Differences in Rat and Human Erythrocytes Following Blood Component Manufacturing: The Effect of Additive Solutions

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Keywords

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Summary

Background: Small animal models have been previously used in transfusion medicine studies to evaluate the safety of blood transfusion products. Although there are multiple studies on the effects of blood banking practices on human red blood cells (RBCs), little is known about the effect of blood component manufacturing on the quality of rat RBCs. **Methods:** Blood from Sprague-Dawley rats and human volunteers ($n = 6$) was collected in CPD anticoagulant, resuspended in SAGM or AS3, and leukoreduced. In vitro quality was analyzed, including deformability, aggregation, microvesiculation, phosphatidylserine (PS) expression, percent hemolysis, ATP, 2,3-DPG, osmotic fragility, and potassium concentrations. **Results:** Compared to human RBCs, rat RBCs had decreased deformability, membrane rigidity, aggregability, and microvesiculation after component manufacturing process. Rat RBCs in SAGM showed higher hemolysis compared to human RBCs in SAGM (rat $4.70 \pm 0.83\%$ vs. human $0.34 \pm 0.07\%$; $p = 0.002$). Rat RBCs in AS3 had greater deformability and rigidity than in SAGM. The number of microparticles/ μl and the percentage PS expression were lower in rat RBCs in AS3 than in rat RBCs in SAGM. Hemolysis was also significantly lower in AS3 compared to SAGM ($2.21 \pm 0.68\%$ vs. $0.87 \pm 0.39\%$; $p = 0.028$). **Conclusion:** Rat RBCs significantly differ from human RBCs in metabolic and membrane-related aspects. SAGM, which is commonly used for human RBC banking, causes high hemolysis and is not compatible with rat RBCs.

Introduction

Blood transfusion is a life-saving treatment for patients with massive blood loss and chronic anemia and a supportive therapy to optimize oxygen delivery and tissue perfusion in critical illness [1, 2]. The clinical benefits of blood transfusion were made possible through the development of techniques to preserve cell viability ex vivo, allowing the blood donation and transfusion to be separated in time and space [3]. In the 1960s, with the introduction of plastic blood bags [4], whole blood transfusion was replaced for specific blood component therapy – red blood cells (RBCs), platelets, plasma and plasma components – translating the life-saving benefits of one whole blood donation to up to four transfusion recipients [5]. Currently packed RBCs (pRBCs), the most highly used blood component, are produced by two common component manufacturing methods: the whole blood filtration method and the buffy coat method [6, 7]. The general procedure is similar for both techniques: whole blood is centrifuged, plasma and RBCs are separated, and RBCs are resuspended in an additive solution, commonly accompanied by leukoreduction [7]. Additive solutions, such as saline-adenine-glucose-mannitol (SAGM) and additive solution 3 (AS3), contain nutrients RBCs need to survive ex vivo and have effectively extended RBC storage to up to 6 weeks [4]. SAGM is widely used in blood banks in Europe, Australia and Canada, while AS3 is mainly used in the USA [8].

Although use of additive solutions extends the storage length of pRBC units, the quality of stored RBCs progressively decreases during hypothermic storage. RBCs undergo a series of biochemical and biomechanical changes, collectively known as the ‘hypothermic storage lesion’ (HSL) [9]. Characteristics of the HSL includes RBC membrane remodeling, decreased metabolites such as ATP and 2,3-DPG, loss of intracellular potassium, oxidative injury of protein structures and lipid peroxidation, membrane loss, vesiculation, and ultimately hemolysis [10–12]. There are increasing con-

cerns regarding the effect of the HSL on hemorheology, including RBC aggregability, deformability and membrane remodeling, effects that could potentially lead to impairment of the oxygen delivery capacity of transfused blood [13–15]. As a consequence, the active debate on the clinical impact of stored RBCs has given rise to large clinical trials about the use of ‘fresh’ versus ‘old’ RBCs [16–18].

