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Citation:

Simonova, G, Tung, J, Fraser, J, Do, H, Staib, A, Chew, M, Dunster, K, Glenister, K, Jackson, D and Fung, Y 2014, 'A comprehensive ovine model of blood transfusion', *Vox Sanguinis*, vol. 106, no. 2, pp. 153-160.

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Version: Accepted Manuscript

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Link to Published Version:

<http://dx.doi.org/10.1111/vox.12076>

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A comprehensive ovine model of blood transfusion

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Supported by the Critical Care Research Group (CCRG), Queensland Emergency Medicine Research Foundation (QEMRF) and the Australian Red Cross Blood Service (ARCBS).

The authors declare that they have no conflict of interest relevant to the manuscript submitted to TRANSFUSION.

Abstract

Background: The growing awareness of the transfusion associated morbidity and mortality necessitates investigations into the underlying mechanisms. Small animal have been the dominant transfusion models but have significant limitations. This study sought to develop and evaluate a comprehensive large animal (ovine) model of transfusion that would encompass: blood collection, processing, storage, compatibility testing and transfusion outcomes.

Study design and methods: Twenty-four units of whole blood were collected from 12 healthy Merino adult male sheep and processed into packed red blood cells (PRBC). Baseline haematological values of sheep blood and PRBC were analysed. Biochemical changes in ovine PRBC packs were monitored and characterized during 42 days of storage. Immunologic compatibility of the blood was confirmed with sera from potential recipient sheep using a saline agglutination crossmatch. Following confirmation of compatibility, 2 units of PRBC were transfused into each recipient sheep (n=12).

Results: Methods for collection, processing, crossmatching and transfusion of sheep blood were established. Ovine RBCs are smaller, higher in number, but have similar mean cell haemoglobin concentration than human RBCs. The ovine PRBC showed improved storage properties in SAGM when compared with previous studies of human PRBC. Although haemolysis was at the upper limit at day 42, ovine PRBC met the human PRBC specifications. In all, 77 compatibility tests were performed and 18.2% were incompatible. No adverse reaction occurred in recipient sheep during blood transfusion.

Conclusion: These findings validate the application of the ovine model for future blood transfusion studies and demonstrate the need of compatibility testing in animal models.

Key Words: ovine model, blood transfusion, storage lesion, compatibility test

Introduction

Blood transfusion is an essential part of modern medicine. While blood transfusion can be life-saving it is not without risk [1]. Specific pathologies associated with transfusion include transfusion-related acute lung injury (TRALI), transfusion related immunomodulation (TRIM), circulatory overload, bacterial infections and haemolytic transfusion reactions are complications that can lead to deleterious outcomes [2-4]. Additionally, there is a growing body of evidence indicating an association between transfusion, especially of aged/stored blood products, and increased levels of morbidity and mortality in patients [5, 6]. This has prompted the development of multicentre clinical trials that aim to answer this question from a clinical perspective. Such trials, however, will not shed any light on the underlying pathological mechanisms.

Ethical issues limit the research that can be undertaken in humans, necessitating the development of clinically relevant animal models of transfusion to investigating the mechanism of adverse transfusion reactions and poor clinical outcomes. However, the value of any information generated from animal models, is defined by the scope as well of limitations of the animal model. Murine models have been used extensively because of their relatively low cost and the ease of accessibility. However, the small size of mice and rats limits the volume of blood samples that can be collected and prevents the use of the standard blood collection packs. In addition, the lifespan of murine red blood cells (RBCs) is about half that of humans [7, 8]. We also noted the absence of pre-transfusion compatibility testing in many animal transfusion studies. These factors fuelled our quest to develop a comprehensive and clinically relevant *in vivo* transfusion model that would support transfusion research investigation by mimicking the human cycle of blood donation, processing, compatibility testing and monitoring of transfusion outcomes.

This study elected to develop a clinically relevant model using sheep because their anatomy, hemodynamic and microcirculatory characteristics and RBCs lifespan are very similar to humans (~120 days) [9-12].

