Title: 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 continuous autotransfusion system (CATS™, Fresenius AG, Germany), or 14-day red cells rejuvenated using Rejuvesol™ solution (Zimmer Biomet, USA) and washed using the CATS™ device. Functional, biochemical, and histological markers of organ injury were assessed for up to 24 hours post transfusion.

Results: Transfusion of 14 day red cells resulted in lung injury (Lung Injury Score (LIS) versus Sham, Mean difference (MD) -0.3 (95% Confidence intervals (CI) -0.6, -0.1, p=0.02), pulmonary endothelial dysfunction and tissue leucocyte sequestration. Mechanical washing reduced red cell derived microparticles but increased cell-free hemoglobin in 14 day red cell units. Transfusion of washed red cells reduced leucocyte sequestration but did not reduce the Lung Injury Score (MD -0.2, 95%CI -0.5, 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 microparticle concentrations without increasing cell-free hemoglobin release. Transfusion of rejuvenated red cells reduced plasma cell-free hemoglobin, leucocyte sequestration, and endothelial dysfunction in recipients, and reduced lung and kidney injury relative to 14 day or Washed 14 day cells.

Conclusion: Reversal of the red cell storage lesion by rejuvenation reduces transfusion associated organ injury in swine.

Introduction

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 microparticles 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 co-incubation with a solution rich in inosine (Rejuvesol™ solution, Zimmer Biomet, IN, USA), activates glycolysis and restores cellular adenosine triphosphate (ATP) and 2,3 diphosphoglycerate (DPG) to normal or even supra-normal levels[8] that is followed by removal of the red cell supernatant in a washing step, prior to transfusion, to 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 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.

Red cell storage: Allogeneic human (from volunteers) and porcine (from eleven adult female Large-White-Landrace crossbred pigs weighing 80-100kg) whole blood was collected in Citrate-Phosphate-Dextrose (CPD), buffy coat removed, leukodepleted and stored in Sucrose-Adenosine-Glucose-Mannitol (SAG-M) using the Leukotrap WB system (Pall Medical, Portsmouth, UK) and stored at 4°C for either 14 days (porcine) or 35 days (human) according to NHS 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 continuous autotransfusion system (CATS™, Fresenius AG, Bad Homburg, Germany) and re-suspended in 0.9% normal saline. Rejuvenation was performed according to the manufacturer's instructions, the cells were washed as described using the CATS™ as above and re-suspended in saline. Red cells were transfused immediately following 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 US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study received local institutional review board approval and was conducted over a 2 year period.

Interventions: Thirty two pigs were allocated to the following groups: **Sham**, neck dissection for great vessel cannulation plus 1000mL crystalloid infusion; **Day 14**, neck dissection plus 4 units (approximately 1000ml) 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. **Rejuvenated**, neck dissection plus 4 units of Rejuvesol-treated washed 14-day old stored porcine red cell units. Post-intervention all animals were recovered, and re-anesthetized and re-evaluated after 24hr. Staff undertaking the animal

experiments were not blinded to allocation, however all laboratory assessments were performed in a blinded manner.

Anaesthesia, analgesia, monitoring, and sample size calculations are described in detail in the Supplemental Digital Content.

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. The Lung Injury Score [13] was calculated from lung compliance, and PaO2/FiO2 ratio measured in-vivo at baseline, 1.5hrs and 24hrs post-intervention using the SERVO-i Universal Ventilator (Maquet Gmbh, Rastatt, Germany) using volume controlled ventilation with a tidal volume of 10ml/kg, FiO2 of 0.5, respiratory rate of 12 breaths per minute and peak end-expiratory pressure of 5 cm/H2O. Chest radiographs were not performed. Leukocyte invasion was assessed in cryosections of porcine lung and renal biopsies with antibodies against CD14 and CD16. All histological methods are described in detail in the Supplemental Digital Content.