The effect of the HSL on RBC quality during storage has also rejuvenated research efforts into novel additive solutions for better preservation of RBC ex vivo quality and function [19]. Animal models are often used as a translational tool to understand the mechanisms behind the ageing of blood cells and possible clinical effects related to the quality of stored blood. [20]. Therefore, understanding biological differences between RBCs from animal model species and humans as well as the effect of blood component manufacturing processes on cell quality are the first steps in animal translational studies. Previous studies have addressed the quality of rat RBCs using citrate-phosphate-dextrose-adenine 1 (CPDA-1) as the additive solution; however CPDA-1 is not widely used in today’s blood component manufacturing. To our knowledge, no study has been done using the current additive solutions for pRBC storage: SAGM or AS3. The differences in quality parameters between human RBCs in SAGM and AS3 are well documented in the literature, but this information is not available for rat RBCs [21]. Also, little is known about the effects of component manufacturing and leukoreduction methods on the parameters important for RBC hemorheology, including deformability, aggregation, and microvesiculation in rat RBCs. Therefore, the aim of this study was to evaluate baseline differences between rat and human RBCs in SAGM, including in vitro quality assays to examine membrane-related and hemorheology parameters. In addition, we investigated the impact of the buffy coat component manufacturing method on rat RBCs and the effects of different additive solutions. Ultimately, our goal is to establish a rat pRBC production method that closely mimics human pRBC techniques currently in use that can be applied in rat models of transfusion studying the HSL and novel RBC biopreservation strategies.

Material and Methods

RBC Collection and Manufacturing

Ethics approval for the study was granted by the Canadian Blood Services and the University of Alberta Research Ethics Boards. Rat whole blood ($n = 6$) was obtained from Biosciences Animal Services (University of Alberta). The Sprague-Dawley rats were all male, between 8 and 9 weeks old, and had an average weight of 300 g. The blood was collected after anesthesia into 10 ml citrate-phosphate-dextrose (CPD) anticoagulant vacuum tubes (Haematologic Technologies Inc., Essex Junction, VT, USA) by cardiac puncture. Harvested blood was centrifuged (2,200 $\times$ g, 10 min at 4 °C), and the plasma and buffy coat were removed by aspiration. The pRBCs were divided into two aliquots. One aliquot was resuspended in SAGM (MacoPharma, Mouvaux, France) and the second aliquot in AS3 (Haemonetics Corporation, Braintree, MA, USA). Both maintained a proportion of 1:2 AS:RBC (vol/vol) and were leukoreduced at room temperature using 10.0 μ m Versapor® membrane syringe filters (Pall Corporation, Ann Arbor, MI, USA). Before leukoreduction, percent hemolysis was measured in both CPD-SAGM RBCs and CPD-AS3 RBCs. Immediately after

leukoreduction, in vitro quality of CPD-SAGM RBCs and CPD-AS3 RBCs was analyzed, including percent hemolysis, ATP, 2,3-DPG, hematological indices, deformability, aggregation and microvesiculation, as described below.

After informed consent, human whole blood from 6 healthy volunteers was collected by venipuncture into CPD anticoagulant tubes. The blood was processed following the same method described above for rat blood. In vitro quality was analyzed within 3 h of collection and included percent hemolysis, osmotic fragility, ATP, 2,3-DPG, supernatant potassium concentrations, hematological indices, deformability, aggregation, and microvesiculation, as described below.

RBC in vitro Quality Assessment

Percent Hemolysis

Percent hemolysis was measured using a Drabkin’s-based method [22]. Briefly, the RBCs were diluted in Drabkin’s reagent (0.61 mmol/l potassium ferri-cyanide, 0.77 mmol/l potassium cyanide, 1.03 mmol/l potassium dihydrogen phosphate, and 0.1% triton X-100). Percent hemolysis was calculated as a ratio of the supernatant hemoglobin (Hgb) to the total Hgb, with the hematocrit (Hct), measured by microhematocrit, used to account for the volume of the supernatant in the sample [23].

Osmotic Fragility

Osmotic fragility was determined using a series of saline solutions with concentrations ranging from 0.0 to 9.0 g/l. In brief, 10 μ l of each RBC sample was added to 1,000 μ l of each saline solution, and the percent hemolysis was determined spectrophotometrically (Molecular Devices Spectramax Plus 384, Sunnyvale, CA, USA). Percent hemolysis in each saline solution was plotted against the saline solution concentration to determine the concentration that produced 50% hemolysis; this parameter was reported as mean corpuscular fragility (MCF) [24].

ATP

ATP concentrations were determined enzymatically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). RBC samples were added to 10% trichloroacetic acid, vortexed, and placed on ice. The supernatants were combined with the substrates (glucose, and NAD⁺) and enzymes (hexokinase and glucose-6-phosphate dehydrogenase) required for the enzymatic reaction to occur. The amount of NADH produced, which is proportional to the amount of ATP within the sample, was measured spectrophotometrically. The amount of ATP in the sample was calculated as μ mol/dl; this was further normalized using the total Hgb concentration (μ mol/g Hgb) [24].

