Transfusion-related acute lung injury in the critically ill: a translational approach

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Supernatant of aged red blood cells causes lung inflammation and coagulopathy in a “two hit” *in vivo* syngeneic transfusion model


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Abstract

Background: Transfusion of red blood cells (RBCs) is associated with increased morbidity in certain patient groups. Storage time of RBCs may contribute to respiratory complications. Using a syngeneic in vivo transfusion model, we investigated whether transfusion of stored rat RBCs cause lung injury in healthy and in lipopolysaccharide-primed rats in a “two hit” model of lung injury.

Methods: Rats were infused with aged rat RBCs (14 days of storage), washed aged RBCs or supernatant of aged RBCs. Controls received fresh rat RBCs (0 days of storage) or saline. In a “two hit” model of lung injury, lipopolysaccharide was used as a “first hit” prior to transfusion. Rat and control human RBC products were analyzed for lysophosphatidylcholine accumulation.

Results: In healthy rats, transfusion of aged RBCs caused mild pulmonary inflammation, but no coagulopathy. In lipopolysaccharide–pretreated rats, transfusion of aged RBCs augmented lung injury by inducing coagulopathy, both in the pulmonary and systemic compartment, when compared to transfusion with fresh RBCs. When transfused separately, supernatant of aged RBCs, but not washed aged erythrocytes, mediated coagulopathy in the “two hit” model. Analysis of the supernatant of aged RBCs (rat and human) showed no lysophosphatidylcholine accumulation.

Conclusions: Transfusion of aged RBCs induces lung injury in healthy rats. In a “two hit” model, injury induced by aged RBCs was characterized by coagulopathy, and was abrogated by washing. Washing of aged RBCs may decrease pulmonary complications in patients with an inflammatory condition who are exposed to a blood transfusion.
Introduction

Transfusion of red blood cells (RBCs) has increased in the past years. This increase may be explained by an aging population and evolving surgical and medical procedures.\(^1\) Annually, almost 14,000,000 RBC units are transfused in the United States.\(^2\) However, it is increasingly recognized that transfusion of RBCs is associated with morbidity and mortality in certain patient populations, including critically ill, post-operative and trauma patients.\(^3\) The age of red blood cells has been implicated as a causative factor in transfusion-related complications.\(^4\)\(^-\)\(^10\) In particular, transfusion of aged RBCs is associated with respiratory complications.\(^8\)\(^,\)\(^11\) The mechanism linking adverse outcomes with RBC storage remains unclear. A decreased deformability capacity, increased adhesiveness of the aged erythrocyte, donor white blood cells and soluble factors such as cytokines and bio-active lipids (i.e. lysophosphatidylcholines) have all been suggested to mediate adverse effects.\(^12\)\(^-\)\(^21\)

Aged blood products have been associated with the occurrence of transfusion-related acute lung injury (TRALI) in the clinical setting\(^18\) and have been used to induce TRALI in “two hit” animal models.\(^17\),\(^22\) In the “two hit” hypothesis, TRALI is the result of endothelial activation, caused by an underlying inflammatory condition (e.g. pneumonia or sepsis), resulting in priming of the pulmonary neutrophils. This “first hit” is followed by activation of the primed neutrophils caused by the “second hit” (transfusion of a blood product), resulting in activation of the primed neutrophils, with subsequent endothelial damage and capillary leak, leading to pulmonary edema. Coagulopathy and decreased fibrinolysis are a distinctive feature of acute lung injury due to other causes,\(^23\),\(^24\) contributing to morbidity and mortality.\(^25\),\(^26\) As the endothelium initiates and regulates coagulation,\(^27\) it can be hypothesized that coagulopathy may also play a role in TRALI. However, data on coagulation during TRALI are absent.

The “two hit” hypothesis has been proposed as a mechanism to explain why critically ill patients, who frequently suffer from an inflammatory condition, are susceptible to a TRALI reaction.\(^28\)-\(^30\) Because transfusion is associated with adverse outcome, at least in certain patient groups including trauma patients and the critically ill,\(^11\),\(^31\)-\(^37\) it is important to study pathways of disease in models that represent the clinical situation. Present TRALI models investigating the role of storage time of blood products are limited by cross species design and modification of transfusion protocols.\(^17\),\(^22\),\(^38\) Currently, no clinically relevant “in species” transfusion model using a clinical preparation protocol has confirmed the hypothesis that aged RBCs contribute to lung injury.\(^39\) We investigated the effect of aged rat RBCs on lung inflammation and coagulation in a syngeneic in vivo RBC transfusion model in healthy rats. As a model of patients with an underlying inflammatory condition, the effect of aged RBCs was also studied in a “two hit” model of lung injury, using
lipopolysaccharide-primed rats. In addition, we evaluated whether washing of RBCs influenced the development of lung injury inflicted by transfusion.