Methods

This study was approved by the University Animal Research Ethics Committee of the Queensland University of Technology and University of Queensland, and all experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Ovine blood collection protocol

Two units of whole blood were collected from each of twelve male Merino sheep aged between 1-3 years and weighing of 40.7 ± 1.7 kg (mean \pm SD) as part of another haemorrhage study (Vox ref). In brief, the sheep were instrumented under ketamine/midazolam anesthesia and monitored for arterial blood pressure, continuous cardiac output, mixed venous oxygen saturation and temperature. Sheep were ventilated throughout the experiment with a Hamilton Galileo ventilator at 12–15 breaths per minute.

Following a period of stabilisation two units of whole blood (400 ± 20 mL per unit) were collected from each sheep via a 8.5G venous sheath placed in the left internal jugular vein at flow rate 80-100 mL/min for 5-8 mins/bag into separate Leukotrap WB blood packs (Pall Medical, UK), containing 63 mL of citrate, phosphate and dextrose (CPD) as an anticoagulant solution each. Additionally, a tube of Ethylenediaminetetraacetic acid (EDTA) blood (Greiner Bio-one Vacuette[®], Thailand) was collected from the diversion pouch of the blood pack for full blood counts.

Processing and storage of donor blood

After 2 hours incubation at room temperature the whole blood was passed through the attached leukofilter. As ovine RBC are significantly smaller the whole blood was centrifuged at 5000g for 45 minutes and separated into plasma and packed red blood cell (PRBC) using a

manual Fenwal® plasma extractor (Baxter, USA). The ovine PRBC (ovPRBC) were suspended in saline-adenine-glucose-mannitol (SAGM) additive solution, and stored under standard storage conditions at 2-6⁰C prior to transfusion.

Quality assessment

The EDTA blood samples (n=12) and a segment from the corresponding prepared ovPRBC units (n=24) were evaluated for pH, potassium, glucose and lactate using a Radiometer ABL825 blood gas analyser (Radiometer, Copenhagen, Denmark). The RBC volume, haemoglobin and haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were also measured using the Beckman and Coulter®Act diff™haematology analyser (Beckman Coulter Inc, Ireland). Subsequently, samples were aseptically collected from ovPRBC units were analysed on days 1, 5, 7, 14, 21, 28, 35 and 42 to determine the effects of storage. The degree of haemolysis in stored samples was determined by measuring supernatant haemoglobin concentration after centrifugation of ovPRBC using a portable HemoCue® Hb 201⁺ Photometer System (HemoCue AB, Ängelholm, Sweden) [13]. The percentage haemolysis was calculated as follows: $(100 - \text{Hct}) \times \text{free plasma Hb (g/L)} / \text{total Hb (g/L)}$.

Compatibility testing

Compatibility testing between donor ovPRBC and recipient sheep RBC was performed to avoid haemolytic transfusion reactions. Compatibility was confirmed by four procedures involving two drops of recipient sheep sera mixed with 1 drop of 5% donor sheep RBC in phosphate buffered saline (PBS) each.

- (i) Immediate centrifugation at 390g for 15sec and examined macroscopically for agglutination. The strength of agglutination was scored on a scale of 0-4 [14].

- (ii) Incubated at room temperature for 30min, then centrifuged as above and examined macroscopically for agglutination.
- (iii) Incubated at 37⁰C for 30 minutes, and then centrifuged and examined.
- (iv) Tube (iii) was then centrifuged at 540g for 1min; the supernatant was removed and replaced by a drop of 10% albumin and incubated at 37⁰C for another 30 minutes before examination macroscopically for agglutination.

The ovPRBC was considered compatible when all 4 tests were negative [Fig 1].

Ovine Blood Transfusion

Two units of compatible ovPRBC were transfused into sheep that had been bled of 30% of their blood volume. This was followed by 4% albumin to achieve volume replacement. Six sheep received fresh (< 5-day old) ovPRBC and another six received stored (35-42 days old) ovPRBC. Transfused sheep were continuously monitored for 4 hours post-transfusion.