2,3-DPG

2,3-DPG concentrations were determined using a commercially available kit and control (Roche Diagnostics GmbH, Germany). After a series of enzymatic reactions, glycerol-3-phosphate is produced from each 2,3 DPG molecule using 2 NADH molecules. NADH absorbs light at 340 nm. Therefore the decrease of absorbance at this wavelength is equivalent to the amount of NADH that has been consumed in the reaction. The concentration of 2,3-DPG is indirectly related to the amount of NADH remaining in the reaction mixture [25].

Supernatant Potassium

RBC samples were centrifuged at 2,200 $\times$ g for 10 min at 4 °C to obtain supernatants. Supernatant potassium concentrations were measured by indirect potentiometry using ion-selective electrodes on a chemistry analyzer (DXC 800, Beckman Coulter, Inc., Fullerton, CA, USA) [26].

Hematological Indices

RBC hematological indices, including RBC count, mean corpuscular volume (MCV), mean corpuscular Hgb (MCH), mean corpuscular Hgb concentration (MCHC), Hgb and Hct, were determined using a Coulter Automated Cell Counter (Coulter Act, Beckman Coulter, New York, NY, USA) [26].

Table 1. In vitro quality parameters of human and rat RBCs^a

RBC quality parameters	Human	Rat
Hemolysis, %	0.34 ± 0.07	4.70 ± 0.83*
Osmotic fragility, MCF	4.9 ± 0.3	5.9 ± 0.3*
ATP, μmol/g Hb	3.1 ± 0.1	2.5 ± 0.2*
2,3-DPG, μmol/g Hb	11.3 ± 1.3	14.1 ± 2.4
Supernatant		
K ⁺ , mmol/l	1.4 ± 0.4	3.5 ± 0.6*
Hematologic indices		
MCV, fl	95.1 ± 1.6	69.5 ± 2.9*
MCH, pg	30.2 ± 0.7	22.6 ± 0.7*
MCHC, g/l	318 ± 7	327 ± 12
Deformability		
EI _{max}	0.57 ± 0.01	0.52 ± 0.02*
RBC rigidity, K _{EI}	2.32 ± 0.51	0.76 ± 0.13*
Aggregation parameters		
AI, %	53.4 ± 2.1	49.2 ± 2.3*
Amp, au	27.4 ± 2.3	13.6 ± 0.7*
t _{1/2} , s	3.3 ± 0.3	4.0 ± 0.6*
Microvesiculation		
MPs/μl	85,377 ± 4,656	67,556 ± 13,318*
RBC-PS, %	0.4 ± 0.0	0.6 ± 0.2
MP-PS, %	6.8 ± 1.4	42.0 ± 5.0*
RBC-MFI	26.0 ± 1.7	86.3 ± 13.1*
MP-MFI	41.2 ± 7.1	187.3 ± 21.9*

^aMean values ± SD for fresh human and rat RBCs resuspended in SAGM post-leukoreduction are shown.
*p < 0.05 compared to humans.

Deformability

RBC deformability was measured using a laser-assisted optical rotational cell analyzer (LORCA; Mechatronics, Zwaag, the Netherlands), as previously described [27]. RBCs were diluted 1:100 in a polyvinylpyrrolidone solution, and subjected to increasing shear stress at 37 °C. The diffraction pattern produced by the scatter of a laser beam at each stress was collected, and subsequently plotted as a deformability curve. Deformability curves were linearized using the Eadie-Hofstee method as published by Stadnick et al. [27]. Two parameters were extrapolated using this linearized function: the maximum elongation index (EI_{max}), as a measure of deformability, and the stress required to reach half of the maximum elongation (K_{EI}), as a measure of rigidity [27].

Aggregation

RBC aggregation was measured by syllectometry using LORCA (Mechatronics). RBCs were washed two times with PBS, and 400 μl of packed washed cells were mixed with 1% dextran (100 kDa) solution to an optimal Hct of 42–46%. The controls were prepared the same way and mixed with PBS (negative) and 3% dextran (positive). Briefly, the cells were subjected to a constant shear rate with rotation to cause complete disaggregation. The aggregation process was started by abruptly stopping the shear rate. The laser backscatter intensity over time was measured (syllectogram), and the following aggregation parameters were generated: aggregation index (AI) in percent, amplitude (Amp) in arbitrary units (au) and aggregation half-life (t_{1/2}) in seconds [28].

