosphate-buffered saline, resuspended in 10 µg/ml sulfo-*N*-hydroxysuccinimide-biotin in phosphate-buffered saline (Thermo Fisher Scientific), and incubated at room temperature for 40 min. The cells were washed with phosphate-buffered saline and resuspended in previously stored supernatant. The biotinylated red blood cell survival was assessed 5, 10, 15, 30, 60, and 120 min and 24 h posttransfusion by flow cytometry (a FACSort flow cytometer driven by Summit V4.3.02 Build 2,451; Beckman Coulter) and FITC-labeled streptavidin.

Biochemical Marker Analysis

Hematocrit and hemoglobin, lactate, and central venous oxygen saturations were performed using the ABL 800 Flex blood gas analyzer (Radiometer, Denmark). Interleukin-6, a marker of myelomonocytic activation, and interleukin-8, a marker of endothelial cell activation,[16,17] were measured in porcine serum samples using enzyme-linked immunosorbent assay (ELISA) kits from R&D Systems (UK). ELISAs were performed using the DS2 two-plate ELISA processing system (Dynex Technologies, USA). Nitric oxide in porcine plasma was measured using a colorimetric R&D Systems kit and the multiplate reader Enspire (PerkinElmer, USA). 2,3-Diphosphoglycerate levels in blood bags were measured with a commercially available kit (Roche Diagnostics, UK) using the multiplate reader Enspire (PerkinElmer). ATP levels were measured with a commercially available kits (ATPlite; PerkinElmer) using a NovoStar reader (BMG LabTech, Germany). For these assays, red blood cell samples were deproteinized with perchloric acid as described previously.[18] Reticulocyte elongation index, the unit of deformability was measured by ektacytometry using Lorrca (Mechatronics, USA) in porcine red cells as previously described.[19] CD235a content in HS and in microvesicle fractions membranes was determined by SDS-PAGE and immunoblotting with specific antibody (clone BRIC 256), supplied by the Bristol Institute for Transfusion Sciences (courtesy of Dr. Rosey Mushens, NHSBT, Bristol Institute for Transfusion Sciences, Filton, UK). Cell-free hemoglobin was estimated as described by Fairbanks *et al.*[20] Briefly, the samples were spun at 1,850*g* for 15 min, and the resulting supernatant was further spun for 5 min at 28,000*g*. The supernatant was diluted 5× with phosphate-buffered saline and absorbance measured at 415, 450, and 700 nm using the multiplate reader Enspire (PerkinElmer). Hemoglobin concentration was calculated as follows: $Hb = 1.58 \times A_{415} - 0.95 \times A_{450} - 2.91 \times A_{700}$. Free iron was measured in plasma and tissue lysates as previously described[21]: 73.5 µl of plasma/lysate was incubated with 1.5 µl of 50 mM ferrous iron chelator bathophenanthroline disulfonate for 15 min. Absorbance was measured at 535 nm and compared against standard curve prepared with ferrous ammonium sulfate.

In Vitro Endothelial Assays. To determine monolayer permeability, human umbilical vein endothelial cells grown on Transwell polyester inserts were incubated with 2 ml of EGM-2 BulletKit medium (Lonza, USA) containing 1 mg/ml hemoglobin, 5 µg/ml lipopolysaccharide, 20% HS, or phosphate-buffered saline and incubated at 37°C for 6 h. Afterward, medium from the top chamber was transferred into a new 6-well plate, filters were placed in corresponding wells, and 50 µl was taken for analysis (0-min time point in Supplemental Digital Content, http://links.lww.com/ALN/B560). One and a half milliliter of medium with 1 mg/ml FITC-dextran was added to the top (filter) chamber and incubated at 37°C for 90 min. Fifty microliters of medium from the bottom chamber was taken for analysis at 20, 40, 60, and 90 min. Fluorescence was measured with NovoStar plate reader (BMG LabTech, Germany). For endothelial activation, human umbilical vein endothelial cells were grown in 96-well plates until confluency and incubated for 12 h with hemoglobin at 1 mg/ml, red cell microvesicles, red cell HS supernatant, 5 µg/ml lipopolysaccharide, and phosphate-buffered saline at 20% of total (3 wells/treatment). The cells were then fixed and processed as described above.

Quantitative Real-time Polymerase Chain Reaction. Total RNA extraction was performed using RNeasy fibrous kit (Qiagen, USA) according to the manufacturer's protocol. The total RNA was quantified using a UV NanoDrop ND-1000 UV (Thermo Fisher Scientific) spectrophotometer and Agilent 2100 Bioanalyzer using the eukaryotic RNA assay with the RNA 6000 Nano LabChip kit (Agilent Technologies, USA). One µg of total RNA was reverse transcribed at 42°C using a Sensifast C-DNA synthesis kit (Bioline, UK) according to the manufacturer's instructions and diluted 1:10 in H_2O. For each transcript, a standard curve was constructed. Single reactions were prepared for each set of primers using Sensifast SYBR Green PCR Master Mix (Bioline). Each reaction included a reverse transcription negative control to confirm the absence of genomic DNA and a nontemplate negative control for primer–dimer. Primers for control housekeeping genes were designed as previously reported,[22] and sequences are listed in the Supplemental Digital Content. The real-time PCR was run on Rotorgene Q (Qiagen), and dedicated software was used to determine the Ct in each reaction.