Materials and Methods

The Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, The Netherlands and the Medical Ethical Committee of Sanquin Blood Bank Foundation approved all experiments. All animals were handled in accordance with the guidelines prescribed by the Dutch legislation and the International Guidelines on protection, care, and handling of laboratory animals.

Preparation of rat RBC products

Male Sprague–Dawley rats (> 250 g, Harlan; The Hague, The Netherlands) were used to obtain blood. Rats were anesthetized with an intramuscular injection of ketamin 45 mg/kg (Eurovet; Bladel, The Netherlands) and medetomidin 0.25 mg/kg (Novartis; Arnhem, The Netherlands). Blood was collected from the vena cava inferior in a syringe containing 1.25 ml citrate-phosphate-dextrose (Fersenius HemoCare GmbH; Bad Homburg, Germany). Approximately 8-10 ml of blood could be obtained from a single rat. Blood of five rats was pooled for component preparation. Prior to pooling, cross-matching was carried out to ensure compatibility.

Blood was handled and stored according to national standards for human blood (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands), with minor changes to adapt for the smaller volumes. After overnight storage at room temperature, blood was centrifuged for 10 min at 1,892 g and 20 °C. Plasma was removed and the buffy coat was separated from the packed red blood cells. Saline-adenine-glucose-mannitol was added to the red blood cells up to a hematocrit of 55-60%. The final products were stored in 50 ml falcons at 4 °C, which were partly open.

Preparation of washed aged RBCs and supernatant rat RBC products

After 14 days of storage, rat RBC products were separated into washed erythrocytes and supernatant. NaCl 0.9% was added to rat RBC products before centrifugation to obtain as much as possible soluble factors from the product. The RBC/NaCl mixture was centrifuged for 15 min at 1,500 g and 4 °C. For the final supernatant used in the experiment, NaCl 0.9% was added to the supernatant up to the original volume of the rat RBC product. The erythrocytes were washed using saline-adenine-glucose-mannitol (1:1) and centrifuged for 15 min at 1,250 g and 4 °C. The supernatant was
removed and the saline-adenine-glucose-mannitol was added to the erythrocytes up to the original volume of the rat RBC product.

In vivo RBC transfusion models
Male Sprague-Dawley rats (275 g) raised on a regular diet were weighed and anaesthetized with pentobarbital 50 mg/kg intra-peritoneally. The tail vein was cannulated with a 24 gauge venflon (Vasofix Certo; B.Braun, Meisungen, Germany) and blood was aspirated to verify intravascular placement and to remove 0.5 ml of blood for cross-matching and baseline measurements. A 10% circulating volume transfusion was administered over 30 minutes using an infusion pump (Harvard Pump 11, Harvard Apparatus; Holliston, MA).

Animals were randomized by an independent researcher into 3 groups (n=6 per group) to receive transfusion with NaCl 0.9%, fresh RBCs or RBCs stored for 14 days (aged RBCs). A storage time of 14 days was chosen, because pilot experiments showed that rat RBCs stored for 14 days showed storage-related changes, which were comparable to storage-related changes found in previous studies comparing human RBCs stored for 28-35 days to rat RBCs stored for 14 days.41 Rats were placed back in their cages to recover and were sacrificed 6 hours after transfusion. In a separate set of experiments, animals were transfused with washed aged erythrocytes or with supernatant of washed aged erythrocytes. For the experiments in the “two hit” RBC transfusion model, rats received 2 mg/kg of lipopolysaccharide (from Salmonella enteritidis, Sigma; St. Louis, MO) intra-peritoneally two hours prior of transfusion. This dose have been used before as a “first hit” in TRALI models, including a model using aged human RBCs, and was shown to result in sequestration of neutrophils in the lungs.17,22,40 Controls received saline (equal volume).

Blood and tissue sampling
After anesthesia with ketamin and medetomidin as described, blood was collected from the vena cava inferior in citrated (0.109 M) vacutainer tubes for analysis and blood culture. The right lung was ligated and the left lung was lavaged three times with 2 ml of saline. After lavage, lungs were weighed and homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK). For cytokine and chemokine measurements, lung homogenates were diluted 1:1 in lysis buffer (150 nmol/l NaCl; 15 mmol/l Tris; 1 mmol/l MgCl₂–H₂O; 1 mmol/l CaCl₂; 1% Triton X-100; and 100 µg/ml pepstatin A, leupeptin, and aprotinin). The right lung was fixed in 4% formalin and embedded in paraffin for histopathology examination. 4 µm sections were stained with hematoxylin–eosin, and analyzed by two researchers who were blinded for group identity. A histology scoring system was used as previously described.42 In short, the following parameters were scored on a scale of 0 – 4: (a)
interstitial inflammation, (b) endothelialitis, (c) bronchitis, (d) edema, (e) thrombus, and (f) pleuritis. The histology score was expressed as the sum of the score for all parameters.