Microvesiculation

The flow cytometry procedure was adapted from Almizraq et al. [24]. Two tagged antibodies were used to label RBCs and RBC microparticles (MPs) from human and rat samples. Fluorescein isothiocyanate (FITC) anti-human CD235a antibody (MHGLA01 or MHGLA01-4; Invitrogen Life Technologies, Burlington, ON, Canada) was used as a marker for RBCs and MPs from human samples, while peridinin chlorophyll protein complex-cyanine 5.5 (PerCP-

Table 2. In vitro quality parameters of rat RBCs in different additive solutions^a

Rat RBC quality parameters	Additive solutions	
	SAGM	AS3
Hemolysis, %	2.21 ± 0.68	0.87 ± 0.39*
ATP, μmol/g Hb	2.6 ± 0.3	3.0 ± 0.3*
2,3-DPG, μmol/g Hb	17.6 ± 2.2	13.8 ± 2.7*
Hematological indices		
MCV, fl	62.9 ± 2.7	60.5 ± 2.0*
MCH, pg	21.3 ± 0.8	21.2 ± 0.7
MCHC, g/l	339 ± 5	350 ± 2*
Deformability		
EI _{max}	0.51 ± 0.02	0.55 ± 0.01*
RBC rigidity, K _{EI}	0.80 ± 0.17	0.98 ± 0.04*
Aggregation parameters		
AI, %	53.8 ± 3.2	49.0 ± 4.2
Amp, au	16.6 ± 3.6	14.8 ± 2.6
t _{1/2} , s	3.2 ± 0.5	4.0 ± 0.9
Microvesiculation		
MPs/μl	67,556 ± 13,318	47,806 ± 3,029*
RBC-PS, %	0.6 ± 0.2	0.2 ± 0.0*
MP-PS, %	42.0 ± 5.0	34.4 ± 3.4*
RBC-MFI	86.3 ± 13.1	74.8 ± 8.3*
MP-MFI	187.3 ± 21.9	173.2 ± 32.5

^aMean values ± SD for fresh rat RBCs resuspended in SAGM and AS3 post-leukoreduction are shown.
*p < 0.05 compared to SAGM.

CyTM 5.5) anti-rat erythroid cells antibody (BD PharMingen, San Jose, CA, USA) was used as a marker for RBCs and MPs from rat samples. APC annexin V (BD PharMingen) was used to label phosphatidylserine (PS) according to manufacturer's instructions in both human and rat samples. RBCs (5 ml) were diluted with annexin-binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl, 2.5 mmol/l CaCl₂, pH 7.4) and 5 μl of each of the fluorescently labeled monoclonal antibodies (FITC, APC, PerCP-CyTM 5.5) were added. After 15 min of incubation in the dark at room temperature, the prepared samples were run on a FACSCalibur (BD Biosciences, San Jose, CA, USA) equipped with a 488 nm argon laser and computer software (CellQuest; BD Biosciences) [24]. Commercial isotype controls directed against glycophorin A (FITC mouse IgG1, k isotype control; BD PharMingen) and rat erythroid cells (PerCP-CyTM 5.5 mouse IgM, k isotype control; BD PharMingen) were used to account for any nonspecific binding of the antibodies. Frozen RBCs served as the positive control for PS expression and microvesiculation [24]. TruCOUNT beads (BD PharMingen) were used to determine the absolute number of MPs/μl. Forward scatter and side scatter, measured on a logarithmic scale, was used to distinguish between RBC and MP populations. Absolute numbers of MPs/μl were calculated using the equation:

$$\text{MPs}/\mu\text{l} = ((\text{number of glycophorin A-positive MP gated events}/\text{TruCOUNT beads-gated events}) \times (\text{number of beads per TruCOUNT tube}/\mu\text{l of resuspension buffer added to TruCOUNT tube})) \times \text{dilution factor (1)}.$$

Statistical Analyses

Statistical analysis was performed using SPSS 20.0 software (IBM, Armonk, NY, USA). Mann-Whitney's U non-parametric test was used to assess differences between rat and human RBCs and Wilcoxon signed-rank test was used to compare samples in different additive solutions (SAGM vs. AS3). Data were expressed as mean ± standard deviation (SD) and p < 0.05 was considered statistically significant.