Statistical Analysis

The study was powered to detect differences in kidney function, as per our previous studies in the model.[4,10] Using existing data, we estimated that a study of 24 animals (8/group) would have 90% power to detect an effect size of 0.7 (equivalent to a difference of 16.4 ml/min between groups), assuming a within group SD of 23.5, with one baseline and two postintervention measures per animal and an assumed correlation between pre- and postmeasures and between postmeasures is 0.9 (estimated from pilot data) and a 5% Bonferroni-corrected statistical significance.

Porcine data were evaluated using the Box–Cox power transformation of each physiologic and immunologic variable and, if required, identified the appropriate transformation function to account for the increased variability of the variable with the corresponding mean. Applied transformations are listed in Supplemental Digital Content, http://links.lww.com/ALN/B556. Each of the transformed (or untransformed) physiologic and immunologic variable was analyzed using a linear mixed model incorporating available baseline variables, group, time, and two-way interaction effect of group and time as fixed effects. The model also included a random intercept for each individual pig. The baseline data for each individual were centered as a deviation from the corresponding population mean of the variable. Estimates of variance were obtained using the restricted maximum likelihood method. The overall statistical significance of the main or interaction term in a linear mixed model was assessed using the global F-statistic (Supplemental Digital Content, http://links.lww.com/ALN/B555). In the presence of a statistically significant ($P < 0.05$) global F-statistic for the main (or the interaction term), we obtained the estimated effects of difference between groups (or the interaction terms of group and time; Supplemental Digital Content, http://links.lww.com/ALN/B556). For some variables, differences between groups were tested using the Welch two-sample t test along with Bonferroni adjustments. Data from the *in vitro* experiments were analyzed using the one-way analysis of variance. If the global F-statistic was significant ($P < 0.05$), we performed the Welch two-sample t test along with Bonferroni adjustments to account for multiple comparisons (summary of the data, effect sizes, CIs along with unadjusted and adjusted P values are shown in Supplemental Digital Content, http://links.lww.com/ALN/B559). All statistical analyses were carried out in the R software environment[23] with appropriate packages (nlme, multcomp, lsmeans, ggplot2).

Results

Anesthesia and Monitoring

There was no difference in measured hemodynamics, with the exception of central venous pressure and pulmonary artery capillary wedge pressure that were comparable between Sham, Day 14, and Washed groups but significantly lower in the Rejuvenated group (see Materials and Methods; fig. 1; Supplemental Digital Content, http://links.lww.com/ALN/B555, http://links.lww.com/ALN/B556, and http://links.lww.com/ALN/B557). Transfused pigs had significantly higher hemoglobin and hematocrit than Sham pigs (Supplemental Digital Content, http://links.lww.com/ALN/B555 and http://links.lww.com/ALN/B556). Twenty-four–hour *in vivo* survival of stored red cells ranged from 68 to 72% (Supplemental Digital Content, http://links.lww.com/ALN/B558). Four pigs did not survive the experiments. Two experiments in the Day 14 group were terminated early for refractory hypoxia, and two pigs in the Rejuvenated group were terminated early for cardiovascular instability, leaving 28 animals in the analysis cohort (Sham, n = 6; Day 14, n = 6; Washed, n = 8; Rejuvenated, n = 8; fig. 1A; Supplemental Digital Content, http://links.lww.com/ALN/B558).

Transfusion and Immune Cell Activation

In prespecified analyses, we demonstrated that transfusion of Day 14 stored red cells had no effect on renal function (creatinine clearance) in swine (Day 14 *vs.* Sham mean difference, 10.5 ml/min; 95% CI, –33.3 to 54.4; $P = 0.62$) but resulted in increased lung injury scores (Day 14 *vs.* Sham mean difference, -0.3 (95% CI, –0.6 to –0.1; $P = 0.02$), capillary leak of proteins into the bronchoalveolar lavage fluid, accumulation of leukocytes in porcine lung and kidney, and serum interleukin-6, a marker of myelomonocytic inflammation (fig. 2, A–E; Supplemental Digital Content, http://links.lww.com/ALN/B556 and http://links.lww.com/ALN/B559). Creatinine clearance was significantly increased in recipients of Rejuvenated *versus* Day 14 red cells (mean difference, 49.8; 95% CI, 7.6 to 92.0; $P = 0.02$; fig. 2A). The lung injury score observed in Day 14 swine was significantly reduced in the Rejuvenated (mean difference, –0.4; 95% CI, –0.6 to –0.1; $P = 0.01$) but not the Washed group (mean difference, –0.2; 95% CI, –0.5 to 0.1; $P = 0.13$; fig. 2B). Bronchoalveolar lavage protein levels were reduced by washing, with a further significant reduction with rejuvenation (fig. 2C). Leukocyte accumulation in porcine lung and kidney and serum interleukin-6 levels were significantly reduced in both the Washed and Rejuvenated groups (fig. 2, D and E). We concluded that removal of the red cell storage supernatant in Washed or Rejuvenated red cells had attenuated leukocyte activation in lungs and kidneys *in vivo*.