**Assays**

Thrombin–antithrombin complexes (TATc; Behring, Marburg, Germany) and fibrin degradation products (Asserachrom D–Di, Diagnostica Stago, Asnières-sur-Seine, France) were measured using Enzyme-Linked Immuno Sorbent Assay. Plasminogen activator activity, and plasminogen activator inhibitor–1 activity were measured by automated amidolytic assays. Tumor necrosis factor, interleukine–6, and cytokine-induced neutrophil chemoattractant–3 were measured by Enzyme-Linked Immuno Sorbent Assay according to instructions from the manufacturer (R&D Systems, Abingdon, United Kingdom). Detection limit was 62.5, 31.25 and 125 pg/ml, respectively.

**Storage-related biochemical changes in rat RBCs.**

RBC samples were collected at the indicated time intervals and analyzed for pH, potassium, sodium, glucose and lactate with a Rapidlab 865 blood gas analyzer (Siemens Medical Solutions Diagnostics; Breda, The Netherlands). Cell counts for leukocytes and red blood cells were done with an Advia 2120 hematology counter, with special software for counting animal blood samples (Siemens Medical Solutions Diagnostics). Supernatants were prepared by centrifugation for 10 minutes at 14,500 g at 4 °C to remove cells and a-cellular debris. Aliquots of supernatants were stored at -80 °C for analysis of lysophosphatidylcholine, phosphatidylcholine and cytokine levels.

**Lipid extraction and lysophosphatidylcholine and phosphatidylcholine measurement**

Lipid extraction of supernatant from stored RBC supernatant was performed using Bligh and Dyer method. In short, 3 ml of CHCl₃:MeOH (1:2) was added to 100 µl of sample and 100 µl of internal standard solution (lysophosphatidylcholine 14:0, 2.5 nmol and phosphatidylcholine 28:0, 10 nmol). 700 µl HAc 0.5%, 1 ml CHCl₃ and 800 µl of HAc 0.5% were added. After each step samples were vortexed for 30 seconds. The final mixture was centrifuged for 10 minutes at 1,892 g at room temperature. After centrifugation, the lower layer of CHCl₃ was separated. This step was repeated two times by adding 1 ml CHCl₃. The separated CHCl₃ layers were combined and dried (N₂, 30°C). Samples were dissolved in 150 µl CHCl₃/MeOH/H₂O/NH₃ 25% (50/45/5/0.01 v/v/v/v) for further analysis.
High Performance Liquid Chromatography tandem mass spectrometry

The relative concentrations of lysophosphatidylcholines and phosphatidylcholine species in supernatant of RBCs were determined using High Performance Liquid Chromatography tandem mass spectrometry (HPLC-MS/MS). 10 µl of extracted lipid sample was injected on the HPLC-MS/MS system. Chromatographic separation was achieved on a modular HPLC system (Surveyor; Thermo Finnigan; San Jose, CA) consisting of a cooled autosampler (T=12°C), a low-flow quaternary MS pump and analytical HPLC column: LichroSpher Si60, 2 ×250 mm column, 5 µm particle diameter (Merck; Darmstadt, Germany). Samples were eluted with a flow rate of 300 µl/minute and a programmed linear gradient between solution B (chloroform-methanol, 97:3, v/v) and solution A (methanol-water, 85:15, v/v); A and B contained 1 ml and 0.1 ml of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient was: T = 0 -10 minutes: 20% A to 100% A; T = 10-12 minutes, 100% A; T= 12-12.1 minutes: 100% A to 0% A; and T= 12.1–17 minutes, equilibration with 0% A. Total run-time, including the equilibration, was 17 minutes. A splitter between the HPLC and MS was used for the introduction of the eluent in the MS by 75 µl/minutes.

MS/MS analyses were performed on a TSQ Quantum AM (Thermo Finnigan; Waltham, MA) operated in the positive ion electrospray ionization mode. The Skimmer Offset was set at 10 V; spray voltage was 3600 V and the capillary temperature was 300°C. In the optimized MS/MS experiments, argon was used as collision gas at a pressure of 0.07 Pa and a collision energy of 40 V. The parent ion scan of m/z 184.1 (m/z 400 - m/z 1000, 1 s) was used for the quantization of the following precursor ions: m/z 468.3 (lysophosphatidylcholine 14:0, I.S.), m/z 496.3 (lysophosphatidylcholine 16:0), m/z 524.3 (lysophosphatidylcholine 18:0/ Platelet Activating Factor (PAF) 16:0), m/z 522.4 (lysophosphatidylcholine 18:1), m/z 482.4 (LysoPAF 16:0), m/z 510.4 (LysoPAF 18:0), m/z 508.4 (LysoPAF 18:1), m/z 678.4 (phosphatidylcholine 28:0, I.S.), m/z 758.4 (phosphatidylcholine 34:2), m/z 782.4 (phosphatidylcholine 36:2).