Results

Differences between Human and Rat RBCs in SAGM Additive Solution

Table 1 describes the differences in RBC in vitro quality parameters between the two species. In this study the samples were filtered using 10.0 μm syringe filters as an adaptation for the small sample volume, while trying to mimic the buffy coat blood compo-

Fig. 1. Percent hemolysis in fresh human RBCs resuspended in SAGM and in rat RBCs resuspended in SAGM and AS3, pre and post leukoreduction. Shown is the mean $\pm$ SD ($n = 6$). *Significant ($p < 0.05$) compared to humans RBCs resuspended in SAGM and **significant ($p < 0.05$) compared to rat RBCs resuspended in AS3.

nent manufacturing process. The level of leukoreduction achieved was $90 \pm 5\%$ for platelets and $71 \pm 9\%$ for white blood cells. The buffy coat blood component manufacturing method caused alterations to rat but not to human RBCs, as demonstrated by increased hemolysis (rat pre-leukoreduction $3.03 \pm 0.64\%$ vs. human pre-leukoreduction $0.24 \pm 0.07\%$, $p = 0.002$; rat post-leukoreduction $4.70 \pm 0.83\%$ vs. human post-leukoreduction $0.34 \pm 0.07\%$, $p = 0.002$; fig. 1). Free Hgb levels were also measured in CPD plasma, where no difference was observed (human 1.13 ± 0.15 g/l vs. rat 0.98 ± 0.18 g/l; $p = 0.157$), and in the SAGM supernatant of pRBCs where the levels in rat were greater than in human (human 1.47 ± 0.07 g/l vs. rat 8.95 ± 0.51 g/l; $p = 0.034$). Rat RBCs were also more sensitive to osmotic stress than human RBCs, as shown by higher MCF values ($p = 0.002$). Rat RBCs had significantly lower levels of ATP compared to human RBCs ($p = 0.004$) while the levels of 2,3-DPG were similar in species ($p = 0.054$). Potassium levels were significantly higher in the supernatant of rat RBCs compared to humans ($p = 0.002$). Rat RBCs had decreased deformability ($EI_{\max}$; $p = 0.002$) and decreased membrane rigidity (K_{EI} ; $p = 0.002$) compared to human RBCs (fig. 2). RBC characteristics, including the hematological indices MCV and MCH, were significantly different between species ($p = 0.002$). Aggregation index and amplitude were significantly higher in human RBCs compared to rats ($p = 0.016$ and $p = 0.004$, respectively) and the aggregation half-life was lower ($p = 0.037$), indicating that aggregation happens faster in human RBCs (fig. 3). The number of MPs/ μl was lower in rats compared to humans ($p = 0.010$). Further analysis showed that there were also differences between the species in terms of MPs expressing PS ($p = 0.004$; fig. 4b) and the mean fluorescence intensity (MFI) for the expression of PS on both RBCs ($p = 0.004$) and MPs ($p = 0.004$).

Fig. 2. Deformability of human and rat fresh RBCs resuspended in SAGM. Shown is the mean $\pm$ SE ($n = 6$) of the elongation indices at different shear stresses after Eadie-Hofstee linearization. The two variables shown ($EI_{\max}$ and K_{EI}) are extrapolated from the lines: $EI_{\max}$ = y-intercept (deformability) and K_{EI} = slope (rigidity).

Fig. 3. RBC aggregation in fresh human RBCs resuspended in SAGM and in rat RBCs resuspended in SAGM and AS3. Sylectograms (light scatter $\times$ time) showing RBC aggregation patterns ($n = 6$). Negative control (PBS) = no aggregation; Positive control (3% dextran) = high aggregation.

Rat RBCs in Different Additive Solutions: SAGM versus AS3

Table 2 describes the effect of different additive solutions on rat RBC in vitro quality parameters. Both EI_{max} (deformability) and K_{EI} (rigidity) were significantly increased when the packed RBCs were resuspended in AS3 compared to SAGM ($p = 0.028$ and $p = 0.046$, respectively). In AS3, percent hemolysis was significantly lower pre and post leukoreduction (fig. 1). ATP concentration was higher ($p = 0.026$) and 2,3-DPG concentration was lower ($p = 0.028$) in AS3 versus SAGM. MCH values were comparable for both additive solutions ($p = 0.288$) while MCV values were lower ($p = 0.028$) and MCHC values were higher ($p = 0.027$) with the use of AS3. No significant differences in the aggregation behavior were observed between the RBCs resuspended in the two additive solutions (fig. 3). The number of MPs/ μ l was slightly lower in rat RBCs resuspended in AS3 compared to SAGM ($47,806 \pm 3,029$ vs. $67,556 \pm 13,318$; $p = 0.046$). The percentage of PS exposure in both RBCs and MPs were significantly lower in the packed cells resuspended in AS3 ($p = 0.028$; fig. 4a, b). MFI for the expression of PS in RBCs resuspended in AS3 was also lower ($p = 0.046$).