MP analysis was performed in citrated plasma samples spun twice at 1500 xg and RBC bags supernatant spun at 1850 xg after 1:1 dilution with PBS. Concentration and size distribution were estimated using NanoSight NS500, nano-particle tracking device (Malvern Instruments, Malvern, UK). Derivation of MV and phosphatidylserine (PS) exposure were determined with FITC-coupled CD235a, and annexin V (PS-coupled) (Affymetrix): 20 – 50 µL samples were labelled with antibodies at 1:20 dilution in annexin V binding buffer for 25 min at RT in total volume 100 µL. The samples were analysed by flow cytometry (CyAn ADP, Beckman Coulter). To separate larger MV, RBC supernatant was further spun at 28,000 xg for 35 min (**eFigure 1A**). The pellet was re-suspended in PBS (1:4 original volume, average MV concentration was 5 – 7 x 10^8 particles/ml) and stored at -80˚C, as was the remaining high speed supernatant (HS sup), for later analyses.

***In vitro* flow assay** Human microvascular pulmonary endothelial cells (HMVEC) or human umbilical vein endothelial cells (HUVECs) 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/cm^2 (**eFigure 1B**). Circulating fresh blood was obtained from healthy donors and anti-coagulated with citrate phosphate dextrose-adenine. Leukocytes were labelled 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 20x, N/A 0.4 objective, Hamamatsu Flash 4.0 camera and Collibri LED illumination system at 10 frames/sec; 301 frames were acquired every 10 min. Images were analysed with ImageJ and Mtrack2 plugin [14]. Region encoding annexin V for blocking experiments was cloned into pTrcHisA bacterial expression vector (ThermoFisher). Protein was isolated from bacterial cultures, adjusted to 1mg/mL and stored at -80˚C. For blocking equal volumes of MV and AV were incubated for 30 min and used in the assays.

Red blood cells survival was measured using a modified version of the methods of Mock et al.[15] Briefly the cells were washed with PBS, resuspended in 10 μg/ml sulfo-N-hy- droxysuccinimide-biotin in PBS (Thermo Fisher Scientific, Rockford, IL) and incubated at room temperature for 40 min. The cells were washed with PBS and resuspended in previously stored supernatant. The biotinylated-RBC survival was assessed 5, 10, 15, 30, 60, 120 min and 24 hr post-transfusion by flow cytometry (a FACSort flow cytometer driven by Summit V4.3.02 Build 2451, Beckman Coulter) and FITC-labelled streptavidin.

Biochemical markers analysis

Hematocrit (Hct) and hemoglobin, lactate and central venous oxygen saturations were performed using the ABL 800 Flex blood gas analyser (Radiometer, Copenhagen, Denmark). IL-6, a marker of myelomonocytic activation, and IL-8, a marker of endothelial cell activation,[16,17] were measured in porcine serum samples using ELISA kits from R&D Systems (Abingdon, UK). ELISA assays were performed using DS2® 2-Plate ELISA Processing System (Dynex Technologies, Chantilly, VA). Nitric

oxide in porcine plasma was measured using a colorimetric R&D Systems (Abingdon, UK) kit and Multiplate reader Enspire (PerkinElmer, Waltham, MA). 2,3-Diphosphoglycerate (2,3-DPG) levels in blood bags were measured with a commercially available kit (Roche Diagnostics, Welwyn Garden City, UK), using Multiplate reader Enspire (PerkinElmer, Waltham, MA). Adenosine triphosphate (ATP) levels were measured with a commercially available kits (ATPlite, PerkinElmer) using NovoStar reader (BMG LabTech, Offenburg, Germany). For these assays, RBC samples were deproteinised with perchloric acid as described previously [18]. Reticulocyte elongation index (EI), the unit of deformability was measured by ektacytometry using (LORCA, Mechatronics) in porcine red cells as previously described [19]. CD235a content in HS sup and in MP fraction 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, Filton, UK). Cell-free hemoglobin was estimated as described by Fairbanks et al [20]. Briefly, the samples were spun at 1,850 xg for 15 min and the resulting supernatant was further spun for 5 min at 28,000 xg. The supernatant was diluted 5x with PBS and absorbance measured at 415nm, 450nm and 700nm using Multiplate reader Enspire (PerkinElmer). Hemoglobin concentration was calculated as follows: $Hb = 1.58 x A_{415} - 0.95 x A_{450} - 2.91 x A_{700}$. Free iron was measured in plasma and tissue lysates as previously described [21]: 73.5µl of plasma/lysate was incubated with 1.5µl of 50mM ferrous iron chelator BPS (bathophenanthroline disulfonate) for 15 min. Absorbance was measured at 535 nm and compared against standard curve prepared with ferrous ammonium sulphate.