Statistics

All data are presented as mean±standard deviation (SD) of the mean and ranges. Significance between groups was determined based on planned comparisons using a 1-way ANOVA. Posterior Bonferroni's test was performed to determine difference between groups and baseline (GraphPad Prism6). *P* values of less than 0.05 were considered to be statistically significant.

Results

Ovine haematology

Baseline EDTA blood samples from 12 healthy male Merino sheep were analysed and compared with healthy human male values (Table1). The red blood cell counts (RBCC) in ovine blood was higher ($9.85-13.52 \times 10^{12}/L$) compared with that of humans ($4.60-6.00 \times 10^{12}/L$). The range of measured haemoglobin (Hb) and haematocrit (Hct) levels in sheep blood overlapped that of humans (115-156g/L vs. 140-180g/L and 35.0-48.4% vs. 40-54% respectively). Mean cell volume (MCV) and mean cell haemoglobin (MCH) values were considerably lower from human (31.6-37.9fL vs. 80-100fL; 10.8-14.1pg vs. 26-32pg respectively). The range of ovine mean cell haemoglobin concentration (MCHC) was similar to that of humans (314-342g/L vs. 320-360g/L).

Quality assessment of ovPRBC units

Twenty-four ovPRBC units were analysed and compared with human PRBC units (Table1). According to current standard requirements by Council of Europe Guideline the Hb level should be higher than 45g and the Hct level between 50-70% in human PRBC units [15]. The Hb level of ovPRBC (136-184g/L) surpassed that requirement but the Hct (39.0-71.3%) was slightly below to the required level. In spite of these differences ovPRBC haematology parameters were proportional to the human equivalent.

Storage-related changes in ovPRBC units

In this study, we also examined the following storage lesion parameters: haemolysis, potassium, glucose, lactate and pH in 10 units of ovPRBC over 42 days. Significant haemolysis was evident in ovPRBC from day 35 ($P < 0.0001$ vs. day 1) and was further increased at day 42 [Fig 2A]. From day 5 the potassium level of sheep PRBC steadily

increased to peak at 6.77mmol/L on day 28 after which it was relatively stable [Fig 2B]. There was a statistically significant decline in glucose levels evident after day 14 of storage ($P = 0.009$ vs. day 1) [Fig 2C]. This corresponded with a significant increase in lactate levels that was evident from day 28 ($P = 0.0028$ vs. day 1) [Fig 2D]. The pH on day 1 was 7.014 ± 0.047 and remained unchanged throughout the 42-day storage period ($P = 0.999$ vs. day 1) [data not shown].

Blood compatibility testing

In the total group, 77 compatibility tests were performed with sera from 24 potential male recipient sheep. Sixty-three of 77 (81.83%) were compatible and 14 (18.18%) were incompatible.

Ovine Blood Transfusion

After compatibility confirmation, 24 units of whole blood were transfused into 12 sheep recipients (2 units per sheep). During the 4 hours of monitoring post-transfusion, there was no evidence of any symptoms of adverse reaction (i.e. fever, tachycardia, hypotension, hypoxia or bleeding).

Discussion

Blood transfusion is a life-saving therapy used widely throughout clinical practice however it is not without risk. An increasing body of evidence has highlighted the non-infectious risks of transfusion, particularly of stored blood products [16, 17]. While clinical trials investigating the possible association between age of blood and poor clinical outcomes will inform changes to clinical practice, clinically relevant animal models of transfusion are necessary to elucidate the underlying mechanisms. Central to the clinical validity of such models are the accurate assessment of the scope and the limitations of each specific animal model. Small animals (mice, rats, guinea pigs) have been used extensively as transfusion models [8, 18-21]. However, they present many anatomical, physiological and immunological differences compared to humans [22]. For example, rodents RBCs have significantly shorter lifespan than human and sheep RBCs (Table 2). For this reason, rodent blood undergoes a more rapid rate of deterioration under storage conditions compared to human erythrocytes and influences the duration of storage [7, 8, 19, 23]. In addition, the smaller size and lower blood volume dictates the use of centrifuge tubes rather than blood packs in collection, processing and storage of rodent blood (Table 2). This and restrictions on sampling volume, repeated measurements and follow-up also limit their clinical relevance.