Next, in exploratory analyses, we investigated the mechanisms by which constituents of the red cell supernatant activated leukocytes *in vivo*. First, to isolate the leukocyte activating factor, we analyzed the storage characteristics of porcine and human red cells. In swine, 14 Day storage was characterized by reduction in 2,3-diphosphoglycerate, an approximate 50% reduction in ATP from baseline, reduced red cell deformability, hemolysis levels less than 1%, and accumulation of red cell–derived microvesicles (Supplemental Digital Content, http://links.lww.com/ALN/B558). Mechanical washing, which is also required for rejuvenation, reduced microvesicles concentrations in stored porcine units (fig. 3A). In parallel, in stored human red cells we demonstrated that microvesicles approximately 200 nm in size, positive for red cells markers (CD235a) and annexin V, increased in stored human red cell units from days 3 to 35 of storage (fig. 3B; Supplemental Digital Content, http://links.lww.com/ALN/B560). In human as in porcine blood bags, washing and rejuvenation significantly reduced the levels of red cell–derived microvesicles *versus* day 41 controls (fig. 3B). We surmised that it was the red cell microvesicles in the 14 Day stored red cells that had resulted in the leukocyte infiltration into the lungs and kidneys of transfusion recipients.
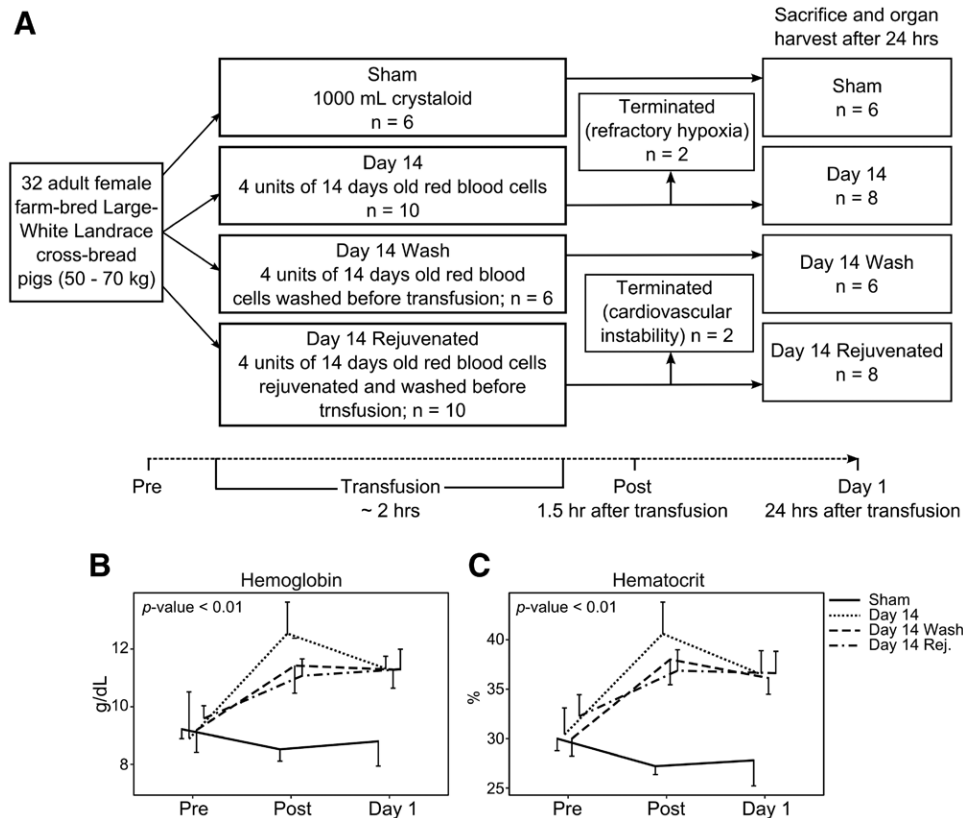
Fig. 1. Experimental design. (*A*) Study schematic. (*B*) Hemoglobin. (*C*) Hematocrit levels in experimental animals. Values represent means ± SD, and the *P* value indicates significant differences between the groups (Supplemental Digital Content, http://links.lww.com/ALN/B555 and http://links.lww.com/ALN/B556, for details). Post = postintervention measure; Pre = preintervention measure; Rej. = rejuvenation.