***In vitro* endothelial assays** To determine monolayer permeability, HUVEC cells grown on transwell PET inserts were incubated with 2 ml of EGM™-2 BulletKit™ medium (Lonza) containing 1 mg/ml hemoglobin, 5 µg/ml LPS, 20 % HS Sup, or PBS, and incubated at 37˚C for 6 hrs. Afterwards, medium from the top chamber was transferred into a new 6-well plate, filters placed in corresponding wells and 50 µl was taken for analysis (0' time point). 1.5 ml medium with 1 mg/ml FITC-dextran was added to the top (filter) chamber and incubated at 37˚C for 90 min. 50 µl 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, Offenburg, Germany). For endothelial activation, HUVEC cells were grown in 96 well plates until confluency and incubated for 12 hr with hemoglobin at 1 mg/ml, red cell MV, red cell HS supernatant, 5 µg/ml LPS and PBS at 20 % of total (3 wells/treatment). The cells were then fixed and processed as described above.

qRT-PCR Total RNA extraction was performed using RNeasy Fibrous Kit (Qiagen) according to manufacturer's protocol. The total RNA was quantified using a UV NanoDrop ND-1000 UV (Thermo Scientific) spectrophotometer and Agilent 2100 Bioanalyzer using the Eukaryotic RNA Assay with the RNA 6000 Nano LabChip® Kit (Agilent Technologies, Santa Clara, CA). One µg of total RNA was reverse transcribed at 42˚C using a Sensifast C-DNA synthesis kit (Bioline, London 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, London UK). Each reaction included a reverse transcription negative control to confirm the absence of genomic DNA and a non-template negative control for primer-dimer. Primers for control housekeeping genes were designed as previously reported in [22] and sequences are listed in the Supplementary Content. The real time PCR was run on Rotorgene Q (Quiagen, Venlo, Limburg, Netherlands) 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 per 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 post intervention measures per animal and an assumed correlation between pre and post measures and between post measures 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 physiological and immunological 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 **eTable 2**. Each of the transformed (or un-transformed) physiological and immunological variable was analysed 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 centred 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 (**eTable 1**). In the presence of 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) (**eTable 2**). 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 analysed 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, confidence intervals along with unadjusted and adjusted p-values are shown in **eTable 5**). All statistical analyses were carried out in the R software environment [23] with appropriate packages (nlme, multcomp, lsmeans, ggplot2).

Results

Anaesthesia and monitoring: There was no difference for measures of cardiovascular function, 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, **Figure 1 and eTable 1 – 3).** Transfused pigs had significantly higher hemoglobin and haematocrit than **Sham** pigs (**eTables 1 – 2**). Twenty four hour *in vivo* survival of stored red cells ranged from 68 to 72% (**eTable 4**). 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, **Figure 1A and eTable 4**).