Discussion

Differences between human and rat RBCs in SAGM, particularly in terms of membrane parameters and hemorheology which play a significant role in the human RBC HSL, are not well documented. One of the objectives of this study was to fill in gaps in the current literature by evaluating the baseline hemorheological differences between human and rat RBCs in SAGM. In addition to

deformability and aggregation, MP analysis is an important tool to assess quality and the effects of new preservation strategies, as MPs have been linked to various biological activities including inflammation, vascular function, and immune response [29]. The concentration of MPs was lower in rat samples compared to human pRBC samples, while the exposure of PS in RBCs from both species was comparable. RBC intracellular ionized calcium concentration in rats ranges from 83 to 105 nmol/l [30] while in humans the physiological range is 20–60 nmol/l [31]. Rat RBCs have also been described as more sensitive to exogenous Ca^{2+} than human RBCs [32]. Considering that PS externalization and microvesicle formation are directly correlated with calcium influx [33–35], we would expect to see a higher number of MPs in rat samples. On the other hand, RBC microvesiculation has also been described as a protective mechanism to avoid erythroptosis [35, 36] or to regulate membrane stability and avoid lysis [33, 35]. In this context, the increased MPs combined with the lower hemolysis values indicates that the regulatory mechanisms in human RBCs are more effective than in rat RBCs, allowing the cells to adapt better to different environments. Willekens and colleagues [34] have demonstrated that in rats RBC-derived MPs have high PS exposure and are easily removed from the circulation by liver Kuppfer cells and that the same clearance mechanism is likely to happen in humans. In this study, the PS exposure in MPs from rat RBCs was higher than in humans. However, further studies are necessary to determine if this high PS exposure plays a role in vivo.

Our results show that the deformability of rat RBCs significantly differs from human RBCs. Lower EI_{max} and K_{EI} values suggests that rat RBCs are less deformable and less rigid than human RBCs. Similar results have been described using ektacytometry

Fig. 4. Percentage of phosphatidylserine (PS) exposure by RBCs (**a**) and MPs (**b**) in fresh human and rat RBCs resuspended in SAGM and in fresh rat RBCs resuspended in AS3. Histogram: region M1 is negative for PS exposure while, region M2 is positive.

[37] and the resistive pulse shape analysis technique in whole blood samples collected in ethylene diamine tetra-acetic acid (EDTA) [38]. Baskurt [38] also showed a direct correlation between deformability and MCV values of various species, suggesting that RBCs with a smaller volume do not have to deform as much to pass through the microcirculation compared to larger RBCs. Our study agrees with this finding, demonstrating that rat RBCs have lower MCV and decreased deformability compared to humans. The aggregation pattern of human and rat RBCs observed in this study is in agreement with previous reports for blood collected in EDTA [39]. The percentage of aggregation is higher in human RBCs, and the cells also aggregate faster and to a greater extent than rat RBCs. Rat RBCs have a higher surface charge compared to human RBCs, as demonstrated by a higher partition coefficient [39] which is expected to influence the aggregation behavior, thereby explaining the different aggregation patterns observed in this study.

Hemolysis is one of the key quality control parameters evaluated in pRBCs before transfusion. It is well established that the percent hemolysis values of human pRBCs in CPD-SAGM at the end of the 42-day storage period should remain under 0.8% [26]. The hemolysis in rat RBCs was more than ten times higher than in human RBCs after the component production process. We investigated whether the hemolysis could have been caused by the CPD anticoagulant, but found that the free Hgb concentration in CPD plasma of rat and human samples was similar. The free Hgb concentration was measured again in the SAGM supernatant of both species, and while in humans the concentration was virtually the same, in rats it had increased eight times. Potassium levels were higher in the supernatant of rat RBCs, in accordance with previously published data for RBCs in CPDA-1 [20]. This is possibly a consequence of the pronounced hemolysis, with more intracellular potassium leaking to the supernatant. Comparison of osmotic fragility in RBCs from different mammalian species has shown that