An ideal and clinically relevant animal model of blood transfusion would have anatomical and size similarities compared to humans, as well as comparative haematological and biochemistry values, analogous collection and processing protocols, not to mention the ease and expanse of care. Ovine models have been used extensively in medical research [24, 25] as they having comparable size and anatomical similarities [26] to humans providing good surgical accessibility and possibilities for multiple sampling and follow-up. They also have comparative plasma and tissue pharmacokinetics [27], pulmonary circulation [28] and a placid nature, which is extremely useful during long *in vivo* studies [29]. Furthermore, as

their size and blood volume is similar to humans it is possible to collect a whole unit of blood in collection bags identical to those used for human blood donors.

This study established normal haematology and biochemistry ranges for the Merino breed of sheep. These values were compared against those reported previously for humans. Ovine RBCs are smaller and more numerous than human RBCs. This corresponded with a lower range for ovine MCH compared to humans. In contrast, the range of ovine Hb, Hct and MCHC overlapped with the human normal range. These results together with the fact that the ovine RBC life span is very similar to that of human reveals some of the advantages ovine models have over other small animal models.

To determine if ovine PRBC would adequately mimic human PRBC in the study of the effects of transfusing stored blood, we also monitored haematological and biochemical properties of ovine PRBC over 42-day storage period (Figure 2). Similar to human PRBC units, the rate of haemolysis evident in SAGM-containing ovPRBC units increased during storage. According to the Council of Europe Guideline [15], the haemolysis rate of human PRBC units at out-date (42 days) should not exceed 0.8%. In this study haemolysis on day 42 was $0.81\% \pm 0.29$, pointing towards a slightly accelerated rate of storage lesion development in ovPRBCs. *In vivo* survival of lamb PRBC after 40 day storage and transfusion [30] have been reported earlier. However, further studies are required to establish the viability and osmotic fragility of adult sheep PRBC during and after 42-day storage period.

Potassium levels in stored human blood products increase over time, which may lead to severe levels of hyperkalemia (potassium > 5.5mmol/L), and this limits the shelf life of stored PRBC [23, 31]. Potassium in PRBC units is derived from RBC leakage, and levels in human PRBC range from 4 to 40mmol/L [32]. Ovine RBCs appear to be more stable as the potassium levels were considerably lower even on day 42.

The glucose levels decreased and lactic acid increased over time in ovPRBC but these changes were milder than what has been reported in human PRBC [23, 33]. In line with this the pH of ovine PRBC was stable over the entire storage period. Together these results suggest that metabolism of ovPRBC is slower. However, detailed investigation of ovPRBC biochemical changes, primarily adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG) level are required to confirm this.

Pre-transfusion compatibility testing and blood typing in humans is vital and routine practise. However, in animal research it is primarily focused on high value animals, such as pets and horses. Our literature search found only rare reports of ovine pre-transfusion testing and the methodology listed contained little detail [34-36]. The diversity of blood groups in sheep and the lack of commercially available blood-typing reagents make complete typing and matching difficult. Therefore, we developed a modified saline agglutination method that can be easily added to the research protocol. Using this technique 18.2% of cross matches were found to be not compatible. This data challenges the presumption that single, unmatched whole blood transfusions are generally safe and well tolerated within an animal species. This unexpected high frequency of incompatibility emphasises importance pre-transfusion compatibility testing especially in transfusion models.

As discussed above an ovine model provides many advantages for the study of transfusion associated injuries, however its main limitation is that ovine RBCs are significantly smaller compared to that of humans [9, 37-40]. The smaller size of ovine RBC equates to a significantly higher surface area to volume ratio, and as a result ovine RBCs do not aggregate or deform as readily as RBCs of other species [41].