To test this hypothesis, we separated the different constituents of the human red cell units using differential centrifugation into red cells, red cell–derived microvesicles (200 nm), and the supernatant fraction (Supplemental Digital Content, http://links.lww.com/ALN/B560). Next, we evaluated the effects of each fraction of the red cell storage lesion on leukocyte activation *in vitro* in a flow assay[24,25] (fig. 3C; Supplemental Digital Content, http://links.lww.com/ALN/B563), in which the attachment of leukocytes circulating in whole blood to a human microvascular endothelial cell monolayer is observed in real time using fluorescent microscopy (Supplemental Digital Content, http://links.lww.com/ALN/B561). We observed that isolated microvesicles but not isolated washed red cells (stored > 22 days) or the microvesicle free supernatant isolated by HS resulted in leukocyte adhesion after 1 h of flow (fig. 3D). In further experiments we demonstrated that blocking phosphatidylserine and other negatively charged lipids on the surface of the microvesicles by coincubation with annexin V prevented leukocyte activation (fig. 3E, Supplemental Digital Content, http://links.lww.com/ALN/B561 and http://links.lww.com/ALN/B562). We concluded that annexin V-recognized lipids on red cell–derived microvesicles that accumulate in stored blood bags are likely responsible for leukocyte activation observed after

transfusion *in vivo* and in *vitro*. These are removed by both mechanical washing and red cell rejuvenation.

Transfusion and Endothelial Dysfunction

Day 14 red porcine cells demonstrated significantly increased membrane rigidity relative to fresh red cells (elongation index, Supplemental Digital Content, http://links.lww.com/ALN/B558), increased release of cell-free hemoglobin into the storage supernatant after mechanical washing (fig. 4A), and increased plasma cell-free hemoglobin as well as serum interleukin-8, a putative biomarker of endothelial activation, in transfusion recipients (fig. 4, B and C). In comparison Rejuvenated units demonstrated red cell deformability comparable to fresh red cells (Supplemental Digital Content, http://links.lww.com/ALN/B558) and significant reductions in cell-free hemoglobin compared to Washed or unwashed Day 14 red cells (fig. 4A). Recipients of Rejuvenated units also demonstrated reductions in plasma cell-free hemoglobin, serum interleukin-8 levels, renal heme oxygenase 1 expression, and clinical biomarkers of acute kidney injury including serum creatinine and urine Neutrophil gelatinase-associated lipocalin (fig. 4, B–E; Supplemental Digital Content, http://links.lww.com/ALN/B556 and http://links.lww.com/ALN/B559). We observed no significant difference between the groups with respect to
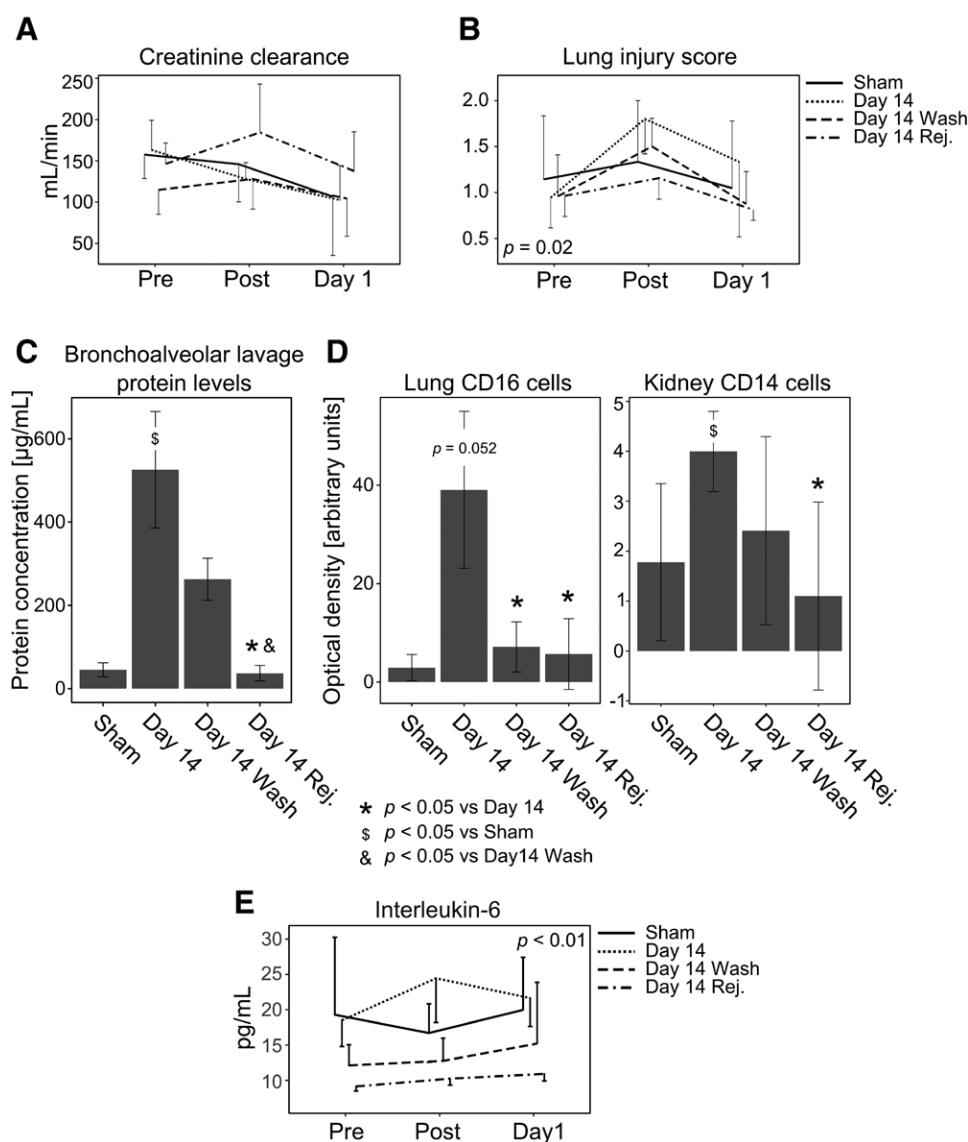
Fig. 2. Effects of red cell washing and rejuvenation *in vivo*. (*A*) Creatinine clearance rates. (*B*) Lung injury score. (*C*) Bronchoalveolar lavage protein levels. (*D*) Quantified images of labeled porcine lung and kidney cryosections. Approximately 20 images were collected from at least 3 sections per animal per group. Tissue samples came from 2 to 4 animals per group. (*E*) Serum interleukin-6 levels. The values represent mean ± SD; the *P* value in (*B*) and (*E*) indicates significant differences between the groups (Supplemental Digital Content, http://links.lww.com/ALN/B555 and http://links.lww.com/ALN/B556, for details). Post = postintervention measure; Pre = preintervention measure; Rej. = rejuvenation.