Storage-related biochemical changes in human RBCs

Healthy adult volunteers (n=5) donated 1 unit of whole blood (500 ml), collected in citrate-phosphate-dextrose (70 ml) and stored for 12 to 18 hours at 20 to 22°C. Leuko-reduced RBCs were prepared by centrifugation for 8 minutes at 2,800 g. After removal of plasma and buffy coat, 110 ml of the standard storage medium saline-adenine-glucose-mannitol was added via the filter to the packed red cells, which were subsequently leukoreduced by filtration. The RBCs were stored at 4 °C according to National Blood Bank standards. Supernatants were collected at day 0, 35 and 42 and prepared by centrifugation for 10 minutes at 14,500 g at 4 °C to
remove cells and a-cellular debris. Aliquots of supernatants were stored at -80 °C for analysis of lysophosphatidylcholine and phosphatidylcholine levels.

**Statistical analyses**

Data are expressed as mean ± SEM. A paired t–test was used to compare the results of RBCs before and after storage. Comparisons between the rat groups were performed using student t-test, one–way ANOVA, followed by post–hoc Dunnett’s. A $p$–value < 0.05 was considered statistically significant. Statistical analyses were performed with SPSS 12.0 (SPSS, Chicago, IL) and Prism 4.0 (GraphPad Software, San Diego, CA).

**Results**

All animals completed the experimental protocol. Blood cultures from the blood products and from the rats collected at the end of the experimental protocol showed no outgrowth of bacteria.

**Effect of transfusion of aged rat RBCs in healthy rats**

Transfusion of aged RBCs resulted in endothelial neutrophil sequestration and edema in lung tissue (fig. 1), with a concomitant higher histopathology score compared to transfusion of fresh RBCs and saline control groups ($p<0.05$, fig. 2). Aged RBCs also caused an increase of interleukine-6 and cytokine-induced neutrophil chemoattractant-3 concentrations in the lung homogenate of healthy animals ($p<0.01$ compared to controls, fig. 3). Aged RBCs did not increase markers of pulmonary coagulation compared to fresh RBCs (TATc (mean±sem): 1.5±0.4 vs. 1.6±0.4 ng/ml, ns; fibrin degradation products: 94±15.3 vs. 99±9.8 ng/ml, ns) nor did aged RBCs impair fibrinolysis by reducing plasminogen activator activity (38%±7.4 vs. 36%±4.5, ns) or increasing fibrinolytic inhibitor plasminogen activator inhibitor-1 (6.9±1.9 vs. 6.4±1.6 ng/ml, ns).

**Effect of transfusion of aged rat RBCs in lipopolysaccharide-primed rats**

To determine whether the “two hit” effect could be reproduced in our syngeneic model, we repeated transfusion with aged RBCs in lipopolysaccharide-pretreated animals. In this experiment, lipopolysaccharide-pretreatment resulted in lung injury, exemplified by neutrophil sequestration in the lung endothelium and pulmonary edema, with an elevated histopathology score ($p<0.001$, fig. 1 and 2) and increased levels of interleukine-6 and cytokine-induced neutrophil chemoattractant-3 in lung homogenate ($p<0.01$, fig. 3) compared to saline controls. Furthermore, lipopolysaccharide-pretreatment increased pulmonary coagulation as shown by
increased thrombin generation (as reflected by TATc, fig. 4) and increased fibrin degradation products levels. In addition, fibrinolysis was impaired, as evidenced by reduced plasminogen activator activity levels (in percentage, %), caused by an increase in the levels of the fibrinolytic plasminogen activator inhibitor-1 compared

Figure 1. Histologic sections of hematoxylin and eosin stained rat lungs at 20x magnification. (a): Saline control, (b): Lipopolysaccharide (LPS) control, (c): Saline + red blood cells (RBCs) day 0, (d): LPS + RBCs day 0, (e): Saline + RBCs day 14, (f): LPS + RBCs day 14. Normal vasculature (arrow) (a and c). Neutrophils sequestrated in the vasculature (arrow) (b,d-f). N=6 per group. For color figure see page 356
to saline controls. Lipopolysaccharide-pretreatment also increased TATc levels in plasma compared to saline controls, indicating increased systemic coagulation.

Transfusion of aged RBCs in lipopolysaccharide-primed animals did not further augment pulmonary inflammation, shown by an unaltered histopathology score (fig. 1 and 2) and a non-significant increase in pulmonary cytokine and chemokine levels compared to lipopolysaccharide-primed rats transfused with saline or fresh RBCs (fig. 3). Aged RBCs worsened pulmonary coagulopathy in lipopolysaccharide-pretreated animals compared to saline controls. Lipopolysaccharide-pretreatment also increased TATc levels in plasma compared to saline controls, indicating increased systemic coagulation.

**Figure 2.** Histology scores on lung injury. Lung injury score was significant increased in lipopolysaccharide (LPS)-pretreated animals compared to saline controls. Aged red blood cells (RBC day 14) increased lung injury compared to fresh RBCs (day 0) in healthy animals but did not further augment injury in LPS pretreated animals. Data are presented as mean±sem. *p<0.05, **p<0.001. N=6 per group. One way-ANOVA analysis, followed by post-hoc Dunnett’s.