In summary, this study presents a comprehensive validated ovine model of blood transfusion. Our results confirm the use of human blood bags for sheep blood collection with analogous blood bag processing, compatibility checking and transfusion procedures. In

addition, we established baseline haematological and biochemical parameters for healthy adult male Merino sheep and ovine PRBC units of various ages, and demonstrated the value of pre-transfusion compatibility testing. Together, these findings validate the application of the sheep model for blood transfusion studies and identify the advantages and limitations of an ovine transfusion model to assist future investigators design and select appropriate *in vivo* transfusion models.

Acknowledgement

This work was funded by the Queensland Emergency Medicine Research Foundation (QEMRF), the Australian Red Cross Blood Service (ARCBS) and the National Health and Medical Research Council (NHMRC). JFF is supported by Health Research Fellowship from the Office of Health and Medical Research, Queensland Health. The authors are deeply grateful to William Sommers and Peter Lindenberg for the skilful sheep handling.

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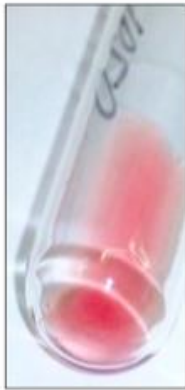
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Figure legends

Figure 1 A range of typical ovine compatibility tests results. 0 = No agglutination, an even red cell suspension; 1 = Small agglutinates, with a lot of free red cells; 2 = Medium size agglutination, clear background; 4 = one solid agglutinate, no free red cells detected.

Figure 2 Characterization of biochemical changes (haemolysis, potassium, glucose, lactate) in ovPRBCs (n=10) during 42 days storage. * $P < 0.05$ vs. day 1 for storage duration. The results are mean \pm SD.