nitric oxide bioavailability that is diminished by intravascular hemolysis, as per the "nitric oxide hypothesis," although recipients of Day 14 and Washed red cells had the lowest levels of nitric oxide (Supplemental Digital Content, http://links.lww.com/ALN/B556). Both Day 14 and Washed, but not Rejuvenated, red cells increased pulmonary vascular resistance index, a measure of pulmonary endothelial dysfunction in swine *in vivo* (fig. 4F). This corresponded well to differences in lung iron levels, a marker of extravascular hemolysis as per the "iron hypothesis" (Supplemental Digital Content, http://links.lww.com/ALN/B559). Overall, our results suggested that elevated plasma cell-free hemoglobin and the accumulation of tissue labile iron in endothelial

dysfunction and organ injury in the swine model were not present in recipients of Rejuvenated cells.

We explored these findings using *in vitro* models. We demonstrated that cell-free hemoglobin as well as the high-speed supernatant fraction of stored red cells, which contains high levels of cell-free hemoglobin, directly induced permeability in cultured monolayers of human umbilical vein endothelial cells (Supplemental Digital Content, http://links.lww.com/ALN/B560). In further experiments, we observed that endothelial activation by hemoglobin did not occur *via* canonical (CD62E) signaling, as observed after exposure to lipopolysaccharide-treated controls (Supplemental Digital Content, http://links.lww.com/ALN/B560), but *via* expression of integrin β1/α5
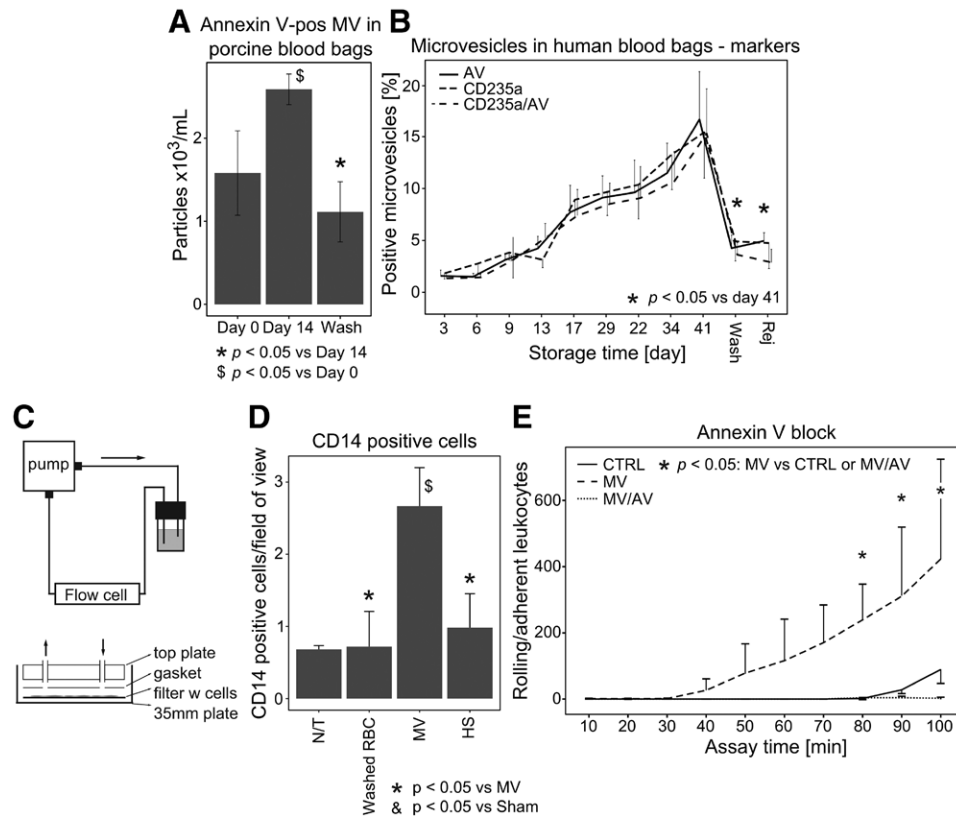
Fig. 3. Microvesicle (MV) analysis in erythrocyte bags. (*A*) Annexin V–positive MVs in aging porcine blood bags (minimum of 3 units analyzed). (*B*) Fractional changes in microvesicle subtypes during storage and after washing and rejuvenation; 8 units were analyzed with indicated antibodies. (*C*) Schematics depicting a flow system used to analyze leukocytes and platelets in real time as described in Materials and Methods. (*D*) Effect of stored blood and MV fractions on leukocyte adhesion under flow *in vitro* (at least three independent repeats). Donor blood was mixed with phosphate-buffered saline (N/T), samples from whole red cell units that were stored for 35 days and washed (Wash), MV fraction (Supplemental Digital Content, http://links.lww.com/ALN/B560), or the high-speed centrifugation supernatant fraction (HS; Supplemental Digital Content, http://links.lww.com/ALN/B560) at 5:1 ratio. The microvesicle fraction was mixed with the donor blood at 1:10 ratio. (*E*) Effect of annexin V (AV) blocking on leukocyte adhesion. The values represent means ± SD. CTRL = control; pos = positive; Rej. = rejuvenation.