**Figure 3.** Levels of interleukin (IL)-6 and cytokine-induced neutrophil chemoattractant (CINC)-3 in the lung homogenate of healthy animals (A and D) and LPS-primed animals infused with aged red blood cells (RBCs day 14), fresh RBCs (day 0), or saline (B and E) (one way-ANOVA analysis, followed by post-hoc Dunnett’s). Additional experiments (C and F) show the effect of infusion of supernatant (Sup) of aged RBC blood products or washed (Wash) aged erythrocytes in lipopolysaccharide (LPS)-primed animals (Student t-test). Data are presented as mean ± sem. *P< 0.01. N=6 per group.
Figure 4. Concentrations of thrombin anti-thrombin complexes (TATc), plasminogen activator activity (PAA%), plasminogen activator inhibitor (PAI-1) and fibrin degradation products (FDP) in the broncho-alveolar lavage fluid (BALF) of lipopolysaccharide (LPS)-primed animals transfused with stored red blood cells (RBCs day 14), fresh RBCs (day 0), saline (control), supernatant (Sup) of aged RBC blood products or washed (Wash) aged erythrocytes. Data are presented as mean ±sem. Aged RBCs and supernatant of aged RBCs activate lung coagulation and decrease fibrinolysis as shown by an increase in TATc level in the BALF and decrease of PAA% and increase of PAI-1 levels in the BALF respectively. *P< 0.05, **P<0.01. N=6 per group. Student t-test (right column) and one way-ANOVA analysis, followed by post-hoc Dunnett’s (left column).
primed animals, by increasing broncho-alveolar lavage fluid levels of TATc compared to lipopolysaccharide controls receiving saline or fresh RBCs (P<0.01, fig. 4). Also, aged RBCs strongly contributed to impaired fibrinolysis in lipopolysaccharide-primed animals, decreasing plasminogen activator activity in the broncho-alveolar lavage fluid and increasing the level of plasminogen activator inhibitor-1 compared to lipopolysaccharide controls receiving fresh RBCs (P<0.05 for both). In addition, aged RBCs further augmented systemic coagulation, by increasing plasma TATc level compared to the lipopolysaccharide controls receiving fresh RBCs (fig. 5, P<0.001).

Effect of transfusion of washed aged erythrocytes vs. supernatant of aged RBCs in healthy and lipopolysaccharide-primed rats

To determine whether lung injury was due to soluble factors in the storage medium or to the aged erythrocyte itself, aged RBCs were washed and separated from supernatant. Using these products, we repeated experiments in healthy and

![Figure 5](image-url)

**Figure 5.** Systemic concentrations of thrombin anti-thrombin complexes (TATc) and plasminogen activator activity (PAA) in the plasma of lipopolysaccharide (LPS)-primed animals transfused with stored red blood cells (RBCs day 14), fresh RBCs (day 0), saline (control), supernatant (Sup) of aged RBC blood products or washed (Wash) aged erythrocytes. Data are presented as mean ±sem. Aged RBCs and supernatant of aged RBCs activate systemic coagulation and decrease fibrinolysis as shown by an increase in TATc level and decrease of PAA% in the plasma respectively. **P<0.01. N=6 per group. Student t-test (right column) and one way-ANOVA analysis, followed by post–hoc Dunnett’s (left column).
lipopolysaccharide-pretreated animals. In healthy rats, transfusion of both washed aged erythrocytes and supernatant of aged RBCs reproduced the findings of the previous experiment, increasing pulmonary cytokine and chemokine levels (data not shown). Washing of the aged RBCs did not prevent the onset of pulmonary inflammation in healthy rats.

In lipopolysaccharide-primed rats, transfusion of supernatant, but not of aged washed erythrocytes, worsened lung inflammation and coagulation, comparable to the previous experiment. Supernatant of aged RBCs increased pulmonary levels of interleukine-6 and cytokine-induced neutrophil chemoattractant-3 compared to rats receiving washed aged erythrocytes (P<0.01, fig. 3), as well as an increase in levels of TATc and decrease in plasminogen activator activity in broncho-alveolar lavage fluid compared to those receiving washed aged erythrocytes (P<0.01, fig. 4). Also, the increase in systemic levels of TATc caused by RBC products was reproduced after transfusion of supernatant but not after transfusion of washed aged erythrocytes (P<0.001, fig.5).