Transfusion and Immune Cell Activation

In pre-specified analyses we demonstrated that transfusion of **Day 14** stored red cells had no effect on renal function (creatinine clearance) in swine; Day 14 versus Sham Mean difference (MD) 10.5 ml/min (95% Confidence intervals (CI) -33.3, 54.4, p=0.62, but resulted in increased Lung Injury Scores; Day 14 versus Sham MD -0.3 (95% Confidence intervals (CI) -0.6, -0.1, p=0.02), capillary leak of proteins into the bronchoalveolar lavage fluid, accumulation of leucocytes in porcine lung and kidney, and serum IL-6, a marker of myelomonocytic inflammation (**Figure 2A-E and eTables 2&5**). Creatinine clearance was significantly increased in recipients of **Rejuvenated** versus **Day 14** red cells; MD 49.8, 95%CI 7.6, 92.0, p=0.02, **Figure 2A**. The Lung Injury Score observed in **Day 14** swine was significantly reduced in the **Rejuvenated**; MD -0.4, 95%CI -0.6, -0.1, p=0.01, but not the **Washed** group; MD -0.2, 95%CI -0.5, 0.1, p=0.13, **Figure 2B**. BAL protein levels were reduced by Washing, with a further significant reduction with Rejuvenation (**Figure 2C**). Leucocyte accumulation in porcine lung and kidney, and serum IL-6 levels were significantly reduced in both **Washed** and **Rejuvenated** groups (**Figure 2D 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 analysed the storage characteristics of porcine and human red cells. In swine **14 Day** storage was characterised by reduction in 2,3DPG, an approximate 50% reduction in ATP from baseline, reduced red cell deformability, hemolysis levels <1% and accumulation of red cell derived microparticles (**eTable 4**). Mechanical washing, which is also required for rejuvenation, reduced microparticles concentrations in stored porcine units (**Figure 3A**). In parallel, in stored human red cells we demonstrated that microparticles approximately 200 nm in size, positive for red cells markers (CD235a) and annexin V increased in stored human red cell units from day 3 to day 35 of storage (**Figure 3B, and eFigure 1A**). In human, as in porcine blood bags, Washing and Rejuvenation significantly reduced the levels of red cell derived microparticles versus Day 41 controls (**Figure 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.
To test this hypothesis, we separated the different constituents of the human red cell units using differential centrifugation into red cells, red cell derived microparticles (200nm), and the supernatant fraction (**eFigure 1B)**. 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] (**Figure 3C**, see supplemental Methods) where the attachment of leukocytes circulating in whole blood to a human microvascular endothelial cell monolayer is observed in real time using fluorescent microscopy (**Movie A**). We observed that isolated microvesicles but not isolated washed red cells (stored >22 day) or the microparticle free supernatant isolated by high speed centrifugation (HS Sup) resulted in leucocyte adhesion after 1 hour of flow (**Figure 3D**). In further experiments we demonstrated that blocking phosphatidylserine and other negatively charged lipids on the surface of the microvesicles by co-incubation with Annexin V prevented leucocyte activation (**Figure 3E**). We concluded that annexin V-

Commented [MGJ(1]: Move this to the Legend: In the flow assay, donor blood was mixed with samples from whole red cell units that were stored for less than 7 days (<7d RBC), or 35 days that was then either washed (Washed) or not (Old), or the high speed centrifugation supernatant fraction at 5:1 ratio. The microparticle fraction was mixed with the donor blood at 1:10 ratio.

recognised lipids on red cell derived microparticles that accumulate in stored blood bags are likely responsible for leukocyte activation observed following 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, **eTable 4**), increased release of cell-free hemoglobin into the storage supernatant following mechanical washing (**Figure 4A**), and increased plasma cell-free hemoglobin as well as serum IL-8, a putative biomarker of endothelial activation, in transfusion recipients (**Figure 4 B,C**). In comparison **Rejuvenated** units demonstrated red cell deformability comparable to fresh red cells (**eTable 4**) and significant reductions in cell-free haemoglobin compared to **Washed** or unwashed **Day 14** red cells (**Figure 4A**). Recipients of **Rejuvenated** units also demonstrated reductions in plasma cell-free hemoglobin, serum IL-8 levels, renal HMOX-1 expression, and clinical biomarkers of acute kidney injury including serum creatinine and urine NGAL (**Figure 4B-E, eTables 2 and 5**). We observed no significant difference between the groups with respect to nitric oxide bioavailability that is diminished by intra-vascular hemolysis, as per the 'nitric oxide hypothesis', although recipients of **Day 14** and **Washed** red cells had the lowest levels of nitric oxide (**eTable 2**). 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* (**Figure 4F**). This corresponded well to differences in lung iron levels, a marker of extravascular hemolysis as per the 'iron hypothesis', (**eTable 5**). 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 and 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 (**eFigure 1C**). In further experiments we observed that endothelial activation by hemoglobin did not occur via canonical (CD62E) signalling, as observed following exposure to lipopolysaccharide treated controls (**Figure D, E**), but via expression of integrin β1/α5 (VLA5) and retention of CS1 fibronectin, a putative non-canonical 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* characterised by alveolar capillary leak and leucocyte sequestration. Red cell derived microparticles that express annexin V-binding lipids accumulate in stored units and result in leukocyte activation *in vitro*. Washing reduces microparticle 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 stabilising red cell membranes and preventing cell-free hemoglobin release post washing. Pigs transfused with **Rejuvenated** red cells had reduced lung injury, reduced levels of leukocyte invasion and serum IL-8 compared to those receiving **Day 14 red cells**, and reduced renal HMOX-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 microparticles and free hemoglobin act via distinct

mechanisms on leucocytes and endothelial cells respectively. These processes could contribute to a putative multi-hit pathogenesis of post transfusion organ injury.[26,27] We demonstrated that transfusion can activate leucocytes through pro-inflammatory lipids present on microparticles that accumulate during storage. We 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/PI3K dependent pathway results in VLA5 (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 hours before re-assessment, 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 extra-vascular hemolysis by tissue macrophages that sense non-compliant 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 NO 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 HMOX-1 expression, and renal injury relative to **Washed** cells, however this may represent the stabilisation 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 hour 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 haemoglobin 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 following 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 days 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.