(very late antigen 5) and retention of CS1 fibronectin, a putative noncanonical endothelial activation pathway. These results suggested that reductions in plasma cell-free hemoglobin could be responsible for the attenuation of endothelial dysfunction and organ injury observed in pigs after transfusion of Rejuvenated red cells.

Discussion

Transfusion of Day 14 stored red cells results in experimental lung injury *in vivo* characterized by alveolar capillary leak and leukocyte sequestration. Red cell–derived microvesicles that express annexin V–binding lipids accumulate in stored units and result in leukocyte activation *in vitro*. Washing reduces microvesicle concentrations in stored units, and transfusion of Washed red cells reduces leukocyte activation *in vivo*. However, washing results in the increased release of cell-free hemoglobin by stored red cells, which directly activates endothelium *in vitro*, and transfusion of Washed red cells causes endothelial activation *in vivo*. These findings suggest that mechanical red cell washing may have benefits and

risks that offset each other. Transfusion with Rejuvenated red cells preserves the benefits of washing but removes the risks by stabilizing red cell membranes and preventing cell-free hemoglobin release postwashing. Pigs transfused with Rejuvenated red cells had reduced lung injury, reduced levels of leukocyte invasion and serum interleukin-8 compared to those receiving Day 14 red cells, and reduced renal heme oxygenase 1 expression, lung iron accumulation, pulmonary endothelial dysfunction, and renal injury compared to those receiving Washed red cells.