Table 1. Storage-related biochemical changes in rat RBCs

<table>
<thead>
<tr>
<th></th>
<th>Whole blood (fresh)(^a)</th>
<th>RBCs day 0(^b)</th>
<th>RBCs day 14(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K(^+) (mmol/l)</td>
<td>5.3 ± 0.2</td>
<td>3.6 ± 0.4</td>
<td>31.2 ± 2.1(^*)</td>
</tr>
<tr>
<td>Na(^+) (mmol/l)</td>
<td>154 ± 4.2</td>
<td>146 ± 1.8</td>
<td>126 ± 2.0(^**)</td>
</tr>
<tr>
<td>pH</td>
<td>6.9 ± 0.1</td>
<td>7.1 ± 0.0</td>
<td>6.6 ± 0.01(^**)</td>
</tr>
<tr>
<td>Lactate (mmol/l)</td>
<td>6.2 ± 1.2</td>
<td>7.0 ± 0.7</td>
<td>16.9 ± 3.1(^**)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>8.7 ± 0.5</td>
<td>18.5 ± 2.7</td>
<td>7.1 ± 0.3(^**)</td>
</tr>
<tr>
<td>Leukocytes (x 10/9/l)</td>
<td>5.4 ± 0.6</td>
<td>4.5 ± 0.8</td>
<td>3.6 ± 0.5</td>
</tr>
<tr>
<td>Hb (mmol/l)</td>
<td>6.0 ± 0.2</td>
<td>9.9 ± 0.3(^†)</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.34 ± 0.01</td>
<td>0.58 ± 0.02(^†)</td>
<td>0.53 ± 0.02(^**)</td>
</tr>
<tr>
<td>LysoPC 16:0 (µM)</td>
<td>91.8 ± 6.9</td>
<td>87.3 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>LysoPC 18:1 (µM)</td>
<td>22.9 ± 2.6</td>
<td>22.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>LysoPC 18:0/PAF 16:0 (µM)</td>
<td>2.9 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>LysoPAF 16:0 (µM)</td>
<td>2.6 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>LysoPAF 18:0 (µM)</td>
<td>2.9 ± 0.2</td>
<td>3.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>LysoPAF 18:1 (µM)</td>
<td>1.2 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>PC 34:2 (µM)</td>
<td>42.6 ± 6.4</td>
<td>42.6 ± 5.3</td>
<td></td>
</tr>
<tr>
<td>PC 36:4 (µM)</td>
<td>16.2 ± 1.7</td>
<td>16.2 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>TNF (pg/ml)</td>
<td>&lt;62.5</td>
<td>&lt;62.5</td>
<td></td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>&lt;31.25</td>
<td>&lt;31.25</td>
<td></td>
</tr>
</tbody>
</table>

RBC=Red Blood Cells, K\(^+\)=potassium, Hb=Hemoglobin in mmol/ml, Hct=hematocrit. LysoPC=lysophosphatidylcholine, PAF=platelet activating factor, PC=phosphatidylcholine. TNF=tumor necrosis factor. Data are presented as mean±SD. \(^*\)p< 0.001, \(^**\)p<0.01, \(^†\)p<0.01 RBCs day 14 compared to RBCs day 0 or RBCs day 0 compared to Whole blood (fresh) (n=5 batches), (a) one way-ANOVA, followed by post-hoc Dunnett’s and (b) paired t-test.
**Effects of storage time of rat RBCs on biochemical changes**

In an effort to determine which factors in the supernatant are causative in inducing pulmonary injury, biochemical changes of RBC products were analyzed. After 14 days of storage, RBCs had significant storage lesions, exemplified by an increase in potassium and lactate levels and a decrease in pH, sodium and glucose compared to day 0 \((p<0.01 \text{ for all, table 1})\). Total hemoglobin concentration remained stable during storage, whereas hematocrit decreased during storage. Concentrations of lysophosphatidylcholine 16:0, lysophosphatidylcholine 18:0/PAF 16:0, LysoPAF 18:0, lysophosphatidylcholine 18:1, LysoPAF 16:0 and LysoPAF 18:1 did not increase during storage. In line with these results, the concentration of the biochemical precursors of lysophosphatidylcholines (phosphatidylcholines) remained stable. Interleukine-6 and tumor necrosis factor were not detectable in the supernatant of stored RBCs at either time point.

**Effect of storage time of human RBCs on lysophosphatidylcholine accumulation**

In contrast to our results, several previous studies found lysophosphatidylcholine accumulation in stored blood products.\(^1\text{5,17}\) Therefore we performed additional studies using human RBCs. Comparable to the results with the rat RBC products, concentrations of lysophosphatidylcholines did not increase in human RBC products stored for 35 and even 42 days when compared to day 0 of storage (table 2). In line with these results, the concentration of the biochemical precursors of lysophosphatidylcholines (phosphatidylcholines) remained stable during storage.