rat RBCs are among the least fragile [40]. In our comparison to human RBCs we observed that rat RBCs are more fragile. Correlations with cell volume have been made, showing that smaller cells are more fragile in hypotonic media [40] and more resistant to hypertonic media [41]. The diffusional permeability of rat RBC membrane to water is higher than that of human RBC membrane [42], which also explains the increased sensitivity observed in hypotonic media and the differences observed in the corpuscular fragility between species. Osmotic behavior is also a key element for cell interaction with additive solutions, especially with hypertonic ones like SAGM [43].

Previously published studies of rat RBCs have used RBCs resuspended in CPDA-1 or EDTA blood, rather than additive solutions that are currently used. With the development of new additive solutions, it is important to know how the RBCs of rats, a species that is often used as a model in storage studies, respond to the additive solutions that are already in use. Human pRBC component manufacturing process is highly regulated, with well-established standards [21]. One limitation of this study is that leukoreduction levels achieved for rat RBCs are below the human blood banking standard of 99%. Data to relate those standards to animal models of transfusion is lacking. This is in part due to a lack of understanding of how slight differences in the final product can benefit or affect the recipient of the RBC transfusion. Studies have already identified how different additive solutions and processes affect human, but not rat, RBCs. Another limitation of this study was that a direct comparison of human RBCs stored in SAGM and AS3 was not performed; however, some quality parameters have been examined before [44]. We have previously reported no difference in ATP levels but increased 2,3-DPG levels with the use of AS3 compared to SAGM in human RBCs [25]. In rat RBCs we observed a significant increase in ATP and a decrease in 2,3-DPG levels using AS3 compared to SAGM. The metabolic aspects seem to follow the same tendency as previously described for RBCs resuspended in CPDA-1, with human RBCs having higher levels of ATP compared to rat RBCs, with no difference in 2,3-DPG levels observed between the two species [20]. The raw values for ATP and 2,3-DPG in rat RBCs using SAGM were comparable to the values described for rat RBCs in CPDA-1.

In terms of hemorheology, we observed that rat RBC deformability improved (EI_{max}), while rigidity increased (K_{EI}), with the use of AS3 compared to SAGM. However, more studies need to be conducted to elucidate the exact mechanism of these differences. It has been shown that increased cellular dehydration caused by increased intracellular Hgb results in greater cell rigidity and increases in the Hgb-spectrin complex, which contributes to rigidity [45, 46]. MCHC was higher for rat RBCs in AS3 compared to SAGM. This increased Hgb concentration in AS3 RBCs may be the cause of the observed increase in rigidity. The aggregation behavior

of rat RBCs in AS3 and SAGM remained the same, with no statistically significant differences observed in aggregation index, amplitude, and kinetics of aggregation between the two additive solutions. Compared to RBCs in SAGM, the number of MPs/ μ l was lower in rat RBCs resuspended in AS3, as was the PS exposure in RBCs and MPs. In vivo, PS exposure is related to increased adherence to the endothelium which leads to hypercoagulability and vascular occlusion [47]. Hemolysis was higher in rat RBCs in SAGM compared to AS3, which might be due to differences in the formulations of the two additive solutions. For example, mannitol, a membrane stabilizer that helps reduce hemolysis in human RBCs [48], is present in SAGM but absent in AS3. Little is known about mannitol's effect on rat RBCs. The second notable difference between the two additive solutions is the NaCl concentration which is 2 times higher in SAGM [21]. The higher concentration of Cl^- in SAGM might generate an imbalance in ion distribution across the RBC membrane, which could lead to activation of K^+/Cl^- co-transport resulting in increased potassium efflux and subsequently hemolysis.

In conclusion, rat RBCs differ from human RBCs in metabolic and membrane-related aspects. This should be taken into account when performing storage studies using a rodent model. Additive solutions play an important role in RBC preservation; however, SAGM, which is commonly used for human RBC storage, is not compatible with rat RBCs, as it causes high hemolysis and increased MP production. Our studies suggest AS3 is a better alternative for rat RBC storage when conducting studies examining the HSL or new preservation strategies for RBCs that might require the use of animal models.

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Ethical Standards

All the aforementioned experiments complied with the current laws of the country in which they were performed.

Disclosure Statement

The authors report no conflict of interest.

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