The study has several strengths, not least the demonstration of parallel processes in two species using complementary *in vivo* and *in vitro* transfusion models. Results from the two models were remarkably consistent, indicating that microvesicles and free hemoglobin act *via* distinct mechanisms on leukocytes and endothelial cells respectively. These processes could contribute to a putative multihit pathogenesis of posttransfusion organ injury.[26,27] We demonstrated that transfusion can activate leukocytes through proinflammatory lipids present on microvesicles that accumulate during storage. We
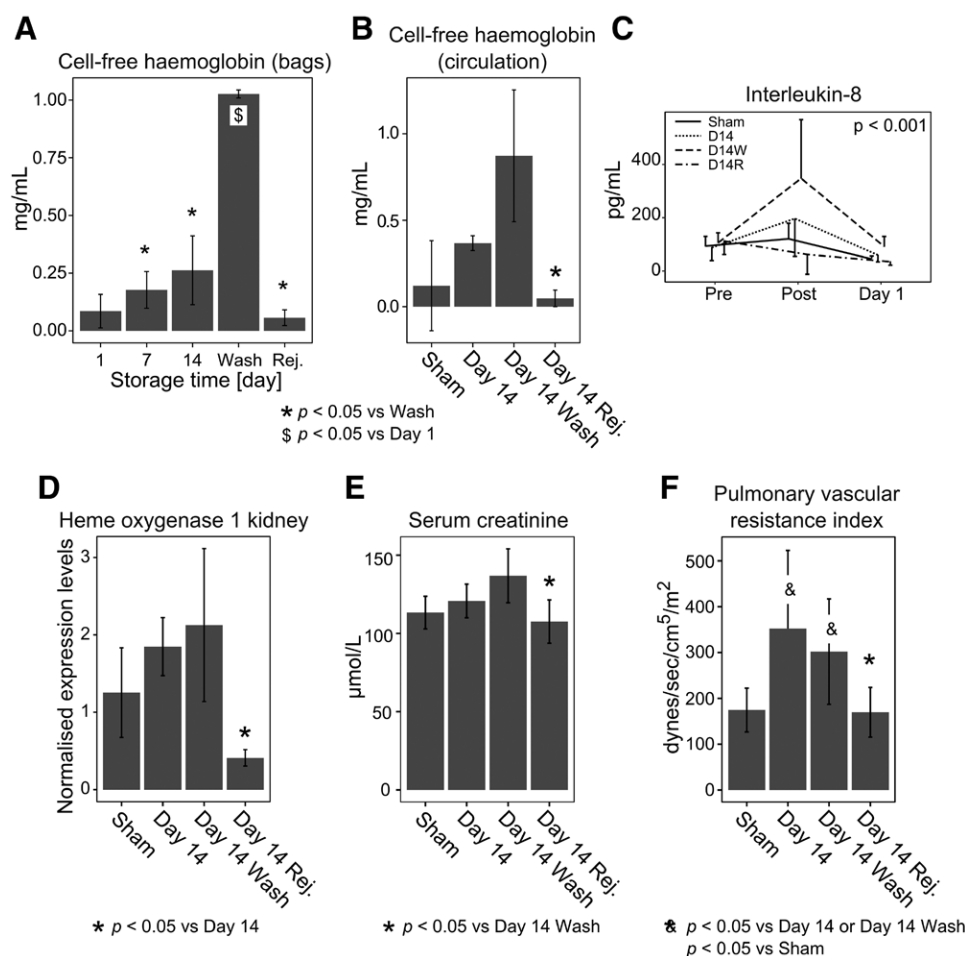
Fig. 4. Endothelial function indicators in swine. (*A*) Cell-free hemoglobin in washed (wash) and rejuvenated (Rej.) porcine red blood cells units. (*B*) Cell-free hemoglobin levels in porcine plasma samples. (*C*) Interleukin-8 levels in porcine samples. (*D*) Heme oxygenase 1 expression in porcine kidney tissue obtained from at least three animals per group. (*E*) Serum creatinine levels. (*F*) Pulmonary vascular resistance index. The values represent means ± SD, and the *P* value in (*C*) indicates significant differences between the groups (Supplemental Digital Content, http://links.lww.com/ALN/B555 and http://links.lww.com/ALN/B556, for details). Post = postintervention measure; Pre = preintervention measure.

also identified cell-free hemoglobin as a component of the storage lesion that can activate endothelial cells *via* a pathway previously only described in human aortic endothelial cells in the presence of minimally modified low-density lipoprotein.[28] Here an alternative cAMP/R-Ras/phosphoinositide-3-kinase-dependent pathway results in very late antigen 5 (integrin-α5/β1) expression, retention of alternatively spliced CS-1 fibronectin on the surface of endothelial cells, and endothelial–monocyte interaction.[29] The recovery component is a strength of the *in vivo* model. We have documented previously that pigs develop hypoxia due to atelectasis during prolonged ventilation.[4,10] Animals in the current study were recovered and ambulatory for up to 24 h before reassessment, reducing the likelihood that atelectasis may have confounded the assessment of lung function. The study also explored alternative hypotheses linking red cell storage to organ injury. The iron hypothesis proposes that transfusion of old blood leads to increased extravascular hemolysis by tissue macrophages that sense noncompliant red cells.[30] Activated

macrophages subsequently phagocytose circulating red cells that results in increased accumulation of iron in tissues and inflammation.[3,31,32] Some of our findings supported this mechanism specifically where lung injury was seen to match changes in tissue iron levels and pulmonary endothelial function. In contrast, we did not detect reductions in nitric oxide bioavailability, as a consequence of intravascular hemolysis, as has been suggested by Baek *et al.*,[2] using a guinea pig model. Rejuvenation did reduce plasma cell-free hemoglobin, renal heme oxygenase 1 expression, and renal injury relative to Washed cells; however, this may represent the stabilization of red cell membranes by rejuvenation as opposed to a reduction in intravascular hemolysis *per se*. It is notable that there were no important differences in 24-h red cell survival between the Washed and Rejuvenated groups.