**Table 2. Storage-related changes in lysophosphatidylcholines in human RBCs**

<table>
<thead>
<tr>
<th></th>
<th>RBC day 0</th>
<th>RBCs day 35</th>
<th>RBCs day 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC 16:0 (µM)</td>
<td>13.4±3.0</td>
<td>10.8±4.4</td>
<td>10.6±5.0</td>
</tr>
<tr>
<td>LysoPC 18:1 (µM)</td>
<td>3.2±0.9</td>
<td>3.1±1.1</td>
<td>2.7±1.2</td>
</tr>
<tr>
<td>LysoPC 18:0/PAF 16:0 (µM)</td>
<td>0.8±0.1</td>
<td>0.6±0.2</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>LysoPAF 16:0 (µM)</td>
<td>0.5±0.2</td>
<td>0.6±0.2</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td>LysoPAF 18:0 (µM)</td>
<td>0.3±0.1</td>
<td>0.2±0.2</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>LysoPAF 18:1 (µM)</td>
<td>0.8±0.1</td>
<td>0.6±0.2</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>PC 34:2 (µM)</td>
<td>25.0±6.5</td>
<td>25.0±7.2</td>
<td>24.0±7.9</td>
</tr>
<tr>
<td>PC 36:4 (µM)</td>
<td>9.8±2.5</td>
<td>10.0±3.0</td>
<td>9.3±2.6</td>
</tr>
</tbody>
</table>

RBC=Red Blood Cells, LysoPC=lysophosphatidylcholine, PAF=platelet activating factor, PC=phosphatidylcholine. Data are presented as mean±SD, \(n=5\) batches. One–way ANOVA, followed by post–hoc Dunnett’s, non significant.
Discussion

We describe a novel in vivo syngeneic rat RBC transfusion model, using a clinical protocol for the preparation and storage of blood products according to National Blood Bank standards. In the model that we believe is clinically relevant, the main findings are as follows: 1) transfusion of aged RBCs resulted in mild lung inflammation in the absence of a priming “first hit”, i.e. in healthy lungs, 2) transfusion of aged RBCs increased lung injury in a “two hit” TRALI model, which was characterized by profound pulmonary and systemic coagulopathy, 3) lung injury in the “two hit” model was abrogated by washing of the aged RBCs before transfusion.

Transfusion of aged RBCs induced lung inflammation in healthy lungs. Notably, the amount of inflammation we found was mild. This may partly be explained by a lack of immunogenicity. However, it can be speculated that the observed mild effects accumulate after repeated transfusions, which may contribute to respiratory complications. In accordance, observational clinical studies show that the number of RBCs transfused is associated with the onset of TRALI as well as with adverse outcome. The clinical relevance of our findings remains to be determined in randomized trials investigating the effect of storage time of RBCs and onset of transfusion related morbidity and mortality. Importantly, although use of a syngeneic model does not reflect allogeneic blood transfusion, our model resembles the clinical situation more closely then the use of cross species or an ex vivo design. Using an “in species” transfusion model, we show that aged RBCs contribute to lung injury.

Previous models that have pointed towards a “two hit” TRALI hypothesis are limited by ex vivo designs, use of blood products which were not manufactured according to clinical protocols or by the use of cross species, including human blood products which were transfused in rat recipients. In our syngeneic transfusion model, we confirm the “two hit” TRALI hypothesis with the use of aged rat RBCs, suggesting that effects of aged RBCs depend on priming status, which is in line with the concept of the threshold model. In this model, a threshold must be overcome to induce a TRALI reaction. Factors that determine the threshold are the clinical condition of the patient (i.e. priming of the lung neutrophils) and the ability of the mediators in the transfusion to cause activation of primed neutrophils. Therefore, in the threshold model, severity of the TRALI reaction depends both on patient- as well as on transfusion-related factors. In accordance, we found that transfusion of aged RBCs induced mild lung inflammation in healthy rats, whereas lung injury increased when a priming hit preceded the transfusion. Of note, not all parameters of inflammation were augmented in the “two hit” model. An explanation for this finding may be that inflammatory reactions, including extravasation of neutrophils, were already elicited by lipopolysaccharide-priming, which could not
be further enhanced by aged RBCs. However, results from our study underline the concept that critically ill patients with an inflammatory response may be susceptible to additional injury following a blood transfusion.\textsuperscript{47,48} If indeed risk factors for acute lung injury of any origin predispose to TRALI, the multiple possible “first events” may explain the increased incidence of TRALI in the critically ill, when compared to the general hospital population.\textsuperscript{29,49-51} Indeed, it is increasingly becoming clear that RBC transfusion is associated with adverse outcome in patient groups which frequently suffer from inflammatory conditions, such as trauma and critically ill patients.\textsuperscript{3} Our results underline the importance of restrictive transfusion protocols in these patient groups.