Figure1



Score=0

COMPATIBLE



Score=1



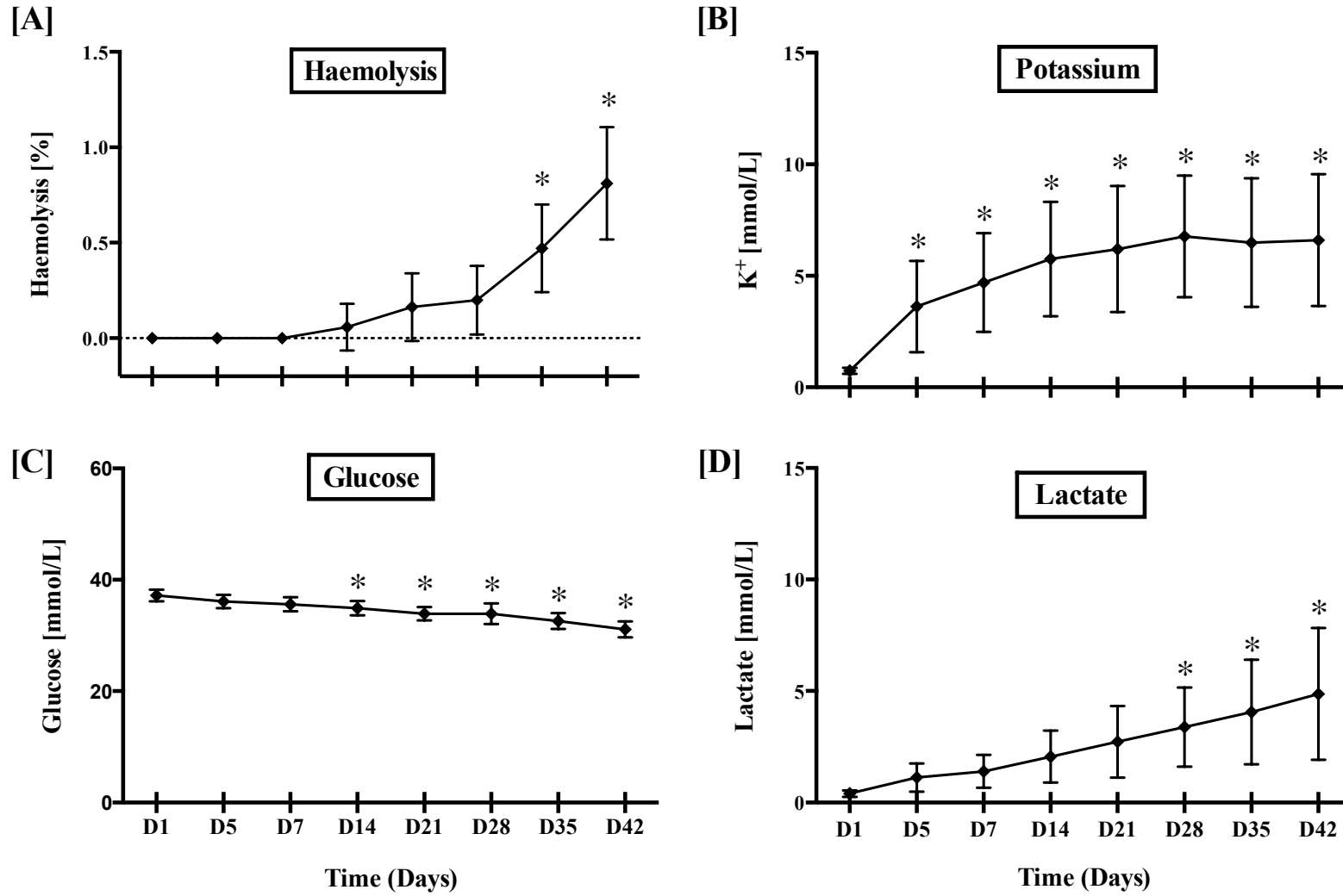
Score=2



Score=4

INCOMPATIBLE

Figure2



Tables

Table 1

Parameters*	Unit	Human blood [#]	Ovine blood (n=12)	Human PRBC units** (n=15)	Ovine PRBC units (n=24)			
		Range	Mean (±SD)	Range	Mean (±SD)	Range		
RBCC	[10 ¹² /L]	4.60-6.00	11.41 (±1.14)	9.85-13.52	6.63 (±0.50)	5.84-7.52	13.74 (±1.87)	11.28-19.07
Hb	[g/L]	140-180	131.27 (±14.4)	115-156	198 (±10.74)	178-215	158.83(±15.37)	136-184
Hct	[%]	40-54	39.93 (±4.46)	35.0-48.4	60.49 (±0.03)	53.9-65.1	48.49 (±7.68)	39.0-71.3
MCV	[fL]	80-100	34.99 (±1.56)	31.6-37.9	91.53 (±5.30)	79.7-102.0	35.25 (±1.63)	32.2-38.2
MCH	[pg]	26-32	11.68 (±0.89)	10.8-14.1	29.97 (±1.63)	26.2-33.9	11.9 (±0.48)	11.2-12.7
MCHC	[g/L]	320-360	327 (±8.25)	314-342	327.73 (±8.30)	310-337	329.08(±27.34)	249-350

Table 1 Normal hematology values for healthy adult male sheep and fresh ovine PRBC. *RBCC = red blood cell count; Hb = hemoglobin; Hct = haematocrit; MCV = mean cell volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration. [#]From Bernadette F. Rodak GAF & Elaine Keohane: *Hematology: Clinical Principles and Applications*, 4th Edition edn: Elsevier. ** Unpublished data provided by Kristen Glenister, Research and Development, Australian Red Cross Blood Service, Melbourne.

Table 2

	Mice	Rat	Guinea pig	Sheep	Human
Size	20-40 g	250-520 g	950-1060 g	30-60 kg	60-80 kg
Blood volume	1.2-2.4 mL	16-32 mL	35-50 mL	2-4 L	4.4-5 L
Red cell life span	42 days [42]	65 days [42]	79 days [42]	~120 days [9-11]	120 days [43]
Blood collection	Microtubes	Microtubes	Small blood bags (150mL)	Blood packs (400-600mL)	Blood packs (400-600mL)
Storage lesion	7 days [7]	7 days [8]	28 days [19]	N/A	42 days [15]

Table 2 Benefits of an ovine model of transfusion**Abbreviations**

TRALI = transfusion-related acute lung injury, TRIM = transfusion-related

immunomodulation, PRBC = packed red blood cell, ATP = adenosine triphosphate,

2,3-DPG = 2,3-diphosphoglycerate