Our results were consistent with two recent studies that evaluated red cell washing in ovine and canine transfusion models. In the ovine model, washing increased plasma cell-free hemoglobin and pulmonary vascular resistance as per

the findings in swine.[33] In the canine model, red cell washing reduced organ injury in recipients of red cells stored for 42 days but increased injury *via* accelerated free hemoglobin release after transfusion of washed 7-day red cells.[6] Canine red cells have limited homology to human red cells after 14 days of storage.[34,35] We speculate that mechanical washing significantly reduced red cell transfusion volume in 42-day cells in that study by lysing all but the youngest and most stable red cells in what is otherwise a very advanced storage lesion, thereby reducing injury, but produced changes in younger red cells akin to those reported in ovine and swine models.

There are several limitations to the study. First, porcine red cell transfusion is not identical to human transfusion, although in the current study we have documented homology between the storage lesion in 14-day stored allogeneic porcine red cells, significant depletion of 2,3-diphosphoglycerate, hemolysis less than 1%, ATP depletion of 50%, and 24-h *in vivo* survival of 72%, homologous to that observed toward the end of storage shelf life in humans. Second, the study was designed and powered to demonstrate differences in kidney injury between groups. The detailed exploratory analyses reported here must therefore be qualified by the observation that differences in other measured outcomes could have occurred due to chance. Third, the study subjects are hemodynamically stable animals with chronic anemia, as is common in farm-bred swine. Almost no blood is lost during the neck dissection. This may not reflect clinical red cell transfusion that often occurs in ill patients or in those experiencing blood loss. Fourth, although acute lung injury (ALI) after transfusion in the swine model meets the consensus criteria for experimental ALI,[12] they do not develop severe ALI, akin to the clinical syndrome transfusion-related acute lung injury that manifests as acute respiratory failure in less than 6 h.[12] However, animal distress due to severe ALI is unacceptable in a recovery model and likely to result in confounding. Our model may be more akin to common but less severe forms of ALI attributable to transfusion, such as transfusion associated dyspnoea.[36–39] Fifth, animals in the Washed and Rejuvenated groups may have received lower volumes of red cells as compared to Day 14 red cells, and the administration of crystalloid to the Sham group will also have led to different changes in intravascular volume as compared to the transfusion of red cells. We must therefore consider the possibility that the lung injury in the D14 group was attributable to circulatory overload. We consider this unlikely; central venous pressures and pulmonary capillary wedge pressures were similar across groups that exhibited significantly different levels of lung injury. It is also unlikely that the marginal differences in the volume of red cells transfused in the Day 14, Washed, and Redjuvenated groups could explain the divergent lung dysfunction, endothelial injury, iron accumulation, and leukocyte sequestration across the groups. Furthermore, in other experiments, we have administered an equivalent volume (4 units) of day 1 stored red cells without evidence of circulatory overload or lung injury.[40] Sixth, four animals died during the experiments, a high rate of attrition. These were terminated approximately 6 h after transfusion. We speculate that these deaths occurred as the

result of latent lower respiratory tract infections that are common in farm bred animals; in our most recent experiments we rejected any animals with respiratory signs before experimentation and have avoided these deaths. We consider it unlikely that latent respiratory tract infection in the surviving animals will have influenced our results; we demonstrated very low levels of pulmonary leukocyte infiltration in three of the four groups, and our results in the Day 14 and Sham groups mirrored those of a previous similar study.[4] Moreover, our analyses suggest that the most likely explanation for the leukocyte invasion observed in the Day 14 group is the elevated concentrations of red cell–derived microvesicles in the transfused units. We did not include the deceased animals in our analyses cohort because we considered any underlying condition a likely confounder. We also consider it unlikely that excluding these animals will have impacted on our findings; a study with six animals/group would still have 80% power to detect the differences specified in the sample size calculation.

The clinical implications of these findings are not yet clear. For example, as per the results of this study, in a recent small clinical trial,[41] we failed to demonstrate any effect of red cell washing on clinical lung or kidney injury in transfused cardiac surgery patients. We await the findings of two similar ongoing efficacy trials to confirm these results (NCT01934907 and NCT02094118). The current study also demonstrated clear benefits for rejuvenation relative to washing or standard care (storage). Recent pragmatic clinical trials (RECESS, ABLE[42,43]) that have failed to demonstrate clinical harms attributable to the storage lesion are limited in that "young" cells in these trials, stored for 7 to 10 days, already have a significant storage lesion. In contrast, rejuvenated red cells will have some properties that are comparable to truly fresh red cells.[8] We suggest that a clinical trial to evaluate the clinical efficacy and safety of rejuvenated red cells *versus* standard care would address residual uncertainty as to the clinical importance of the storage lesion.

Competing Interests

Professor Murphy; Drs. Woźniak, Cardigan, and Wiltshire; and Tracy Kumar declare that they have received grant funding for a randomized trial of inosine solution treated allogeneic red cells in cardiac surgery patients from Zimmer Biomet, the manufacturer of the inosine solution. The trial will be sponsored by the University of Leicester. The results of the current study have not been influenced by and this report has not been assisted in any way by any represen-

tative or employee of Zimmer Biomet. The other authors declare no competing interests.

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