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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 2,3DPG, hemolysis <1%, ATP depletion of 50% and 24 hour *in vivo* survival of 72%, homologous to that observed towards 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 anaemia, 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) following transfusion in the swine model meets the consensus criteria for experimental ALI [12] they do not develop severe ALI, akin to clinical syndrome TRALI that manifests as acute respiratory failure <6 hours.[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 for example 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 leucocyte 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 (Patel NN, Lin H, Abidoye A, et al. Washing of stored red cells prevents transfusion related acute lung injury but not transfusion associated coagulopathy. Circulation. 2012;126:A18252). Sixth, four animals died during the experiments, a high rate of attrition. These were terminated approximately 6 hours following 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 reject any animals with respiratory signs prior to 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 leucocyte infiltration in 3 of the 4 groups, and our results in **Day 14** and **Sham** groups mirrored those of a previous similar study [4]. Moreover, our analyses suggest that the most likely explanation for the leucocyte 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 as 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 6 animals per group would still have 80% power to detect the differences specified in the sample size calculation.

Our results may have clinical implications. For example, as per the results of this study, in a recent small clinical trial[40] 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, NCT02094118). The current study also demonstrated clear benefits for **Rejuvenation** relative to **Washing**, or Standard care (storage). Recent pragmatic clinical trials (RECESS, ABLE [41,42]) that have failed to demonstrate clinical harms attributable to the storage lesion are limited in that 'young cells' in these trials, stored for 7-10 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.

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Figure legends

Figure 1 – Experimental design: A – Study schematic; **B** – Hemoglobin and **C** – Hematocrit levels in experimental animals. Values represent means +/-SD and *p*-value indicates significant differences between the groups (see eTables 1 – 2 for details).

Figure 2 – Effects of red cell washing and rejuvenation in vivo: A – Creatinine clearance rates, **B** – Lung injury score, **C** - BAL protein levels, **D** – Quantified images of labelled porcine lung and kidney cryosections. Approximately 20 images were collected from at least 3 sections per animal per group. Tissue samples came from 2 – 4 animals per group. **E** – Serum IL-6 levels; Values represent mean +/-SD; *p*-value in **B** and **E** indicates significant differences between the groups (see eTables 1 – 2 for details).

Figure 3 – MP analysis in red blood cell bags: A – Annexin V positive MP in aging porcine blood bags (min 3 units analysed). **B** – Fractional changes in MP subtypes during storage and after washing and rejuvenation – 8 units were analysed with indicated antibodies. **C** – Schematics depicting a flow system used to analyse leukocytes and platelets in real time as described in Materials and Methods. **D** – Effect of stored blood and MP fractions on leukocyte adhesion under flow *in vitro* (at least 3 independent repeats). Donor blood was mixed with samples from whole red cell units that were stored for less than 7 days (<7d RBC), or 35 days that was then either washed (Washed) or not (Old), or the high speed centrifugation supernatant fraction at 5:1 ratio. The microparticle fraction was mixed with the donor blood at 1:10 ratio. **E** – Effect of annexin V blocking on leukocyte adhesion. Values represent mean +/-SD.

Figure 4 – Endothelial function indicators in swine: A – Cell-free hemoglobin in Washed and Rejuvenated porcine RBC units; **B** – Cell-free hemoglobin levels in porcine plasma samples; **C** – IL-8 levels in porcine samples; **D** – HMOX-1 expression in porcine kidney tissue obtained from at least 3 animals per group; **E** – Serum creatinine levels; **F** – Pulmonary vascular resistance index. Values represent mean +/-SD and *p*-value in **C** indicates significant differences between the groups (see eTables 1 – 2 for details).