Our study extends previous findings, showing for the first time that aged RBCs cause increased coagulation and impaired fibrinolysis in the presence of primed neutrophils. RBCs are often considered passive bystanders in coagulation. However, it has long been known that aged RBCs have pro-coagulant activity,\textsuperscript{52} which may result via increasing thrombin generation,\textsuperscript{53,54} and activation of coagulation factors.\textsuperscript{55} Our results suggest that in the presence of a “first hit”, coagulopathy may be an important pathway in mediating lung injury after transfusion of aged RBCs. Of note, histopathologic examination of the lungs did not reveal evident thrombosis. This is in line with histopathologic findings in lungs of patients with acute lung injury due to other causes, in which thrombi are not a frequent finding, even though, coagulopathy is abundant.\textsuperscript{56} Taken together, we suggest that lung injury induced by transfusion is comparable with the pathogenesis of acute lung injury/acute respiratory distress syndrome,\textsuperscript{56,57} with regard to neutrophil extravasations and coagulopathy. Moreover, morbidity and mortality in critically ill patients developing TRALI may be comparable to patients with acute lung injury/acute respiratory distress syndrome, as evidenced by recent studies.\textsuperscript{29,49,58} We suggest that TRALI should be regarded as a form of acute lung injury and not as a separate entity.

Proposed mechanisms of the induction of lung injury by storage of RBCs have included white blood cell derived mediators, soluble factors in the supernatant, in particular lysophosphatidylcholines, or erythrocytes as the causative agents.\textsuperscript{15,17,38} Our study suggests that the supernatant of the stored RBCs and not the aged erythrocyte itself caused inflammation in primed lungs. We found no increase in levels of lysophosphatidylcholines or other pro-inflammatory factors after storage. As these findings are in contrast with previous studies,\textsuperscript{15-18} we performed additional experiments with human RBC blood products, which also did not show lysophosphatidylcholine accumulation. Blood product preparation for animal models varies considerably between laboratories, including use of storage solutions and leucoreduction, which may account for different study outcomes.\textsuperscript{17,22,38,40,59-62} Also clinical studies on the association between lysophosphatidylcholine concentration in transfused blood products and TRALI,\textsuperscript{63} show conflicting results.\textsuperscript{18,29}
An alternative explanation for the mechanism of the detrimental effects in our model may be biochemical deterioration of the blood product. An increase in potassium, lactate and a decrease in pH in transfused products have been associated with morbidity and mortality in the pediatric patient.\textsuperscript{64,65} Whether biochemical deterioration of blood products is able to induce coagulopathy, is not answered by our study. Possibly, mediators produced by residual white blood cells may have contributed to lung injury. However, plasma from stored RBCs that were leukoreduced before storage were shown to induce lung injury in a 2-event transfusion model,\textsuperscript{22} rendering this hypothesis unlikely. Further research on the factor in the supernatant that elicits coagulopathy is required.

Of note, results suggest that whereas supernatant is the causative factor in primed lungs, both aged cells as well as supernatant elicit inflammation in healthy lungs. This interesting finding calls for further experiments with aged RBCs in various storage conditions. Also, it should be noted that the comparison of supernatant with aged RBCs in this study, does not account for possible interactions between aged cells and aged supernatant. Furthermore, separating the products may have introduced other variables, such as a change in blood viscosity.

Our finding that washed aged RBCs inhibited lung injury in the “two hit” model, may have implications for the preparation and storage of red blood cells. Washing of red blood cells may reduce respiratory complications. Washing of stored blood without disturbing integrity of the aged erythrocyte seems a feasible procedure.\textsuperscript{66,67} Alternatively, transfusion of fresh cells only may reduce pulmonary complications. A retrospective study suggested that cardiac surgery patients transfused with fresh RBCs (<14 days) compared to patients receiving aged RBCs (>14 days) had a reduced ventilation time and suffered less from respiratory insufficiency.\textsuperscript{11} However, other clinical trials have not confirmed this finding.\textsuperscript{68,69} Although it is clear that RBC products deteriorate over time, a specific cut-off point in the risk/benefit ratio in transfusion related to the age of RBCs remains to be determined. Our data, however, suggest that in certain patient populations (e.g. the critically ill), washing of aged RBCs before transfusion or transfusion of fresh RBCs only may be a rational approach in reducing respiratory complications.

In conclusion, we show that transfusion of the supernatant of aged RBCs but not washed aged RBCs, causes lung injury in a clinically relevant transfusion model, an effect which was modulated by the presence of a priming hit. In primed lungs, red blood cell-induced lung injury was characterized by increased inflammation and coagulation and impaired fibrinolysis. The findings in the lipopolysaccharide-primed rats suggest that washing procedures of aged RBCs may decrease pulmonary complications after a blood transfusion. Given that critically ill and trauma patients...
are the patients who are most often transfused and that transfusion is the most common event preceding the development of acute lung injury\(^\text{70-72}\) and an independent risk factor for acute lung injury\(^\text{32,36,45,73}\) efforts to reduce the adverse relation of blood transfusion and outcome are mandatory. Whether transfusion of fresh RBCs or washed aged RBCs reduces the increased risk for acquiring lung injury deserves further clinical studies.
Reference List


44. Vlaar AP, de Korte D, Juffermans NP. The aged erythrocyte: key player in cancer progression, but also in infectious and respiratory complications of blood transfusion? Anesthesiology 2009;111:444.


